Understanding The Cellular Role Of Prolyl Oligopeptidase

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SUMMARY OF THESIS:

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The PO homologue, DpoA, was identified in the cellular slime mould *Dictyostelium Discoideum* following a lithium screen. Loss of DpoA confers resistance to both lithium and valproic acid (VPA), through regulation of inositol phosphate pathways.

The intracellular substrate and cellular role of PO remain uncertain. However, it is known to inhibit dephosphorylation of the higher order inositol phosphates IP_5 and IP_4 to IP_3, a reaction catalysed by Multiple Inositol Polyphosphate Phosphatase (MIPP), by an unknown mechanism.

In this study it has been determined that DpoA is highly similar to the mammalian enzyme. It has also been demonstrated that while some variation was observed at the sequence level there is clear homology around the active site and the catalytic triad is conserved. Enzyme activity and inhibition studies reveal similar K_M and K_i values in the presence of known specific inhibitors. Thus making it a relevant model for the mammalian enzyme.

Characterisation of the direct effects of three mood stabilisers on PO activity has revealed no effect of lithium, carbamazapine or valproic acid at therapeutic concentrations. However, VPA while exerting no clear effect *in vivo* was able to inhibit PO *in vitro* at increased concentrations. This inhibition was also seen using VPA analogues lacking either the carboxylic acid domain or branching structure.

Significant to the elusive intracellular role for PO is the identification of a clear inhibitory effect of PO on MIPP activity *in vitro*. While identification of PO presence within the nucleus as well as distributed throughout the cytoplasm may also be significant to its role in inositol signalling.

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Contents

Abstract

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Abbreviations

σ	Standard Deviation
α-MSH	α-Melanocyte-Stimulating Hormone
$[PP]_2$-IP	Bis-Diphosphoinositolphosphate
[PP]-IP	Diphosphoinositolphosphate
1°	Primary
2°	Secondary
5-HT	5-Hydroxytryptamine
aa	Amino Acid
Ab	Antibody
Ac	Acetyl
ACA	Adenylyl Cyclase
AP	Acylaminoacyl Peptidase
Ala	Alanine
AMC	7-amino-4-methylcoumarin
APP	Amyloid Precursor Protein
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine Triphosphate
AVP	Arginine-Vasopressin
BD	Bipolar Disorder
BOC	Butoxycarbonyl
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ion
CBC	Cerebellar Granule Cell
cDNA	Copy DNA
ChAT	Choline Acetyltransferase
CMF	Conditioned Medium Factor
CNS	Central Nervous System
CTP	Cytidine Triphosphate
DAG	Diacylglycerol
ddH_2O	Double Distilled Water

DFP	Diisopropylfluorophosphate
DhkA, B, C	*Dictyostelium* Histidine Kinase A, B, C
DIPP	Diphosphoinositol Polyphosphate Phosphohydrolase
dpoA	*Dictyostelium* prolyl oligopeptidase gene
DpoA	*Dictyostelium* prolyl oligopeptidase protein
DPPIV	Dipeptidyl Peptidase IV
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
FPLC	Fast Protein Liquid Chromatography
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GM-CSF	Granulocyte-Macrophage Colony Stimulatory Factor
GnRH	Gonadotropin-Releasing Hormone
GSH	Glutathione
GSSG	Glutathione disulfide
GTP	Guanosine Triphosphate
His	Histidine
HMIT	H^+/*myo*-inositol symporter
IL	Interleukin
IL-IRA	Interleukin-1 Receptor Agonist
IMPase	Inositol Monophosphatase
INF-γ	Interferon-γ
ino1	Inositol Monophosphate Synthase gene
IP	Inositol Monophosphate
IP_2	Inositol Bisphosphate
IP_3	Inositol Trisphosphate
IP_4	Inositol Tetrakisphosphate
IP4P	Inositol Polyphosphate-4-Phosphatase
IP_5	Inositol Pentakisphosphate
IP5P	Inositol Polyphosphate-5-Phosphatase
IP_6	Inositol Hexakisphosphate
IP_6K	Inositol Hexakisphosphate Kinase
IPK1	I(1,3,4,5,6)P_5 2-kinase

IPMK	Inositol Phosphate Multi-Kinase
IPP	Inositol Polyphosphate Phosphatase
IPSynthase	Inositol Monophosphate Synthase
IPTG	Isopropyl β-D-Thiogalactopyranoside
JTP-4819	(S)-2-[[(S)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N-(phenylmethyl)-1-pyrrolidinecarboxamide
kDa	Kilo Daltons
K_{ic}	Inhibition constant (competitive inhibition)
K_{iu}	Inhibition constant (uncompetitive inhibition)
K_M	Michaelis constant
LHRH	Luteinising Hormone Releasing Hormone
Li^+	Lithium ion
mAb	Monoclonal Antibody
mAChR	Muscarinic Acetylcholine Receptor
MAPs	Microtubule-Associated Proteins
MCA	7-Amino-4-Methyl Coumarin
mEH	Microsomal Epoxide Hydrolase
MEL	Murine erythroleukemia
MeOH	Methanol
MIPP	Multiple Inositol Polyphosphate Phosphatase
mRNA	Messenger RNA
nt	Nucleotides
ONO-1603	(S)-1-[N-(4-chlorobenzyl)succinamoyl]pyrrolidine-2-carbaldehyde
PA	Phosphatidic Acid
PCP	Prolyl Carboxypeptidase
PhdA	Pleckstrin Homology Domain Containing Protein A
pH_i	Intracellular pH
PI	Phosphoinositol
PI3K	Phosphatidylinositol-3-Kinase
PIKK	Phosphatidylinositol-3-Kinase related Kinase
PIP	Phosphoinositolphosphate
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-Trisphosphate

PKB	Protein Kinase B, also known as Akt
PKC	Protein Kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonylfluoride
pNA	p-Nitroaniline
PO	Prolyl Oligopeptidase
PREPL A	Prolyl Endopeptidase Like A
Pro	Proline
PSF	Pre-Starvation Factor
PTEN	Phosphatase and Tensin Homologue
Rpo	Rat prolyl oligopeptidase protein
rRNA	Ribosomal RNA
S17092	(2S,3aS,7aS)-1([(R,R)-2-phenylcyclopropyl]carbonyl)-2-[(thiazolidin-3-yl)carbonyl]octahydro-1H-indole)
SEM	Standard Error of the Mean
Ser	Serine
SMIT1	Na^+/*myo*-inositol cotransporter
SP	Substance P
SUAM-1221	1-[1-(4-phenylbutanoyl)-L-prolyl]pyrrolidine
TCA	Trichloroacetic Acid
THA	Tetrahydroaminoacridine
Tm	Melting Temperature
TRH	Thyrotropin Releasing Hormone
U or mU	Enzyme Unit or milliUnit (μmoles or nmoles of substrate cleaved min^{-1})
v	Velocity (enzyme activity)
V_{max}	Maximum Velocity
Z	benzyloxycarbonyl
Z-321	1-[3-(2-indanylacetyl)-L-thiopropyl]pyrrolidine
ZIP	Z-Pro-prolinal-insensitive Z-Gly-Pro-MCA hydrolyzing peptidase
Z-Gly-Pro-AMC	Z-Gly-Pro-7-amido-4-methylcoumarin
Z-Pro-prolinal	Benzyloxycarbonyl-prolyl-prolinal
ZTTA	N-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetyl

1 INTRODUCTION

1.1 Introduction

Prolyl oligopeptidase (PO, Prolyl Endopeptidase, PE, PEP) is a member of the serine protease protein family. It also shares structural similarity with the family of α/β hydrolase fold enzymes, such as, oligopeptidase B, dipeptidyl peptidase IV and acylaminoacyl peptidase (Polgar 2002). PO also contains a seven-bladed beta-propeller domain that confers selectivity for small peptide substrates, which PO cleaves specifically after a proline residue (Fulop et al. 2000).

PO homologues have been identified in multiple species from archaea, bacteria and eukaryotes. In the model organism *Dictyostelium discoideum* the PO homologue, DpoA, was identified following a lithium screen. Loss of DpoA is able to confer resistance to treatment with both lithium (Williams et al. 1999) and valproic acid (VPA) (Williams et al. 2002).

Lithium and VPA are both drugs commonly prescribed to treat Bipolar disorder. Both have multiple intracellular targets, however both affect the inositol phosphate pathway, causing inositol depletion, suggesting a common therapeutic mechanism.

The intracellular substrate and cellular role of PO remain uncertain. Inhibition of PO results in an increase in intracellular IP_3 through increased dephosphorylation of higher order inositol phosphates. PO is also able to cleave a number of extracellular neuropeptides however there is strong evidence that this enzyme is not secreted from the cell but localised throughout the cytoplasm (Dresdner et al. 1982; Goossens et al. 1995; Williams et al. 1999; Schulz et al. 2005).

Dictyostelium is a highly tractable model organism. A sequenced genome and multi-staged life cycle, which provides clear mutant phenotypes, makes it an ideal model organism for molecular genetic and biochemical studies of cellular processes. Thus

Dictyostelium may provide a suitable model for understanding the cellular role of prolyl oligopeptidase.

1.2 Prolyl Oiigopeptidase

1.2.1 Enzymes Related To PO

1.2.1.1 The Serine Protease Family

The serine proteases include several families of enzymes involved in a wide variety of roles throughout the cell. This family contains examples of both divergent and convergent evolution, showing conservation or development of the same proteolytic mechanism. As implied by the name, the serine proteases are characterised by a catalytic serine residue at the active site. This residue attacks the carbonyl carbon atom of a peptide bond, resulting in peptide bond hydrolysis and cleavage of the peptide on the carboxyl side of the residue attacked.

Despite showing an identical catalytic site the three serine protease classes; chymotrypsin, subtilisin and serine carboxypeptidase, exhibit no secondary or tertiary structure similarity (Perona and Craik 1995). Thus the serine protease family show a wide range of specificities with preferences for small peptides or large proteins and different target sequences at and around the cleavage site.

1.2.1.2 Proline Specific Peptidases

1.2.1.2.1 Proline is a unique amino acid

Out of the 20 amino acids proline is unique; it has a cyclic structure whereby the side chain forms a covalent bond with the nitrogen of the peptide bond thus forming an imino acid structure. This has several consequences; restriction of the proline backbone conformation, restriction of the conformation of the residue preceding proline and an inability to act as a hydrogen donor (Williamson 1994). Because of this

the presence of a proline residue results in a kink in the peptide chain (MacArthur and Thornton 1991). It also affects the formation of an α-helix structure as the proline nitrogen atom is unable to form the normal hydrogen bonds involved in the helical structure. This proline induced distortion of the α-helix probably plays a structural or functional role as it is highly conserved (Barlow and Thornton 1988). Proline also results in pre-disposition of the X-Pro peptide bond to be in the cis rather than trans position (10-30% compared to <1% of other amino acid's), because of this isomerisation the peptide-proline bond often acts as the limiting step in protein folding (Brandts et al. 1975).

1.2.1.2.2 Function of proline residue

Because of its unique structure proline plays an important role in degradation, protection and maturation of a variety of biologically active peptides. The presence of a proline residue may prevent degradation by non-specific amino peptidases however, in some conditions a proline residue causes a conformational change such that the peptide becomes susceptible to hydrolysis, for example; proline-directed arginyl cleavage (Schwartz 1986; Vanhoof et al. 1995).

A large proportion of neuropeptides, cytokines and growth factors have a proline residue at the second amino acid of the mature peptide, e.g. Substance P, Interleukin II, V and X and Tumour necrosis factor β. This proline prevents further degradation by the proteases involved in formation of the mature peptide as these proteases cannot cleave the X-Pro bond (Mentlein 1988; Vanhoof et al. 1995). A number of neuro- and vaso-active peptides also contain proline residues which may confer both resistance to non-specific proteases and conformation required for activity (Yaron and Naider 1993; Vanhoof et al. 1995). Proline rich regions show additional functions, acting as

spacers between functional protein moieties and non-specific binding regions important in protein-protein interactions (Williamson 1994).

1.2.1.2.3 Specific cleavage at proline residues

A family of proline specific peptidases able to cope with cleavage of peptides containing proline residues have been identified. This family consists of a variety of enzymes able to cleave at the carboxyl or amino side of a proline residue in a wide range of sequences and positions. They have been attributed roles in protein activation, bioactive peptide degradation and catabolism.

Members of this family include: Prolyl oligopeptidase; a serine protease which cleaves small peptides at the carboxyl side of proline. Dipeptidyl peptidase IV; another serine protease which exists in a membrane bound or soluble form, it is involved in activation of immune cells and removes X-Pro N-terminal dipeptides (Heins et al. 1988). Dipeptidyl peptidase II; has a lysosomal localisation and shows similar specificity to Dipeptidyl peptidase IV (Mentlein and Struckhoff 1989). Aminopeptidase P; a glycoprotein with a possible role in cardiovascular and pulmonary function, classed as a metalloprotease, it cleaves the N-terminal amino acid where the penultimate residue is proline (Fleminger et al. 1982; Hendriks et al. 1991; Ward et al. 1991). Prolidase; this enzyme shows a similar activity to Aminopeptidase P but will only cleave dipeptides, it has an important role in proline recycling (Yoshimoto et al. 1983; Freij et al. 1984). Proline Iminopeptidase; an aminopeptidase which can release an N-terminal proline residue from a peptide of any size (Fujimura et al. 1985). Prolinase; an enzyme thought to have a similar role to prolidase, it is highly specific for dipeptides with an N-terminal proline (Akrawi and Bailey 1976; Imai et al. 1982). Prolyl carboxypeptidase; cleaves the carboxyl terminal amino acid following a proline residue, and may be involved in metabolism of

angiotensin (Yang et al. 1968). Carboxypeptidase P; shows a similar specificity to Prolyl carboxypeptidase (Hedeager-Sorensen and Kenny 1985). Finally, Human Immunodeficiency Virion-1 protease; cleaves X-Pro bonds within a protein, and is necessary for HIV-1 virion maturation (Graves et al. 1988; Meek et al. 1989) (Figure 1-1) (Summarised in Cunningham and O'Connor 1997).

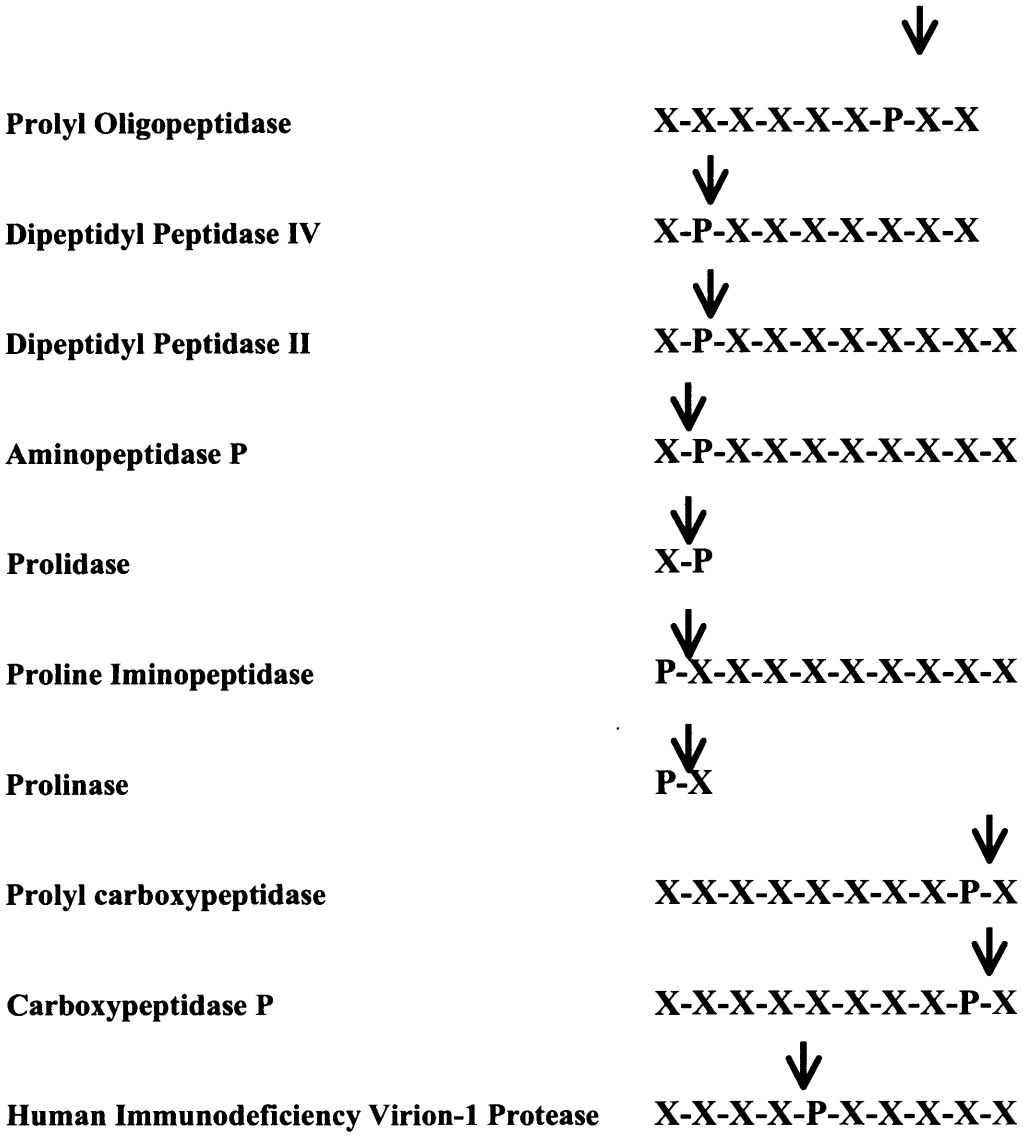

Figure 1-1 Cleavage Sites of Proline Specific Peptidases

A number of peptidases cleave small peptides specifically at or near a proline residue each of these peptidases shows slightly different cleavage site specificities. Arrow indicates the peptide bond that is specifically cleaved, X indicates any amino acid, P indicates position of proline.

1.2.1.3 The Prolyl Oligopeptidase Family

PO is a key member of the S9 family of serine proteases, which is also known as the Prolyl Oligopeptidase family. This family also contains, dipeptidyl peptidase IV, acylaminoacyl-peptidase, dipeptidyl peptidase B, oligopeptidase B (Rawlings et al. 1991) and the more recently identified prolyl endopeptidase like A (PREPL A) (Szeltner et al. 2005). These are not all proline specific enzymes but can be placed into the same evolutionary family by virtue of their sequence homology around the active triad (Rawlings et al. 1991). Sequencing of the human prolylcarboxypeptidase (PCP) provided an evolutionary link between the prolyl oligopeptidase family and the serine carboxypeptidases by showing clear sequence homology, especially around the active site, between both families of enzymes (Tan et al. 1993). In fact, despite sharing many characteristics with the serine carboxypeptidase family, the sequence surrounding the active serine residue of human PCP fits with the consensus sequence of the prolyl oligopeptidase family (GXSXGGZZ, X=any aa, Z=hydrophobic aa).

1.2.2 Structure Of Prolyl Oligopeptidase

1.2.2.1 Identification Of PO

Walter et al. first reported potential prolyl oligopeptidase activity in 1971. They identified an enzyme activity in human uterine tissues, which cleaved the prolyl-leucyl bond of the hormone oxytocin thus releasing leucylglycinamide (Walter et al. 1971). This was the first identification of endopeptidase activity directed specifically at the Pro-X peptide bond.

1.2.2.1.1 Size of PO

PO was purified from lamb kidney by Koida and Walter in 1976 and estimated to have a molecular weight of 57kDa. They further characterised the post-proline cleaving activity reporting maximal activity at pH 7.5-8.0. They also observed preferential cleavage of Pro-X bonds where X is a hydrophobic residue and Pro is not the N-terminal residue, a lower rate of cleavage for basic then acidic X residues and complete inhibition by Z-Pro-X dipeptides (Koida and Walter 1976). PO has since been determined to have a molecular mass of approximately 80kDa (Rennex et al. 1991). Human PO was first cloned and sequenced in 1994 from human lymphocyte mRNA. The resulting cDNA was 2562 nucleotides (nt) long and encoded a 710 aa protein sharing 97% identity with the PO sequence from porcine brain (Vanhoof et al. 1994).

1.2.2.1.2 Specificity for proline and short peptides

A potential PO activity was identified in the brain by partial purification of an enzyme, referred to as Brain kininase B, able to cleave the Pro-Phe bond within Bradykinin (Oliveira et al. 1976). Koida and Walter also identified in vitro cleavage of the bio-active peptides; Insulin, Oxytocin, Angiotensin II, Bradykinin and Bradykinin potentiating factor, all of which were cleaved at the carboxyl bond of a proline residue (Koida and Walter 1976). The specificity of PO towards oligopeptides was identified by its inability to cleave the substrate bradykinin when covalently attatched to a larger peptide; this is in contrast to other serine protease family members, trypsin and chymotrypsin whose activity was unaffected by the larger substrate size (Camargo et al. 1979). PO was subsequently renamed Prolyl

Oligopeptidase from Prolyl Endopeptidase, as it was originally called, in order to reflect this property (Polgar 2002).

1.2.2.2 Catalytic Domain

PO consists of two distinct structural domains. Partial trypsin proteolysis revealed the catalytic domain at the C-terminal with a proteolytically susceptible link between residues Lys196 and Ser197 (porcine PO). Following cleavage of this linker domain the two fragments remained associated revealing a clear close interaction between the two domains in the functional protein (Polgar and Patthy 1992). For a protein structure diagram of PO see Figure 1-2.

The structure of the α/β hydrolase fold, which forms the catalytic domain of the prolyl oligopeptidase (S9) family of enzymes and the carboxylase serine proteases, was first modelled in wheat serine carboxylase. It was found to consist of a central 11-stranded β-sheet with 15 α-helices arranged on either side. The active triad was located within a hydrophobic pit and arranged in such a way as to suggest the existence of two active diads (His-Asp and His-Ser) rather than a single active triad (Liao and Remington 1990; Liao et al. 1992).

Analysis of the primary and secondary structure of PO from human lymphocytes revealed poor sequence homology yet striking structural similarity between PO, dipeptidyl peptidase IV (DPPIV) and acylaminoacyl peptidase (AP). The sequence of α and β structures formed by the last 250 residues are consistent with the α/β hydrolase fold and form 8 β-strands interconnected by five α-helices (Goossens et al. 1995). The residues of the catalytic triad are all located in loop regions and an interdomain linking oligopeptide was predicted around residue 200 (agreeing with the

proteolytic susceptibility observed between residues 196 and 197 (Polgar and Patthy 1992)) (Goossens et al. 1995).

This sequence predicted structure was confirmed by the X-Ray crystal structure of the porcine PO. This revealed an α/β hydrolase fold consisting an eight stranded β-sheet and six α-helices formed from residues 428-710, as predicted and an additional two β strands and two α helices formed from residues 1-72. The N-terminal region interacts with the C-terminal domain via twenty three hydrogen bonds and salt bridges as well as several hydrophobic interactions (Fulop et al. 1998).

NMR studies carried out by Kahyaoglu et al. on Prolyl Oligopeptidase and Oligopeptidase B identified resonance spectrums indicating the presence of two non-catalytic histidine residues participating in strong hydrogen bonds (low barrier hydrogen bonds, LBHB). It also showed an absence of clear hydrogen bonds between the catalytic His and Asp residues, in contrast to the chymotrypsin and subtilisin families of serine proteases (Kahyaoglu et al. 1997b).

1.2.2.3 β-Propeller Domain

The second functional domain of PO is a 7-bladed β-propeller domain. β-propeller domains, of between four and eight β-sheets, are present in a number of different proteins including G-protein β-subunits and collagenase (Li et al. 1995; Wall et al. 1995; Fulop and Jones 1999). Identified in PO following x-ray crystal structure analysis, the β-propeller domain is formed by seven 4-stranded antiparallel β-sheets arranged around a central tunnel. The β-propeller of PO is unusual in that the first and last β-sheets are not physically joined to close the tunnel (Figure 1-2) (Fulop et al. 1998). In other β-propellers this gap is closed by disulphide bridges (Faber et al. 1995) or formation of one β-sheet from strands of both the C and N-terminus (Neer

and Smith 1996; Baker et al. 1997). The non-closing of the PO tunnel may play an important role in the function as introduction of an additional cysteine residue, resulting in disulphide bonding across the gap, greatly reduces the enzyme activity (Fulop et al. 2000).

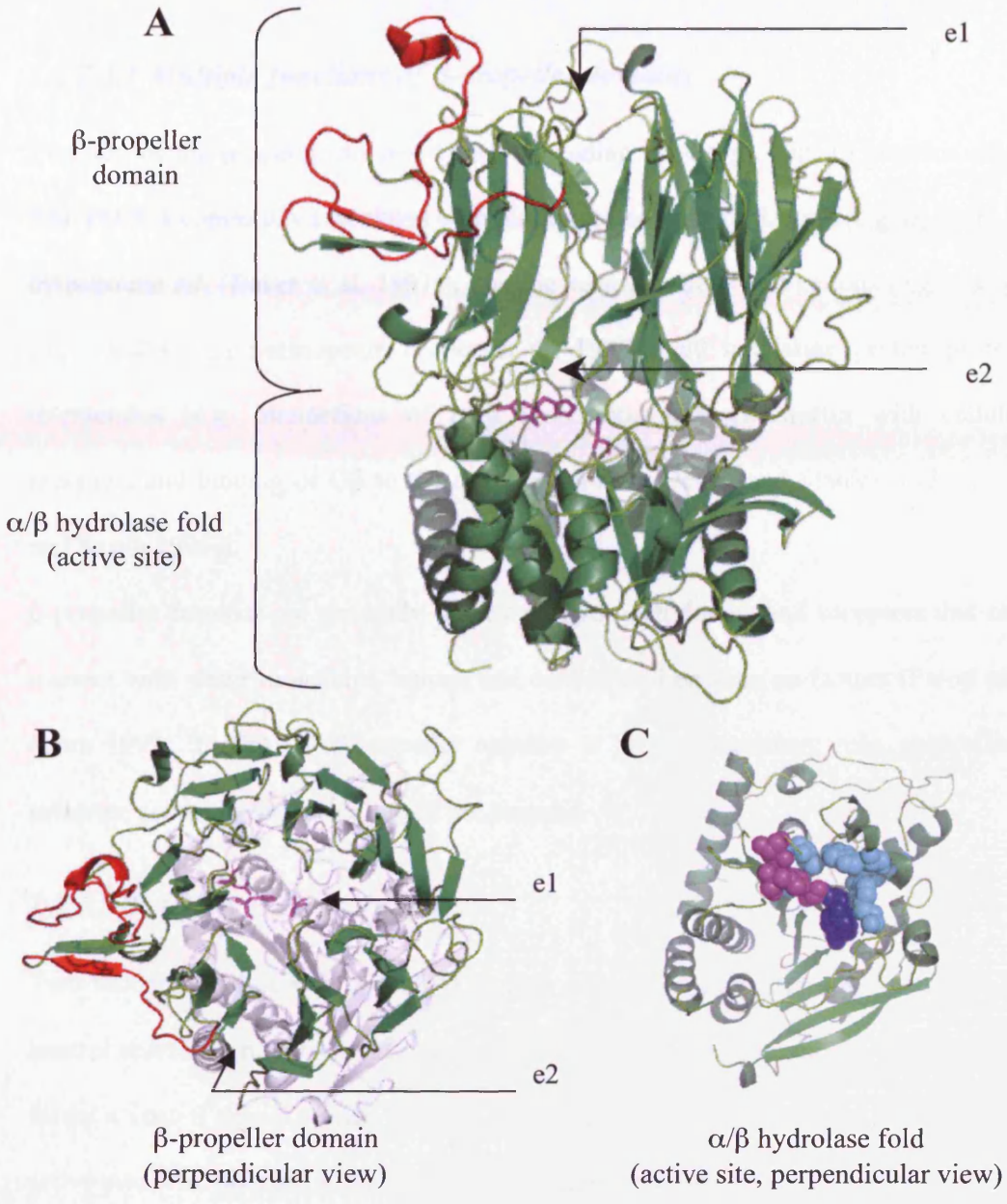

Figure 1-2 Structure of DpoA

The structure of *Dictyostelium* PO, DpoA, as modelled by SwissModel using prolyl oligopeptidase from porcine brain, accession no. 1h2w, as a template. Images were produced using MacPyMol software. Additional sequence not present in the mammalian β-propeller domain (see section 3.2.1.7) is highlighted in red. Active site residues are shown in purple. **A. Ribbon representation of entire protein structure**. e1 and e2 indicate the 2 potential routes of substrate entry to the active site. Active site residues are represented by purple stick structures. **B. Perpendicular view of the β-propeller domain.** The catalytic domain is coloured in white for simplicity, active site residues are represented by purple stick structures. **C. Perpendicular view of the catalytic domain**. The active site residues are represented by purple space filling models. Also shown as space filling models are the proline binding residues (light blue) and the oxyanion binding residues (dark blue).

1.2.2.3.1 Multiple functions of β-propeller domains

The role of the propeller domain varies depending on the protein. In proteins other than PO it is commonly associated with binding of enzyme co-factors (e.g. d_1 haem in cytochrome cd_1 (Baker et al. 1997)), binding various anions and cations (e.g. Na^+ or Mg^{2+} and Cl^- in haemopexin (Faber et al. 1995)) and mediating protein-protein interactions (e.g. interactions of both hemopexin and vitronectin with cellular receptors and binding of Gβ to the α and γ subunits (Jenne and Stanley 1987; Neer and Smith 1996)).

β-propeller domains are generally lined with hydrogen donors and acceptors that can interact with water molecules, anions and cations and enzyme co-factors (Fulop and Jones 1999). In PO the β-propeller appears to play a regulatory role, controlling substrate access to the active site of the enzyme.

1.2.2.3.2 Access to the active site

Two models have been proposed to explain how the β-propeller domain is able to control access of the substrate to the active site of PO. In the first model, the propeller forms a central tunnel, which lines up with and was thought to control access to the active site. The opening at the bottom of the tunnel is covered by flexible side chains. It is too narrow, in the steady state, to allow substrate entry therefore, may be responsible for the rate determining conformational change involved in PO activity. The tunnel is large enough to theoretically accommodate peptides larger than 30aa, however, it is thought that such peptides would contain secondary structures thus excluding them from entering (Fulop et al. 1998). Regulation of substrate entry by a conformational change is supported by the inability of the fluorescent substrate Abz-Gly-Phe-Gly-Pro-Phe-Gly Phe(NO_2)-Ala-NH_2 to bind to the enzyme in the presence

of disulphide bonds between the 1st and 7th blades of the propeller domain or between the propeller and catalytic domains (Szeltner et al. 2004).

In the PO family member DPPIV a similar mechanism is unlikely. This protein contains a much looser 8-bladed β-propeller domain as well as a large gap or 'cave' at the base of the propeller, thus, allowing access to the active site without entering via the tunnel of the propeller (Hiramatsu et al. 2003). It is possible that substrate binding to PO may also occur, not via the β-propeller tunnel, but via a gap between the two domains.

This second model is supported by; the inhibitory effect of disulphide bonds between the two domains and the highly stable structure of the β-propeller region when expressed without the peptidase domain, despite the absence of a closing mechanism (Szeltner et al. 2004; Juhasz et al. 2005). However, it is of note that the β-propeller region is less stable when present as part of the complete enzyme. Also, the Cys residue involved in formation of the interdomain disulphide bond is the Cys close to the active site, which, affects substrate binding in the presence of thiol inhibitors (Polgar 1991). It is therefore difficult to make a distinction between the effect of the disulphide bond on domain motility and a steric effect at the active site.

Subsequent determination of the crystal structure of PO from the bacterium *Sphingomonas capsulata* revealed an open configuration of the active site, providing strong evidence for the interdomain entry model (Shan et al. 2005). Shan et al compared this structure with the closed structure obtained from *Myxococcus xanthus* PO complexed with the inhibitor Z-Ala-prolinal. They proposed a model involving a conformational change, induced by the incoming peptide, resulting in opening of a large cavity leading to the active site. The specificity for oligopeptides is thought to be mediated by limited surface area for binding at the active site. The nature of this

conformational change has been characterised using molecular dynamics and FIRST (Floppy Inclusion and Rigid Substructure Topology) simulations on porcine PO. Identification of highly flexible regions within the enzyme has revealed a tunnel formed by; the N-terminal region (residues10-40), a hydrophilic loop of the β-propeller domain (residues 192-205) and some C-terminal residues including the active site Asp (637-641, 645-647, 650, 674) that is able to expand following disruption of a salt bridge between Asp35 (N-terminal region) and Lys196 (hydrophilic loop) (Fuxreiter et al. 2005).

1.2.2.4 Variations

PO has now been cloned from a number of sources revealing a number of enzyme variations between different organisms, in particular those of microbial origin.

1.2.2.4.1 Cleavage of larger proteins

PO from the hyperthermophilic archaeon *Pyrococcus furiosus* is active at 85°C and shows evidence of sequential halide binding which may cause a conformational change at the interdomain hinge region. Despite retaining both the α/β-hydrolase fold region and β-propeller domain structures, the *P.furiosus* enzyme is able to cleave large proteins and shows increased activity towards Ala-X peptide bonds compared with its mammalian counterparts (Harris et al. 2001). An ability to cleave large proteins, specifically fibronectin and collagen, has also been reported for the parasitic *Trypanosoma cruzi* PO (Tc80). Structural models of this protein have revealed that binding of a large protein, such as the collagen helix, would not significantly affect the overall structure of the enzyme (Bastos et al. 2005).

In addition, identification of a bacterial PO in *Flavobacterium meningosepticum* has uncovered a 20aa signal motif which is responsible for a periplasmic distribution of PO and is cleaved to form the mature enzyme (Chevallier et al. 1992).

1.2.2.4.2 ZIP

Existence of another enzyme also able to cleave the PO specific substrate Z-Gly-Pro-MCA, but insensitive to the specific inhibitor Z-Pro-Prolinal, has been reported and named Z-Pro-prolinal insensitive Z-Gly-Pro-MCA hydrolyzing peptidase (ZIP) (Birney and O'Connor 2001). ZIP was isolated from Bovine serum and despite sharing a similar pH profile, specificity for oligopeptides and propensity for cleaving Pro-X peptide bonds, ZIP was unable to cleave PO substrates bradykinin, substance P, vasopressin and neurotensin. ZIP also exhibited different inhibition profiles with a number of serine protease inhibitors and exists as a homodimer more than twice the size of PO.

1.2.3 Mechanism Of PO Catalysis

In the more traditional members of the serine protease family; chymotrypsin and subtilisin, the rate of enzyme reaction is dependent on a general base/general acid chemical catalysis step. In PO it appears that this is not the case. Studies of the rate of acyl-enzyme formation, using substrates with a variety of leaving groups, suggest that the rate is independent of the leaving group (Polgar 1992c). This is different to observations made on the other serine proteases, where, leaving groups which were readily hydrolysed were degraded several orders of magnitude faster than those with leaving groups which were not readily hydrolysed (nitrophenyl ester compared to corresponding amide) (Zerner and Bender 1964). This difference led Polgar to conclude that the rate limiting step is in fact physical, rather than chemical, and may

rely on either diffusion controlled binding of the substrate or a conformational change during catalysis (Polgar 1992c).

1.2.3.1 General Serine Protease Catalysis

The serine residue at the active site forms part of a catalytic triad along with (in the majority of cases) aspartic acid and histidine. Serine reacts with the substrate via its hydroxyl group. This nucleophilic attack is facilitated by stabilisation by the other residues in the triad.

1.2.3.1.1 Interaction of catalytic triad residues

Two models have been put forward to describe the peptidase action at the molecular level. The first is the charge relay model, described by Polgar and Bender in 1969. This model involves proton transfer from the serine residue to the substrate leaving group, via the imidazole ring of the histidine residue, to explain the reactivity of the hydroxyl group (Polgar and Bender 1969). This also explains stabilisation of the protonated imidazole ring, by proton transfer between the non-protonated N atom of the imidazole ring and the carboxyl group of the, catalytically important, aspartic acid residue (Blow et al. 1969). This model is supported by NMR and proton inventory studies (Hunkapiller et al. 1973; Pollock et al. 1973; Hunkapiller et al. 1976; Elrod et al. 1980).

The second model favours formation of a hydrogen bond between the imidazole ring and aspartate residue rather than complete proton transfer and is also supported by NMR and neutron diffraction studies (Robillard and Shulman 1974a, 1974b; Bachovchin and Roberts 1978; Kossiakoff and Spencer 1980, 1981).

It is beleived that the role of the aspartate ion is to stabilise the imidazole-tetrahedral intermediate (Kollman and Hayes 1981; Umeyama et al. 1981). The hydrogen bond

between the imidazole ring and the aspartate residue plays a role only during the tetrahedral transition state (Jordan and Polgar 1981). The His residue moves during catalysis, forming a hydrogen bond with Ser during formation of the tetrahedral intermediate, then shifting position to form a hydrogen bond with Asp assisting formation of the acyl enzyme (Bachovchin 1986).

1.2.3.1.2 Role of the oxyanion hole

The tetrahedral intermediate is further stabilised by formation of hydrogen bonds between the negative oxyanion of the substrate and two hydrogen atoms in the protein (Robertus et al. 1972). This 'oxyanion hole' in the protein plays an important role in the specificity of the catalytic site, as has been shown by studies comparing binding of substrates and their enantiomers (DeTar 1981). Serine proteases differ from the catalytically similar cysteine proteases in this way as the oxyanion hole does not appear to be essential in the latter (Asboth et al. 1985).

Mutational analysis also places great importance on the structure around the active site especially the oxyanion hole, to hold the peptide substrate in a conformation favourable to hydrolysis (Corey and Craik 1992).

1.2.3.2 Catalytic Triad

The catalytic triad is arranged differently within the sequence of the three serine protease families, chymotrypsin has His···Asp···Ser, subtilisin has Asp···His···Ser while prolyl oligopeptidase has Ser···Asp···His.

1.2.3.2.1 Identification of the catalytic triad in PO

Using the porcine enzyme, the active site serine residue was identified by the use of radiolabelled diisopropylfluorophosphate [3H]DFP, a potent serine protease inhibitor

which binds to the active serine residue (Rennex et al. 1991). The His residue of the active site was identified in a similar manner. His 680 was alkylated following treatment with the radiolabled chloromethane [^{3}H]acetyl-Ala-Ala-Pro-CH_2Cl a competitive inhibitor of PO (Stone et al. 1991).

The final residue of the catalytic triad to be identified was the aspartate residue. Asp529 and Asp 642 of the porcine enzyme were put forward as candidates due to their conserved presence throughout the prolyl oligopeptidase family (Rawlings et al. 1991). The latter of these two was presumed correct following identification of a striking homology between the catalytic triad and surrounding sequence between lipases, especially the microbial lipases, and prolyl oligopeptidase family enzymes (Polgar 1992a).

Active site residues in human PO are Ser554 and His680 (Vanhoof et al. 1994), as identified by sequence homology within the PO family, and Asp641, as confirmed by structural modelling of the catalytic domain (Goossens et al. 1995). Crystal structure of the human PO has revealed the presence of the active site residues exposed on loop or bend structures within a large hydrophobic cavity. Ser, in particular, is held in an energetically unfavourable conformation. This results in exposure of the OH group to both the substrate and the His residue imidazole ring, which in turn is located in the same plane as the Asp oxygen atom thereby favouring hydrogen bond formation (Fulop et al. 1998).

1.2.3.2.2 A non-catalytic cysteine is present at the active site

The presence of a cysteine residue near, but not in, the catalytic site has been identified by studies of PO purified from porcine muscle in the presence of both bulky and small thiol reagents. The latter results in a much smaller inhibition suggesting a steric hindrance effect rather than direct interaction with the active site residues

(Polgar 1991). Szeltner et al. identified this cysteine residue as Cys255 on the fourth blade of the β-propeller domain. Substitution by threonine or alanine at this position ablated the inhibition by thiol-reagents, interestingly it also inactivated the high pH form of the enzyme suggesting a role in catalysis (Szeltner et al. 2000a). The presence of a cysteine residue close to the active site has been reported in other members of the prolyl oligopeptidase family (Medrano et al. 1998).

1.2.3.3 pH Dependence

PO may exist in two pH dependent catalytically competent forms which interconvert around pH6.1-6.9, as is demonstrated by a sigmoidal distribution of pH dependence using purified PO from porcine muscle (Polgar 1991).

1.2.3.3.1 Two pH dependent forms of PO

Acid/base catalysis is the rate determining step only for the apparent low pH form thereby implying a conformational change is rate-limiting for the higher pH form. Polgar put forward the following equation for the pH dependence;

EH(ImH)XH	↔	**EH(Im)XH**	↔	**EH(Im)X**	↔	**E(Im)X**
EH3		**EH2**		**EH**		**E**
(inactive)		**(active)**		**(active)**		**(active)**

Where E= Enzyme, Im=Imidazole ring of Histidine and X=Additional enzymic group.

When the imidazole ring is protonated at a low pH the enzyme is inactive as the imidazole ring is unable to accept a proton from the catalytic serine (Polgar 1991).

Substrates with varying side chains may show pronounced preferences for the high or low pH forms. Positively charged substrates are hydrolysed much more effectively by the high pH form which, presumably has a negatively charged active site. Negatively charged substrates are generally less favourable and show a preference for the low pH form of the enzyme (Polgar 1992b).

Studies using a thiono-substituted (reactive oxygen atom replaced by a sulphur atom) substrate showing slow binding to PO suggest that the rate limiting conformational change is substrate dependent rather than a change in the free enzyme (Polgar et al. 1993).

1.2.3.4 Ionic Effect

There is also a clear ionic effect on PO activity. In the presence of 0.5M NaCl PO activity is enhanced with a much more significant increase observed at pH8 than pH6 (Polgar 1991). However, a NaCl concentration above 1-2M results in a significant decrease in bacterial or mammalian PO activity (Polgar 1991; Kabashima et al. 1998). A compound, usually a salt, able to cause a protein to precipitate out of solution may be defined as a salting out agent. NaCl can act as a weak salting out agent and can also stabilise a protein structure by resulting in preferential hydration of the protein and preferential exclusion of NaCl from the water shell formed around the protein (Arakawa and Timasheff 1982). The pH dependence of PO unfolding, inactivation and urea denaturation in the presence and absence of NaCl demonstrate that NaCl destabilises PO, suggesting that NaCl is able to interrupt the water shell around PO and bind sites on the protein (Polgar 1995). This is contradictory to earlier observations that the linker peptide between PO's two functional domains was protected from trypsin in the presence of salt (Polgar and Patthy 1992), unless the slightly unfolded protein becomes a less favourable substrate.

PO has an isoelectric point of pH4.8-4.9 (Wilk 1983), therefore, the low pH form will have a charge close to 0 while the high pH form has a highly negative charge. This may explain the increased effects of salt destabilisation at increased pH. This also suggests that the looser structure is more catalytically active, following observation that reaction rates, particularly for the high pH form, are increased in the presence of NaCl (Polgar 1991).

It has been suggested that PO may be regulated by the intracellular redox state; PO activity in Murine erythroleukemia cells (MEL cells) is inactivated by treatment with the oxidising agents menadione or diamide, both of these treatments result in elevated intracellular glutathione disulfide (GSSG) and decreased glutathione (GSH) levels (Tsukahara et al. 1990). PO activity was also decreased by the addition of GSSG to cell lysates, but only following heat treatment, this inhibition was prevented by the addition of GSH but activity could not be rescued by GSH once lost. Another suggestion to the redox effect on PO activity is that PO is denatured by a high accumulation of GSSG.

1.2.3.5 Substrate Binding

The X-Ray structure of porcine PO in complex with the specific inhibitor Z-Pro-prolinal has identified the residues involved in the specificity of substrate binding by this enzyme (Fulop et al. 1998).

1.2.3.5.1 Binding to the proline residue

Proline binding is mediated by a hydrophobic pocket formed by residues Trp595, Phe476, Val644, Val580 and Tyr599 which also results in ring stacking of the proline residue and indole ring of the Trp residue (Figure 1-2). The oxyanion binding site, provided by the backbone amide groups of two residues in other serine proteases is in

fact provided by one backbone amide and the OH group of a Tyr residue in PO (Fulop et al. 1998).

In contrast to chymotrypsin and subtilisin mechanisms of substrate binding, the orientation of the proline residue means that hydrogen bond formation between the P1 amide (the amide group of the residue forming the tetrahedral intermediate) and the S1 carbonyl oxygen of the enzyme is impossible. By replacing the Gly residue of the effective substrate Z-Gly-Pro-Nap with the corresponding thiono substrate it appears that prolyl oligopeptidase instead relies on a P2S2 interaction between the neighbouring residue to proline and the enzyme as this substitution ablates all observed activity (Polgar et al. 1993).

1.2.3.5.2 Binding of adjacent residues

Interactions with the rest of the peptide chain are also important. Unlike other serine endopeptidases PO shows a preference for shorter peptide substrates and is unable to cleave further than 3-4 residues from the end of a peptide, suggesting limited access to its binding site (Polgar 1992b).

The crystal structure revealed; hydrogen bonding between the enzyme and the residue before proline (P2), a non-polar environment preferring a hydrophobic residue before that in the peptide (P3) and a cysteine residue close to the active site, as was previously predicted, (Fulop et al. 1998). Studies using a Tyr473Phe enzyme variant suggest the role of the OH group in substrate binding is very much dependant on the substrate and ionic conditions. It also plays an important role in stabilization of the transition state (Szeltner et al. 2000b).

Complexes of an S554A inactive variant of the porcine PO with an octapeptide substrate determined substrate binding relies on residues P3-P2' only, with residues outside this region remaining unbound by the enzyme. It also identified formation of a

catalytically important intramolecular hydrogen bond between the P1'(NH) and P2(OH) residues, as well as hydrogen bonding between the P2' residue and His680 (Nε2) during decomposition of the transition state which, facilitates proton transfer to the P1' residue (Fulop et al. 2001). Despite the majority of substrate-enzyme interactions being between the substrate backbone groups, the charge of the non-binding side-chains can affect the rate of substrate binding and de-acylation by affecting the electrostatic environment at the active site (Szeltner et al. 2003).

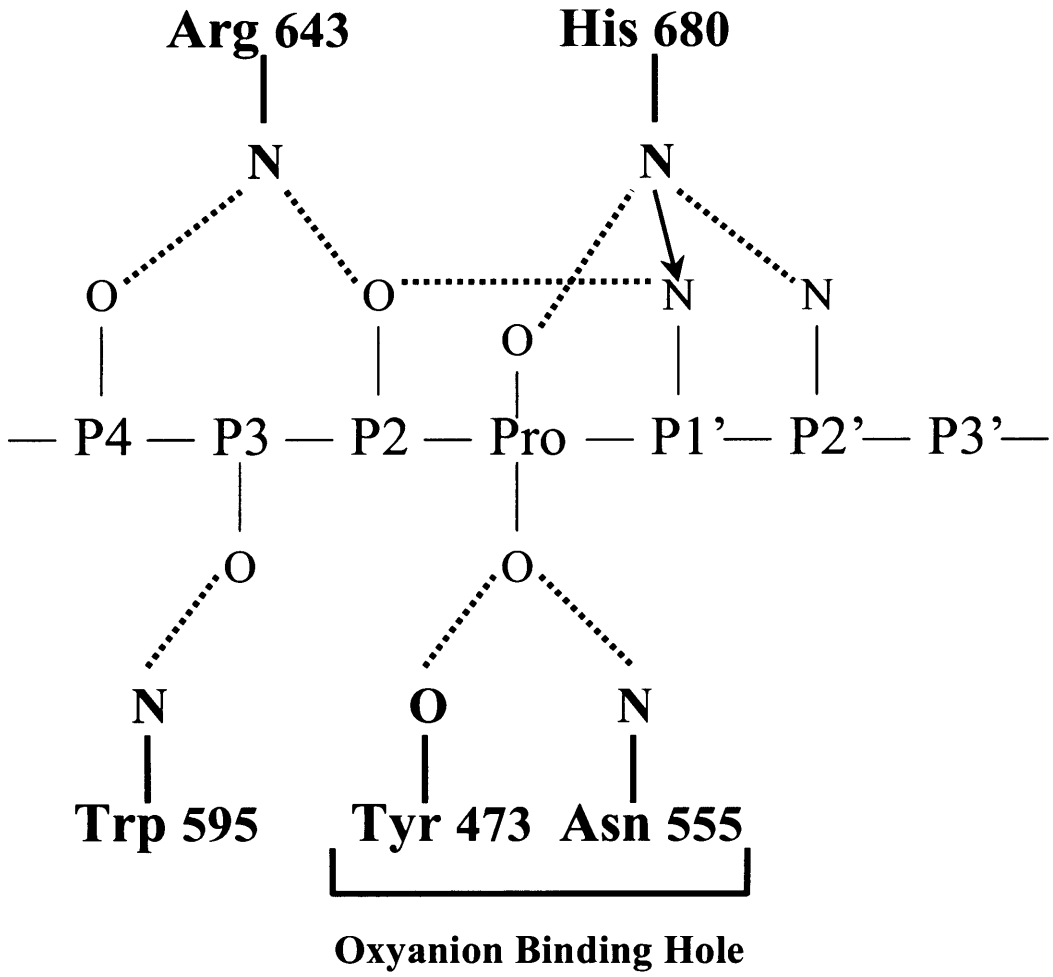

Figure 1-3 Hydrogen Bonds Formed Between PO and Bound Substrate

During substrate binding hydrogen bonds are formed between the highlighted residues of PO and the peptide substrate as well as between individual residues within the peptide. The dashed lines indicate hydrogen bonds and the solid line with arrow indicates proton transfer. Residues of PO and their position are indicated in bold while peptide residues and their functional groups are drawn in regular type.

1.2.3.5.3 Synthetic PO substrates

Several different synthetic substrates have been used to monitor PO activity in vitro. Studies of these various substrates have identified the importance of the substrate composition on enzyme activity as well as the variable effect of individual residues on activity depending on the strength of the bond to be cleaved (Szeltner et al. 2002).

The preferred substrate of PO is Yaa-Pro-Xaa where Yaa is not an N-blocked peptide or an additional Proline residue and Xaa is hydrophobic rather than basic or acidic. Synthetic fluorimetric and colourimetric substrates based on this structure are available for use in assaying specific activity of PO. Studies using the two fluorogenic substrates, Z-Gly-Pro-AMC and Suc-Gly-Pro-AMC revealed that while Z-Gly-Pro-AMC followed regular enzyme kinetics, Suc-Gly-Pro-AMC was able to bind non-specifically to the S1' position resulting in substrate inhibition, making this an unreliable substrate for studying the kinetics of this enzyme (Venalainen et al. 2002).

PO may also cleave some peptides after an alanine residue. PO purified from bovine lens was able to cleave the carboxyl residue after an alanine residue depending on the nature of the surrounding amino acids, though it retained a strong preference for cleavage following proline residues (Sharma and Ortwerth 1994).

1.2.3.6 Inhibitors

A large majority of the PO inhibitors, both specific and non-specific, identified are based around modifications of the proline amino acid and peptide bond that form the natural substrate for PO. For a summary of PO inhibitors identified and developed to date, see Table 1-1.

1.2.3.6.1 Synthetic inhibitors

PO inhibitors have been synthesized in the form of peptidyl acetals (Augustyns et al. 1995), aminodicarboxylic acid pyrrolidides (Demuth et al. 1993), acyl-prolyl-pyrrolidines (Wallen et al. 2002b) and dicarboxylic acid bis(prolyl-pyrrolidine) amides (Wallen et al. 2002a; Wallen et al. 2003).

The majority of these inhibitors are designed to mimic the proline residue, peptide bond and adjacent amino acid structure. Binding to the enzyme in the S1, S2 and S3 positions requires specific structures. Subtle changes or substitutions of different moieties of the inhibitor structure can significantly alter enzyme inhibition. For example, Venalainen et al. reported increased K_i by up to two orders of magnitude by adding a CHO, CN or $COCH_2OH$ functional group at the P1 position of the inhibitor isophthalic acid bis(L-prolyl-pyrrolidine) (Venalainen et al. 2004).

Replacement of the P1 pyrrolidine with a thiazolidine moiety retains inhibitory potency of the compound however replacement with larger heterocycles results in a loss of inhibition (Portevin et al. 1996). Acylisoxazole and acylisoxazoline groups at this position resulted in an inhibitor specific to the *T.cruzi* parasitic enzyme with very low toxicity towards mammalian cells (Bal et al. 2003).

It is also possible to replace the proline moiety of these inhibitors with a similar structure, cyclopent-2-enecarbonyl, without causing a significant decrease in inhibition. Replacing proline in this way may increase the bioavailability of the inhibitor for use in vivo (Jarho et al. 2004). The group present at the P3 position also affects the inhibition constant given for the compound. Studies of different functional groups at this position and extending away from the proline moiety have revealed two potential binding modes depending on the hydrophobicity of the compound. It is probable that such a flexibility aids binding to multiple, varied substrates (Jarho et al.

2005). This ability to bind a number of varied structures at the P3 end of the inhibitor has enabled recent development of a fluorescent PO inhibitor, containing a fluorescein moiety linked to the inhibitor at the P3 position, which may have several applications in studying the role of PO *in vivo* (Venalainen et al. 2005).

1.2.3.6.2 Natural inhibitors

Inhibitory substances have been isolated from sources as diverse as the mushroom *Polyozellus multiplex* (Song and Raskin 2002), the alpine plant *Rhodiola sachalinensis* (Fan et al. 2001), green tea (Kim et al. 2001) and various red and white wines (Yanai et al. 2003). A number of substances isolated from microbial sources (several of which also act as antibiotics) have been found to inhibit PO including; Staurosporine, isolated from *Streptomyces staurosporeus* (Kimura et al. 1990), Poststatin isolated from *Streptomyces viridochromogenes* (Aoyagi et al. 1991), Eurystatins A and B isolated from *Streptomyces eurythermus* (Toda et al. 1992), Lipohexin isolated from the funghi *Moezia lindtneri* and *Paecilomyces* (Christner et al. 1997) and Propeptin isolated from the mycelium of *Microbispora* (Kimura et al. 1997).

1.2.3.6.3 Endogenous inhibitors

Serine proteases are regulated in vivo by their own natural protein inhibitors. Studies of these protease inhibitor complexes have revealed approximately 20 structural families of inhibitors, varying from small proteins up to proteins of 500 amino acids representing a wide variety of structures (Krowarsch et al. 2003). An endogenous inhibitor of PO has been reported to be present in mammals; Yoshimoto et al. (1982) identified and partially purified a 6.5KDa protein from porcine pancreas that acted as a specific competitive inhibitor of prolyl oligopeptidase. This protein was found to be

widely distributed in both rat and porcine organs. More recently Salers has reported that the inhibitor present in the pancreas; co-localises with PO in the cytosol of the cell, is absent from secretory vesicles and, importantly, is not susceptible to PO degradation (Salers 1994). The two polyamines, spermine and spermidine, act to reverse PO inhibition possibly by formation of a complex including the endogenous inhibitor (Soeda et al. 1986).

1.2.3.6.4 PO inhibitors as drugs

The potential role of PO in the pathophysiology of many disorders has resulted in a large number of studies into the development and identification of PO inhibitors as potential drugs. The first synthetic inhibitor of PO to be reported was Z-Pro-Prolinal. Several compounds consisting of a modified form of Z-Pro-Prolinal have since been synthesized, for example; Z-Pyr-prolinal, Z-Val-prolinal and Z-Gly-Pro-CH_2Cl (Cunningham and O'Connor 1997) as well as ZTTA, which is similar to Z-Pro-prolinal but contains a sulphur atom in each of the proline rings (Shishido et al. 1996). The PO specific inhibitor Z-Pro-Prolinal (a transition state analog) forms a hemiacetal adduct with the active site (Kahyaoglu et al. 1997a).

JTP-4819 ((S)-2-[[(S)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N-(phenylmethyl)-1-pyrrolidinecarboxamide), developed as a PO inhibitor, also has a structure based around two proline residues (Toide et al. 1995a; Toide et al. 1997) and acts as a tight-binding competitive PO inhibitor (Venalainen et al. 2002). S17092 ((2S,3aS,7aS)-1([(R,R)-2-phenylcyclopropyl]carbonyl)-2-[(thiazolidin-3-yl)carbonyl]octahydro-1H-indole)) is a specific highly potent PO inhibitor with a K_i as low as 0.3nM for purified human PO. S17092 is also cell permeant in HEK293 cells inhibiting intracellular PO with a K_i of 30nM (Barelli et al. 1999). Clinical trials with this inhibitor have revealed rapid PO inhibition, maximum inhibition was observed between 30minutes and

widely distributed in both rat and porcine organs. More recently Salers has reported that the inhibitor present in the pancreas; co-localises with PO in the cytosol of the cell, is absent from secretory vesicles and, importantly, is not susceptible to PO degradation (Salers 1994). The two polyamines, spermine and spermidine, act to reverse PO inhibition possibly by formation of a complex including the endogenous inhibitor (Soeda et al. 1986).

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2hours after treatment and lasted for at least 12hours. The half life of S17092 *in vivo* varied between 7 and 31hours (Morain et al. 2000).

Other specific inhibitors studied include; Z-321 (1-[3-(2-indanylacetyl)-L-thiopropyl]pyrrolidine) (Miura et al. 1997) and ONO-1603 ((S)-1-[N-(4-chlorobenzyl)succinamoyl]pyrrolidine-2-carbaldehyde) (Katsube et al. 1999). A number of these inhibitors have entered in vivo studies looking at potential therapeutic benefits, these are discussed in section 1.2.5.3.

Table 1-1 Summary of Prolyl Oligopeptidase Inhibitors

Inhibitor	Source	Organism	K_i/*IC50	Reference
Specific Inhibitors				
Z-Pro-Prolinal	**Synthetic**	**Rabbit brain**	**14nM**	**(Wilk 1983)**
ZTTA	**Synthetic**	**Rat brain**	**2.9μM**	**(Shishido et al. 1996)**
JTP-4819	**Synthetic**	**Rat brain**	**0.83nM***	**(Toide et al. 1995b)**
		F.meningosepticum	**5.43nM***	**(Toide et al. 1995b)**
Z-321	**Synthetic**	**Canine brain**	**0.5nM***	**(Tanaka et al. 1994)**
S17092	**Synthetic**	**Human brain**	**1nM**	**(Barelli et al. 1999)**
		Rat brain	**1.3nM***	**(Portevin et al. 1996)**
SUAM-1221	**Synthetic**	**Bovine brain**	**190nM***	**(Portevin et al. 1996)**
ONO-1603	**Synthetic**	**Rat brain**	**12nM**	**(Katsube et al. 1996)**
Val-Glu-Ile-Pro-Glu	**Wine**	***F.meningosepticum***	**17.0μM***	**(Yanai et al. 2003)**
Tyr-Pro-Ile-Pro-Phe	**Wine**	***F.meningosepticum***	**87.8μM***	**(Yanai et al. 2003)**
N-[N-benzyloxycarbonyl-(S)-prolyl]-(S)-prolinal dimethylacetal	**Synthetic**	**Human blood**	**120nM***	**(Augustyns et al. 1995)**
BOC-Glu(NH-O-Ac) pyrrolidide	**Synthetic**	**Human placenta**	**28nM**	**(Demuth et al. 1993)**
Ac-Ala-Ala-Pro-CH_2Cl	**Synthetic**	**Porcine muscle**	**0.55μM**	**(Stone et al. 1991)**
Lipohexin	***M.lindtneri***	**Human placenta**	**3.5mM***	**(Christner et al. 1997)**
		F.meningosepticum	**25mM***	**(Christner et al. 1997)**
Non-specific Inhibitors				
Oleic acid	**Synthetic**	***F.meningosepticum***	**26.7μM**	**(Park et al. 2006)**
Linoleic acid	**Synthetic**	***F.meningosepticum***	**51.0μM**	**(Park et al. 2006)**
Arachidonic acid	**Synthetic**	***F.meningosepticum***	**91.3μM**	**(Park et al. 2006)**
Eicosapentaenoic acid	**Synthetic**	***F.meningosepticum***	**247.5μM**	**(Park et al. 2006)**
Docosahexaenoic acid	**Synthetic**	***F.meningosepticum***	**89.0μM**	**(Park et al. 2006)**
Prostatin	**Microbispora**	***F.meningosepticum***	**0.70mM**	**(Kimura et al. 1997)**
Serine Protease Inhibitors				
DFP	**Synthetic**	**Lamb kidney**		**(Yoshimoto et al. 1977)**
		T.cruzi	**60nM***	**(Joyeau et al. 2000)**
PMSF				
Cysteine Protease Inhibitors				
PCMB	**Synthetic**	**Porcine muscle**		**(Polgar 1991)**
Non-competitive Inhibitors				
Epigallocatechin gallate	**Green tea**		**142nM***	**(Kim et al. 2001)**
Epicatechin gallate	**Green tea**		**10.2μM***	**(Kim et al. 2001)**
Gallocatechin gallate	**Green tea**		**109nM***	**(Kim et al. 2001)**
Kynapsin-24	***P.multiflex***	***F.meningosepticum***	**1.14μM***	**(Song and Raskin 2002)**
1,2,3,6-tetra-O-galloyl-β-D-glucose	***R.sachaliensis***	***F.meningosepticum***	**15nM**	**(Fan et al. 2001)**
1,2,3,4,6-penta-O-galloyl-β-D-glucose	***R.sachaliensis***	***F.meningosepticum***	**0.15μM**	**(Fan et al. 2001)**
Rhodionin	***R.sachaliensis***	***F.meningosepticum***	**23μM**	**(Fan et al. 2001)**
Rhodiosin	***R.sachaliensis***	***F.meningosepticum***	**28μM**	**(Fan et al. 2001)**
3-O-galloylepigallocatchin-(4β-8)-epigallocatechin 3-O-gallate	***R.sachaliensis***	***F.meningosepticum***	**0.17μM**	**(Fan et al. 2001)**
Rosiridin	***R.sachaliensis***	***F.meningosepticum***	**56μM**	**(Fan et al. 2001)**
6-(8'Z-pentadecenyl)salicylic acid	***G.biloba***		**0.87mM**	**(Lee et al. 2004a)**
6-(10'Z-heptadecenyl)salicylic acid	***G.biloba***		**0.80mM**	**(Lee et al. 2004a)**
Staurosprorine	***S.staurosporeus***	***Flavobacterium***	**0.70μM**	**(Kimura et al. 1990)**
Poststatin	***S.viridochromogenes***	**Porcine kidney**	**55μM***	**(Aoyagi et al. 1991)**

1.2.4 Localisation And Distribution

1.2.4.1 Tissue Distribution

Initially identified in the uterus, PO was first cloned from the brain and is now thought to be ubiquitously expressed throughout mammalian tissues. Northern analysis revealed the presence of mRNA encoding PO in brain, heart, muscle, liver. kidney and ovarian tissues (Rennex et al. 1991). PO activity has also been reported in bone marrow (Cavasin et al. 2004) and macrophages (Green and Shaw 1983).

A number of reports have found PO expression or activity levels to vary between tissues or tissue regions. Elevated enzyme activity has been reported in muscle, testes, kidney, submandibular gland and the cerebral cortex (Kato et al. 1980). Venalainen et al. (2002) also reported the concentration of PO in the brain, determined in porcine brain homogenate, to be relatively high. A significant increase in PO activity in the brain compared to other tissues was also reported for both rat and teleost enzymes (Agirregoitia et al. 2005).

Identification of PO in the bovine lens revealed an intriguing distribution of maximal activity in the outer cortical region, decreased activity in the inner cortex and a complete lack of activity in the nuclear region. It was postulated that this decrease in activity may be due to an alteration in the redox state of the cells moving through the cortex, alternatively, PO may be important in clearing peptide fragments generated during the differentiation of epithelial cells to fiber cells (Sharma and Ortwerth 1994).

1.2.4.2 Intracellular Localisation

The subcellular localisation of PO is generally believed to be cytosolic, as has been confirmed in studies of a variety of tissues and species, e.g. rabbit brain (Dresdner et

al. 1982), human lymphocytes (Goossens et al. 1995), human glial cells (Schulz et al. 2005) and *D. discoideum* (Williams et al. 1999).

1.2.4.2.1 Secretion of PO

The majority of β-propeller domain containing proteins are secreted, however, there does not appear to be any evidence that PO is a secreted enzyme. Another example of a non-secreted β-propeller protein is the G-protein β-subunit (Baker et al. 1997).

There are some exceptions, bacterial POs discovered in *Flavobacterium meningosepticum* and *Sphingomonas capsulata* are targeted to the periplasm by a signal sequence that may later be cleaved (Chevallier et al. 1992; Kabashima et al. 1998). Another bacterial PO was identified in a screen for outer membrane proteins of *Flavobacterium columnare* G_4 and found to have a potential transmembrane region (Xie et al. 2004). A secreted or extracellular PO has also been reported in the parasite *Trypanosoma cruzi* (Bastos et al. 2005) and the fungi *Lyophyllum cinerascens* (Yoshimoto et al. 1988) and *Agaricus bisporus* (Sattar et al. 1990).

Another conundrum is the presence of PO activity in the serum. As PO is not thought to be secreted PO activity is expected to be absent from the serum, however this is not the case. In the study of rat and teleost tissues PO activity was reported in the serum but at 30-100 fold less than that observed in the other tissues (Agirregoitia et al. 2005). Significant levels of PO activity in serum or blood plasma have also been reported in a number of studies reporting altered PO levels in a number of disease states (Maes et al. 1995; Maes et al. 1998; Maes et al. 1999b; Maes et al. 1999a; Maes et al. 2001).

If it is true that PO is not secreted one explanation for this activity could be enzyme release by cell lysis in the blood. Another possibility is that these studies have in fact been measuring activity of the enzyme ZIP, which shows a similar specificity to PO.

The latter option has not been ruled out by inhibitor studies in any of the mentioned reports, resistance to the PO specific inhibitor Z-Pro-prolinal is the accepted method of ZIP identification (Birney and O'Connor 2001). Observations of serum activity in the presence of Z-Pro-prolinal revealed a 40% decrease implying ZIP activity accounts for 60% of the PO activity observed in the serum (Breen et al. 2004). It is of note that no ZIP activity was reported in human lymphocyte extracts and the combined serum activity was 4-8 fold less than that of the cell extracts. In addition Cunningham and O'Connor have reported the purification of an enzyme from bovine serum which is biochemically similar to PO, sensitive to Z-Pro-prolinal as well as several other PO specific inhibitors, yet has a larger molecular weight and lower specificity towards a number of biological peptides than the intracellular enzyme (Cunningham and O'Connor 1998).

1.2.4.2.2 Membrane bound form of PO

In eukaryotic cells PO is a cytosolic enzyme, however, a membrane-associated form has been reported in both Bovine brain (O'Leary et al. 1996) and Ovine hypothalamus (Lew et al. 1994). In the latter the membrane-associated activity was significantly less than that observed in the cytoplasm and was diminished by sequential washing of the membrane fraction suggesting an association with membrane proteins rather than integration into the membrane. The membrane associated form reported in bovine brain however accounted for a much more significant proportion of the cellular PO activity (~30%) and showed localisation to the synaptosomal membrane fragment. Other studies have observed between 10 and 30% PO activity associated with the particulate fraction rather than the soluble fraction (Dresdner et al. 1982; Agirregoitia et al. 2005).

1.2.4.2.3 Other intracellular localisations

Neither a membrane bound nor secreted form of PO has been reported in human cells and there is currently only one human PO encoding sequence present in the NCBI database. Millennium Pharmaceuticals recently applied for patents on two sequences claimed to encode novel human prolyl oligopeptidases (Patent No.s 21163 and 21953), however, sequence alignments revealed these two potential proteins lack both the PO conserved domain and β-propeller domain (data not shown).

Schulz et al. have reported localisation of PO in human glial and neuronal cell lines, as well as rat primary glial and neuronal cells, to the perinuclear region and a limited co-localisation to the microtubules, which breaks down towards the cell periphery. They have identified a PO-interacting C-terminal fragment of α-tubulin which also includes the microtubulin-associated proteins (MAPs) binding site (Schulz et al. 2005). In addition PO has also been observed inside the nucleus of both insect cells and Swiss 3T3 cells (Ohtsuki et al. 1997; Ishino et al. 1998).

1.2.5 Role of Prolyl Oligopeptidase

1.2.5.1 Degradation/regulation of bioactive peptides

Prolyl Oligopeptidase is able to cleave a number of neuroactive and bioactive peptides. However, the physiological role of this ability is somewhat questionable due to the cytosolic location of PO and the mainly extracellular location of the peptides cleaved. Despite reports of PO activity in serum there is no evidence of PO secretion or extracellular localisation within tissues. The potential substrates for PO include; Neurotensin (Checler et al. 1986b), Angiotensin I and II (Chappell et al. 1990), Arginine-Vasopressin (AVP) (Miura et al. 1995), Oxytocin (Walter et al. 1971), Bradykinin (Oliveira et al. 1976), Substance P and Thyrotropin-Releasing Hormone

(TRH) (Wilk 1983). Cleavage of these peptides would result in a role for PO in a number of cellular functions.

There is some evidence in support of a physiological role for PO in bioactive peptide regulation. Treatment of rats with a selection of PO inhibitors resulted in decreased levels of the anti-fibrotic peptide Ac-SDKP, this peptide is produced in vitro by PO hydrolysis of thymosin-β (Cavasin et al. 2004). Oral doses of PO inhibitor also resulted in decreased AVP stimulated protein synthesis in rat brain slices (Shishido et al. 1999b). It is of note that a membrane-bound form of PO identified in the synaptosomal membrane fraction of bovine brain homogenates shows higher affinity for neuropeptides angiotensin II, LHRH, Neurotensin, Bradykinin and TRH than its tissue counterpart (O'Leary et al. 1996). However the question remains as to how or when this intracellular, largely cytosolic enzyme would come into contact with these secreted peptides.

PO activity has also been related to a number of inflammatory disorders. Elevated expression of PO has been seen in atopic dermatitis, a chronic relapsing inflammatory skin disease, using real-time pcr (Seo et al. 2006). Altered PO levels have been linked to arthritis (Kamori et al. 1991), systemic lupus erythematosus (Aoyagi et al. 1985; Aoyagi et al. 1987) and autoimmune and inflammatory responses (Shoji et al. 1989). In addition lowered PO levels also show correlation with increased interleukin-6 and 10 (IL-6 and IL-10), interferon-γ (INF-γ), interleukin-1 receptor antagonist (IL-1RA) and granulocyte-macrophage colony stimulatory factor (GM-CSF) (Maes et al. 1999b). A study of the proteinases present in inflammatory foci of, *Mycobacterium tuberculosis* induced, delayed-type allergic-inflammation in mice revealed significant amounts of PO and cathepsin B compared to a number of other proteases (Kakegawa et al. 2004). Though this case raises the question of the origin of the enzyme activity,

several bacteria secrete PO during infection thus it is possible that this enzyme activity may be secreted from the *M.tuberculosis* rather than the host (this is discussed further below).

Conversely, PO is currently emerging as a potential treatment for Celiac sprue, an immune disease involving an inflammatory response to ingested wheat gluten. Treatment with recombinant PO greatly reduces the presence of immunostimulatory peptides in wheat gluten already treated with pepsin and a number of pancreatic peptides (Marti et al. 2005). Bacterial PO enzymes able to function at extreme pH and therefore work effectively in stomach acid are presently being investigated as potential oral supplements for sufferers of this disease (Stepniak et al. 2006).

Many of the bioactive fragments generated by PO *in vitro* may be produced *in vivo* by a number of different enzymes. A candidate for such a role would be the neurotensin-degrading peptidase, a metallopeptidase isolated from rat brain synaptic membrane, able to cleave neurotensin at the carboxyl side of the proline residue (Checler et al. 1986a). Also human PCP (or Angiotensinase C) is able to cleave a C-terminal residue following a proline residue so is therefore able to cleave the Pro-Phe bond releasing angiotensin-(1-7) providing angiotensin I has first been cleaved by a deamidase enzyme (Tan et al. 1993). This action may be hard to detect as PCP may also be inhibited by the specific PO inhibitor Z-Pro-prolinal. In addition to this, Chappell et al. reported that Z-Pro-prolinal treatment only inhibited angiotensin-(1-7) formation by 30% implying another enzyme was also involved (Chappell et al. 1990). Endopeptidases other than PO are able to cleave bradykinin in the brain and other tissues (Camargo et al. 1973; Oliveira et al. 1976; Camargo et al. 1979).

PO may not target these peptides under physiological conditions; a number of studies have found no change in TRH or LHRH degradation in rat brain homogenates, slices

or in vivo following treatment with Z-Pro-prolinal. This suggests another enzyme is responsible for degradation of these two peptides in the brain (Friedman and Wilk 1986; Charli et al. 1987; Mendez et al. 1990).

1.2.5.2 General Proteolysis.

A number of peptidases involved in the extracellular degradation and regulation of neuropeptides and hormones are also found in the cytosol of the cell. These enzymes may play a role in regulation of intracellular peptides, which in turn regulate specific protein interactions playing a part in numerous cellular processes including; cell signalling, protein targeting and metabolism (Ferro et al. 2004).

Rates of intracellular proteinase activity are altered following cell ageing or during differentiation. PO activity was found to decrease in murine erythroleukemia cells (MEL cells) following DMSO induced differentiation, a decrease was not seen in DMSO resistant cells (Tsukahara et al. 1991). Another decrease in PO activity has been reported following heat shock treatment of HeLa cells. This was a result of enzyme inhibition/inactivation. No loss of protein was observed and activity was not diminished following heat treatments of cellular extracts (Pratt et al. 1989)

Tsukahara et al. also reported decreased PO activity in MEL cells following treatment with the oxidising reagents menadione or diamide or heat shock at 45°C for 10minutes. This inactivation was independent of other protease action or synthesis of new proteins or polypeptides (Tsukahara et al. 1990).

1.2.5.3 Neuroprotection

Ischemia induced neuronal cell death in rats can be significantly reduced by treatment with the neuropeptides vasopressin-(4-9) and TRH, the same effect is achieved by treatment with Z-Pro-prolinal. The neuroprotective properties of the PO inhibitor or

vasopressin treatment are both lost in the presence of a vasopressin receptor agonist, implying the protective effect of Z-Pro-prolinal is mediated by preventing degradation of the neuroactive peptides (Shishido et al. 1999a).

A neuroprotective role has also been reported for the PO inhibitors ONO-1603 and THA(Schulze et al. 1993; Katsube et al. 1999). These inhibitors delay age-induced apoptosis in cultured central nervous system (CNS) neurons. The mechanism remains unclear though may involve a decrease in expression of glyceraldehyde-3-phosphate dehydrogenase (a glycolytic enzyme that binds specifically to the carboxy terminal of the APP).

Specific PO inhibitors were reported to reverse scopolamine induced amnesia in rats (Yoshimoto et al. 1987). The PO specific inhibitor JTP-4819 is able to prevent degradation of neuropeptides by purified PO *in vitro*. Treatment of rats with this inhibitor at ineffective dose was also able to reverse scopolamine induced amnesia when accompanied with substance P, AVP or TRH (Toide et al. 1995a). Another study showed an improvement in the impaired spatial memory of aged rats following administration of a PO inhibitor (JTP-4819). This study also reported an improved central cholinergic function, possibly due to; increased acetylcholine release from nerve terminals, normalized choline uptake and choline acetyltransferase (ChAT) activity (Toide et al. 1997). JTP-4819 may also increase various neuropeptide levels in both the cerebral cortex and hippocampus via PO inhibition (Toide et al. 1995a; Shinoda et al. 1999). A similar neuroprotective effect is seen with ONO-1603, which prevents neurodegeneration at low potassium concentrations, upregulates m3-muscarinic acetylcholine receptor (mAChR) mRNA in cerebellar granule cells (CBCs) and increases mAChR mediated phosphoinositide hydrolysis (Katsube et al. 1996).

Miura and colleagues reported an improvement in the potentiation of synaptic transmission following treatment with a PO inhibitor (Z-321). They also confirmed the role of PO inhibition in this effect by the use of a stereoisomer (D-Z-321) with a negligible effect on PO (Miura et al. 1997). Another PO inhibitor, S17092, has entered clinical trials as an effective treatment for amnesia and other cognitive disorders associated with ageing (Morain et al. 2002). Treatment of healthy volunteers with S17092 showed an improvement in verbal memory tests with no significant adverse effects (Morain et al. 2000). Following acute S17092 treatment of rats, PO levels in the rat brain were significantly decreased and increased levels of the two memory enhancing neuropeptides Substance P (SP) and α-melanocyte-stimulating hormone (α-MSH) were seen in the frontal cortex and hypothalamus. However, following chronic treatment for 8 days the increase in SP and α-MSH was no longer seen despite continued PO inhibition (Bellemere et al. 2003).

1.2.5.4 Inositol Signalling

Inositol(1,4,5)trisphosphate (IP_3) is a cellular second messenger involved in a number of cell signalling pathways. IP_3 is generated, along with diacylglycerol (DAG), by phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and by dephosphorylation of higher order inositol polyphosphates (mainly IP_5 and IP_4) by a, potentially Ca^{2+} sensitive, multiple inositol polyphospate phosphatase (MIPP) (Van Dijken et al. 1995; Van Dijken et al. 1997; van Haastert and van Dijken 1997). Further dephosphorylation of IP_3 recycles myo-inositol for the synthesis of phosphatidylinositol and inositol phosphates. Inositol signalling is discussed in detail later.

A *Dictyostelium* mutant lacking PO expression has a significant increase in IP_3, this mutant shows an increased rate of IP_5 breakdown to IP_3 as well as an increase in IP_3 breakdown. A similar increase in IP_3 was observed following Z-Pro-prolinal treatment (Williams et al. 1999). Schulz et al. also observed increased IP_3 levels in U343 astroglioma cells following treatment with specific PO inhibitors or decreased PO expression by use of an antisense vector (Schulz et al. 2002).

Treatment of neural growth cones with lithium, valproic acid or carbamazepine, three mood stabilising drugs, results in growth cone spreading. This effect is reversed by either addition of inositol or treatment with PO inhibitors, in agreement with the inverse correlation of PO activity with cellular IP_3 levels (Williams et al. 2002).

1.2.5.5 Mood Disorders

A number of studies have reported altered inositol levels in a variety of mood disorders. However, inositol signalling in the cell is regulated by a multitude of different enzymes as discussed later.

In addition to the role of PO in inositol signaling several naturally occurring peptide substrates of PO (see section 1.2.5.1) are neuropeptides that play a role in the pathophysiology of depression. In 1995 Maes and colleagues reported decreased levels of plasma PO activity in depressed patients while observing an increased plasma PO activity in both manic and schizophrenic patients. They also found that the plasma PO activity was significantly increased in depressed patients by treatment with the anti-depressant fluoxetine and decreased in manic patients by the use of valproate (Maes et al. 1995). An increase in serum PO levels has also been linked with both stress-induced anxiety and post-traumatic stress disorder (Maes et al. 1998; Maes et al. 1999a), while decreased levels of PO are observed in patients with anorexia and bulimia nervosa (Maes et al. 2001).

A decrease in serum PO activity has also been reported following Interferon-α treatment. IFN-α treatment is associated with neuropsychiatric side-effects and it is postulated that altered serum PO may play a role in these neuro-psychoses, however, a decrease in leukocyte, lymphocyte and platelet count was also reported-all of which are known to express PO (Van Gool et al. 2004). An alternative theory to account for neuropsychiatric effects of IFN-α treatment is an increased platelet monoamine oxidase-B activity, this enzyme oxidatively deaminates amines such as dopamine, noradrenaline and serotonin (Bannink et al. 2005).

1.2.5.6 Alzheimers Disease

PO activity has been related to β-amyloid plaque formation and Alzheimer's disease. Rossner et al. observed an increase in PO expression in the hippocampus of aged adult mice that was not observed in other regions of the brain. An increase in hippocampal PO activity was also observed in non-aged adult transgenic Tg2576 mice compared to non-transgenic littermates. Tg2576 mice are characterized by a 5-7 fold over expression of human Amyloid Precursor Protein APP695 carrying a K670N and M671L mutation (Swedish mutation), they develop β-amyloid plaques around 11 months. Both PO increase and hippocampus-dependant memory deficits precede the appearance of β-amyloid plaques (Rossner et al. 2005). Rossner observed that inhibition of PO resulted in a significant increase in secretion of β-amyloid peptides. Petit et al reported no degradation of synthetic β-amyloid peptide by purified human PO thus disputing any direct interaction between PO and Aβ (Petit et al. 2000). Johnston et al also reported no change in Aβ peptide levels following significant inhibition of PO (Johnston et al. 1999).

An alternative explanation for the effect of PO inhibition on β-amyloid secretion has been put forward by Schulz et al. (2005) who found PO to play a role in protein secretion. They observed co-localisation of PO with microtubules, involved in trafficking vesicles to the membrane. Decreased PO activity following addition of inhibitor or in an anti-sense cell line resulted in a significant increase in overall protein secretion. This explanation is also disputed by Petit et al. who observed no change in Aβ secretion from HEK293 cells over expressing wild type or Swedish mutated APP in the presence of PO inhibitors (Petit et al. 2000).

1.2.5.7 Infection

A secreted PO from *T.cruzi* is able to degrade the host extracellular matrix proteins, collagen types I and IV and fibronectin (Santana et al. 1997), and has been found to be essential for parasite entry into mammalian host cells. This parasite has elevated levels of PO during the infective trypomastigote phase of its life cycle compared to the non-infective epimastigote stage, treatment with a PO inhibitor was able to impede entry to host cells (Grellier et al. 2001; Bastos et al. 2005).

In both *F.meningosepticum* and *T.denticola* PO activity is localised to the periplasm. This localisation along with the ability of the bacterial PO to hydrolyse several human bioactive peptides led to suggestion of a role in exploitation of the host's bioactive peptides for use by the bacteria or interference with normal bioactive peptide levels, e.g. those involved in inflammation, to create favourable conditions for the microorganism (Makinen et al. 1994). PO has been found in the non-pathogenic bacteria *Sphingomonas capsulata* (Kabashima et al. 1998) as well as a number of pathogenic bacteria; *F.columnare, F.meningosepticum, Aeromonas hydrophila* and *Xanthomonas sp.* (Yoshimoto et al. 1991; Szwajcer-Dey et al. 1992; Kanatani et al.

1993; Xie et al. 2004) suggesting bacterial PO has cellular functions in addition to a potential role in infection.

1.2.6 DpoA; *Dictyostelium* PO homologue

A *Dictyostelium* homologue of the human prolyl oligopeptidase enzyme was identified in a lithium resistance screen (Williams et al. 1999). At high Li^+ concentrations *Dictyostelium* aggregation and spore formation is severely retarded. In carrying out this screen Williams screened for aggregation in 10mM LiCl, and isolated a total of 13 Li^+ resistant mutants. One of these mutants, lithium suppressor A (LisA), was discovered to lack the PO homologue, given the name *dpoA*.

Williams et al (1999) went on to show that DpoA activity is not directly affected by Li^+ nor does it interact with GSKA (the *Dictyostelium* homologue to GSK-3). However, loss of DpoA leads to a significant (3-fold) increase in cellular IP_3; an effect also observed following addition of PO inhibitors. The increase in IP_3 appears to be due to an increase of IP_5 conversion to IP_3. It is possible that a loss of DpoA results in removal of PO inhibition of MIPP, the Multiple Inositol Polyphosphate Phosphatase, which catalyzes this reaction. PO is also an inhibitor of IP_3 degradation. Only one PO homologue has been found in the *Dictyostelium* genome, *dpoA*, and its disruption eliminates all activity. No secreted PO activity is observed (Williams et al. 1999).

1.3 Lithium

Lithium, an alkali metal, is a monovalent ion (Li^+). It has been used to treat mood disorders, in particular Bipolar Disorder (previously known as manic depression) since the 1860s (Williams and Harwood 2000). Since then it has experienced periods of unpopularity due to potentially fatal side effects and a lack of information on the therapeutic mechanism of action. Despite this it is currently one of the most common treatments for bipolar disorder (Heit and Nemeroff 1998; NIMH 2001; Jope 2003). Our understanding of the molecular mechanisms of these disorders and the mechanism by which lithium acts to alleviate these symptoms is still very limited.

1.3.1 Bipolar Disorder

Depression affects 121 million people worldwide and is thought to be responsible for close to 1 million deaths by suicide every year (World Health Organisation figures). Bipolar disorder (BD) is thought to account for 10-20% of all cases of depression and affects 1% of the American population over 18years of age (NIMH 2001). BD is characterised by cycling between periods of depression and periods of mania often with periods of normal behaviour in between. Cycling between these extremes may occur over a time span of weeks to a single day depending on the disease progression and how well it is controlled by mood stabilising drugs (NIMH 2001). A clear genetic component to BD has been identified, a number of potential associations have been described though no genes have been definitively linked to the disorder yet. It is also clear that a genetic susceptibility to BD will not necessarily result in manifestation of the disorder and an environmental trigger is often associated with the onset of symptoms (Hayden and Nurnberger 2006).

1.3.2 Other Mood Stabilisers

Lithium is the most common treatment for Bipolar Disorder, also popular are the two antiepileptics valproic acid (VPA) and carbamazapine (CBZ). Possible treatments for BD involve a number of mood stabilising drugs including atypical antipsychotics (e.g. clozapine, olanzapine, and risperidone) and antidepressants (e.g. imipramine and fluoxetine), which are often given in combination with one another to achieve the desired therapeutic effect. Lithium in particular has a very small window of therapeutic use and must be closely monitored to find an effective non-toxic dose (Young and Newham 2006). A number of negative effects such as increased mania and rapid cycling between mania and depression have been associated with antidepressant treatment (Sachs and Thase 2000).

The majority of mood stabilising drugs have several unwanted side effects including weight gain, lack of co-ordination, tremor, hypothyroidism, dry mouth and anxiety (NIMH 2001; Freeman and Freeman 2006), the most significant of which is that many, including VPA and CBZ, are highly teratogenic and therefore are undesireable during pregnancy. A number are also recommended to be avoided during breastfeeding (Burt and Rasgon 2004). It is hoped that identification of common mechanisms of action of multiple drugs will help identify common therapeutic targets.

1.3.2.1 Potential Common Therapeutic Mechanism

Mood stabilising drugs have a multitude of effects on cells which can be assigned to interference in a number of signalling pathways, the majority of these are unique to each drug. The ability of VPA to decrease the number of axons may be ascribed to its histone deacetylase (HDAC) inhibitory action, while the ability of Li^+ to increase the

number of axons and affect microtubules may be ascribed to its inhibition of GSK3 (Williams et al. 2002). Neither VPA or CBZ inhibit GSK3 activity in primary cultured neurons (Ryves et al. 2005). However, previous studies report that, while unable to directly inhibit GSK3 *in vitro* VPA may indirectly inhibit GSK3 in some neuronal cell lines (Chen et al. 1999; Hall et al. 2002). Both Li^+ and VPA are able to promote neural survival in specific cell lines, due to interplay with the ERK/MAPK signalling pathway, however this is not seen with CBZ treatment (Di Daniel et al. 2005).

A study looking at the effect of Li^+, VPA and CBZ on dorsal root ganglia neurons from rats has revealed that while the three drugs had differing effects on axon branching and microtubule organisation all three resulted in enlarged growth cones. This effect was reversed by addition of inositol, identifying inositol signalling as a potential therapeutic target in the treatment of mood disorders (Williams et al. 2002). The potential role of inositol phosphates in mood disorders is supported by a number of neurological studies showing altered IP levels in the brain (Shimon et al. 1998; Beacher et al. 2005). It is important to understand IP signalling in the context of mood disorders. PO has been identified in a Li^+ screen for proteins involved in inositol signalling and has also been associated with a number of mood disorders (see above). Growing interest in the inositol signalling pathways has revealed a complex and highly regulated network of inositol and phosphatidylinositol phosphorylation and dephosphorylation. It will be interesting to understand the role of PO in the inositol phosphate signalling network.

1.4 Inositol Signalling

1.4.1 Basic Signalling Pathways

Inositol $C_6H_6(OH)_6$ is a six carbon ring which can be phosphorylated at single or multiple positions by a number of cellular enzymes to produce a number of biologically active molecules. The position and number of phosphates around the inositol ring plays an important role in its function and is controlled by a number of enzymes with varying specificities. Naming of inositol phosphates indicates the position and number of bound phosphate groups (Figure 1-4).

It has been suggested that inositol-3-phosphate (I3P) was the first inositide to evolve, produced through the conversion of glucose-6-phosphate and converted in turn to inositol and eventually phosphatidylinositol (Irvine 2005). Prokaryotes may contain inositol monophosphate and phosphatidylinositol however no polyphosphate inositols have been identified. The eukaryotes have a much more complex inositide biochemistry, we are currently aware of seven polyphosphoinositol lipids and over thirty inositol phosphates with an ever-expanding list of cellular functions.

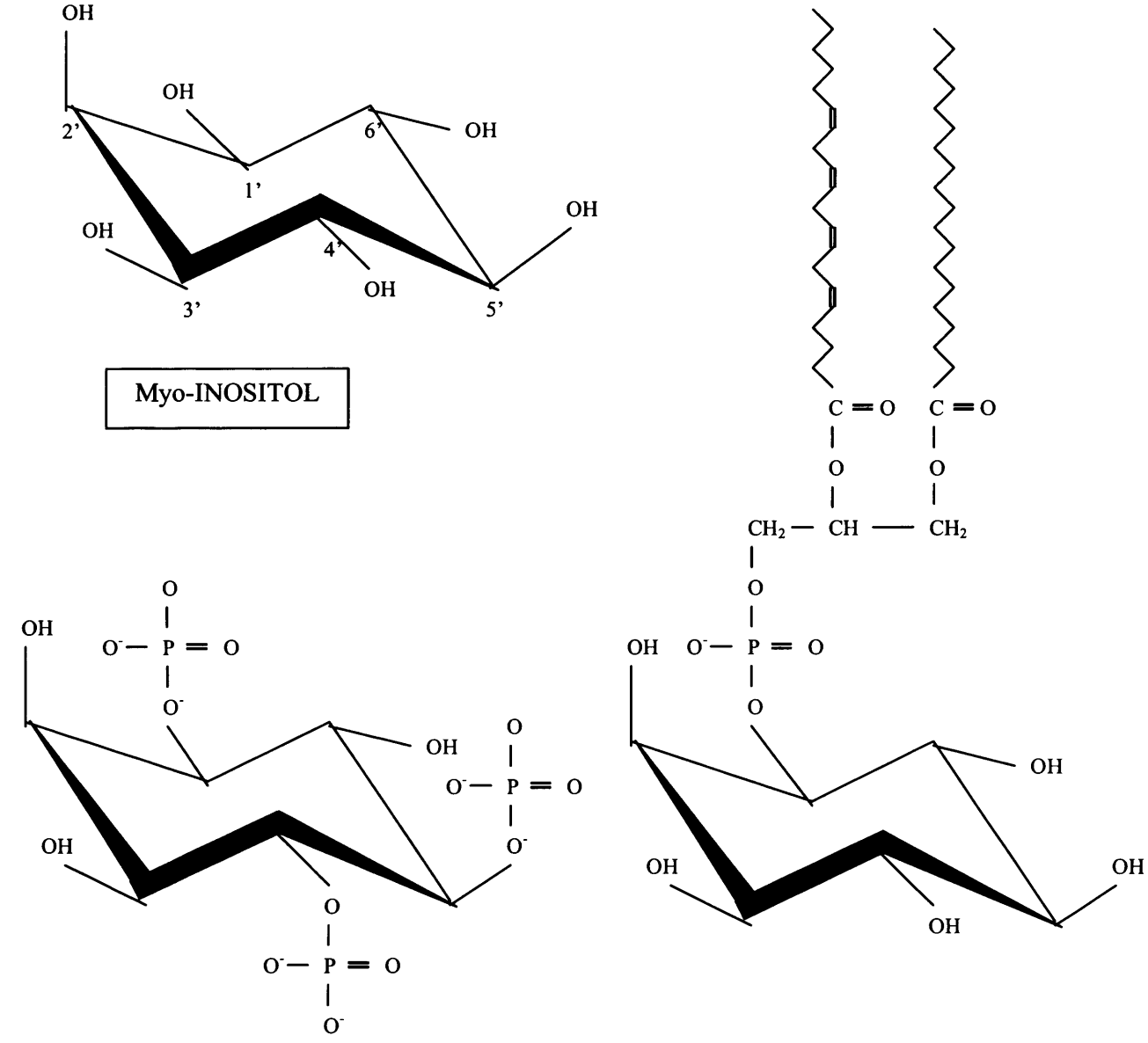


Figure 1-4 Structure and Numbering of Myo-Inositol, Inositol (1,4,5)-Trisphosphate and Phosphatidylinositol

Myo-inositol represents the inositol form generally present in biological systems. Numbering of carbon atoms in the inositol carbon ring indicates the position of attachment of phosphate groups.

1.4.1.1 PIP_2 Is Hydrolysed To IP_3 And DAG

1.4.1.1.1 IP_3 release

IP_3 is rapidly released from phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$) following activation by an external stimulus e.g 5-hydroxytryptamine (5-HT) or carbachol. The rapid increase in IP_3 and IP_2 observed in [^{3}H]inositol labelled blowfly salivary glands following 5-HT treatment, along with a corresponding decrease in PIP_2 and to a lesser extent phosphatidylinositol 4-phosphate (PI(4)P) but not phosphatidylinositol (PI), provided the first evidence that this stimulus causes hydrolysis of PIP_2 rather than PIP or PI (Berridge 1983; Berridge et al. 1983). Phospholipase C (PLC) catalyses the hydrolysis of $PI(4,5)P_2$ to produce DAG and IP_3 both of which act as second messengers in cell signalling (Berridge and Irvine 1984; Nishizuka 1988).

1.4.1.1.2 Roles of IP_3 and DAG

IP_3 is an important cellular second messenger. We first began to understand the importance of this molecule when Berridge proposed the role of IP_3 as a second messenger in calcium signalling, following observation of increased Ca^{2+} release from intracellular stores when permeabilised pancreas cells were treated with IP_3 (Streb et al. 1983). Following this report the same effect was observed in a number of different cells and tissues (reviewed in (Berridge and Irvine 1984)) and the IP_3 receptor and its Ca^{2+} channel activity has been identified and characterised (Mikoshiba 1993). The role of IP_3 as a second messenger has been identified in a number of cellular processes.

DAG activates the conventional and novel but not the atypical subtypes of protein kinase C an enzyme involved in a multitude of signalling pathways; regulating cell survival, development, migration and gene expression (for reviews see Tan and Parker 2003; Guo et al. 2004; Oka and Kikkawa 2005; Martelli et al. 2006).

1.4.1.1.3 Replenishing PIP_2 levels

The PIP_2 hydrolysed in response to receptor activation is thought to represent a distinct pool of PI from the total cellular PI (Fain and Berridge 1979; Monaco and Woods 1983). DAG is recycled to replenish $PI(4,5)P_2$ at the plasma membrane via phosphatidic acid (PA) and cytidine monophosphate-phosphatidic acid (CMP.PA) which recombines with inositol to form PI. PI undergoes sequential phosphorylation to PI(4)P then $PI(4,5)P_2$. PI is phosphorylated by type II PI kinase to produce PI(4)P (Chang and Ballou 1967) and type I PI kinase producing PI(3)P, the latter representing just a small fraction (1-12%) of intracellular PIP (Whitman et al. 1988).

1.4.1.2 Inositol To IP_3

Intracellular inositol levels are maintained via three distinct pathways; intracellular transport, de novo synthesis from glucose and recycling of inositol phosphates.

1.4.1.2.1 Intracellular inositol transport

Inositol is pumped into the cell by two transporters, which operate under different conditions. The Na^+/*myo*-inositol cotransporters (SMIT1 and 2) are expressed in a number of tissues including kidney, brain (neuronal and non-neuronal cells) and placenta. They play an essential role in maintenance of intracellular inositol levels under normal and stress conditions (Kwon et al. 1992; Inoue et al. 1996; Guo et al. 1997; Coady et al. 2002; Berry et al. 2003). The H^+/*myo*-inositol symporter is

expressed mainly in neurons in the brain. It is present on intracellular vesicles and appears to be transiently localised to the plasma membrane at growth cones and synapses, especially the postsynaptic region, during times of upregulated inositol phosphate signalling, PKC activation and cell depolarisation enabling sustained phosphatidylinositol synthesis (Uldry et al. 2001; Uldry et al. 2004).

1.4.1.2.2 Do novo inositol synthesis

Inositol is produced in the cell by dephosphorylation of inositol monophosphate (IP) to inositol, catalysed by inositol monophosphatase (IMPase). IMPase is a homodimeric enzyme which requires Mg^{2+} for activity and is inhibited in an uncompetitive manner by Li^{+} with a K_i between 0.2 and 1mM depending on the IP isomer being dephosphorylated (Gee et al. 1988). IP is generated in the cell from two sources representing the second and third pathways of inositol production. One pathway involves de-novo synthesis of IP from glucose-6-phosphate. Glucose-6-phosphate is circularised by inositol phosphate synthase to form inositol 1-phosphate (I(1)P) (Culbertson et al. 1976).

1.4.1.2.3 Inositol recycling

Finally, inositol is also recycled from the second messenger $I(1,4,5)P_3$ and the other IP_3 present in the cell $I(1,3,4)P_3$ (see below). Inositol polyphosphate-5-phosphatases type I and II (IP_3/IP_45P) are responsible for dephosphorylation of $I(1,4,5)P_3$ to $I(1,4)P_2$, type I IP_3/IP_45P is both cytosolic and membrane associated while the type II IP_3/IP_45P shows cytosolic and mitochondrial localisation (Mitchell et al. 1989; Laxminarayan et al. 1993; Laxminarayan et al. 1994; Speed et al. 1995; Communi et al. 2001). Both enzymes require Mg^{2+} ions for activity and are inhibited by D-2,3-bisphosphoglycerate (Hansbro et al. 1994). Dephosphorylation of IP_3 to IP_2 occurs

relatively rapidly (within seconds) following a stimulus induced increase in IP_3 (Berridge 1983; Berridge et al. 1983).

Further dephosphorylation to I(4)P is catalysed by inositol polyphosphate phosphatase (IPP), this enzyme also requires Mg^{2+} for its activity and is inhibited by the other divalent cations Ca^{2+} and Mn^{2+}. IPP activity is increased in the presence of monovalent sodium and potassium ions, however, lithium ions show uncompetitive inhibition of the enzyme with a K_i of approximately 0.75mM (Inhorn and Majerus 1987). IPP is also able to remove the 1-phosphate from $I(1,3,4)P_3$ to produce $I(3,4)P_2$, the affinity of IPP for $I(1,3,4)P_3$ is less than that for $I(1,4)P_2$ (K_M ~20μM compared to ~4μM) however both reactions show the same maximal activity. IPP is unable to dephosphorylate I(1)P, $I(1,3)P_2$, $I(1,4,5)P_3$ or $I(1,3,4,5)P_4$ (Inhorn and Majerus 1987). Another enzyme, inositol polyphosphate-4-phosphatase (IP4P), is required for removal of the 4-phosphate from $I(1,3,4)P_3$ and $I(3,4)P_2$ to produce $I(1,3)P_2$ and I(3)P respectively while subsequent dephosphorylation of the $I(1,3)P_2$ produced is catalysed by the inositol polyphosphate-3-phosphatase to form I(1)P. Both of these enzymes activities are Mg^{2+} independent and not inhibited by Li^+ ions (Bansal et al. 1987). All three IP isoforms (I(1)P, I(3)P, I(4)P) are dephosphorylated by the Li^+ sensitive, Mg^{2+} dependant enzyme IMPase (Ackermann et al. 1987; Atack et al. 1995)(Figure 1-5).

1.4.1.3 Higher Order Inositol Phosphates

IP_3 also undergoes further phosphorylation to generate a network of higher order inositol phosphates (Balla et al. 1989a). Following receptor stimulation resulting in elevated levels of IP_3 subsequent increases in the higher order inositol phosphates IP_4 and IP_5 are observed. Different isomers appear at varying intervals implying a complex pathway of phosphorylation/dephosphorylation reactions (Balla et al. 1989b).

1.4.1.3.1 IP_4, IP_5 and IP_6

Four possible isoforms of IP_4 have been identified in vivo, I(1,4,5,6)P_4, I(1,3,4,5)P_4, I(1,3,4,6)P_4 and I(3,4,5,6)P_4 (Balla et al. 1989a). Two of these, I(1,4,5,6)P_4 and I(1,3,4,5)P_4 are phosphorylation products of I(1,4,5)P_3 by Inositol Polyphosphate Multiple Kinase (IPMK) and Inositol(1,4,5)trisphosphate-3-kinase respectively (Takazawa et al. 1989; Takazawa et al. 1990; Nalaskowski et al. 2002). The latter two are produced in a seemingly more convoluted manner. I(1,3,4,6)P_4 is produced following dephosphorylation of I(1,3,4,5)P_4 at the 5-position by IP_3/$IP_4$5P to I(1,3,4)P_3 and subsequent phosphorylation of the 6-position by I(1,3,4)P_3 5/6-kinase/I(3,4,5,6)P_4 1-kinase (Balla et al. 1989a). I(3,4,5,6)P_4 is not produced from IP_3 but by dephosphorylation of I(1,3,4,5,6)P_5, this dephosphorylation and subsequent rephosphorylation at the 1'position are both catalysed by I(1,3,4)P_3 5/6-kinase/I(3,4,5,6)P_4 1-kinase (Yang and Shears 2000; Ho et al. 2002).

I(1,3,4,5,6)P_5 is also generated by IPMK action on I(1,4,5,6)P_4 and I(1,3,4,6)P_4 but not I(1,3,4,5)P_4, phosphorylation of I(1,4,5,6)P_4 can be reversed by the enzymes Phosphatase and Tensin Homologue (PTEN) or Multiple Inositol Polyphosphate Phosphatase (MIPP) (Craxton et al. 1995; Caffrey et al. 2001; Deleu et al. 2006). Metabolism of these higher order inositol phosphates may be controlled independently from receptor stimulated IP_3 production (Balla et al. 1989b; Pesesse et al. 2004; Choi et al. 2005).

1.4.1.3.2 Role of MIPP in inositol phosphate dephosphorylation

MIPP (Initially known as I(1,3,4,5)P_4 3-phosphatase), dephosphorylates IP_6, I(1,3,4,5,6)P_5 and I(1,3,4,5)P_4 at the 3' position. MIPP preferentially dephosphorylates IP_4 (V_{max} 4,250nmol/mg/min) over IP_5 (V_{max} 211nmol/mg/min) or

IP_6 (V_{max} 12nmol/mg/min). Both IP_5 and IP_6 act as competitive inhibitors for IP_4 metabolism (Hodgson and Shears 1990; Nogimori et al. 1991). MIPP is also able to remove the 6'phosphate from $I(1,4,5,6)P_4$ and any phosphate from IP_6 though is unable to hydrolyse any of the IP_3 or IP_2 isoforms (Craxton et al. 1997). This enzyme is located principally within the rough and smooth endoplasmic reticulum (ER) thus questioning its physiological role (Ali et al. 1993; Craxton et al. 1995). This argument is supported by a report showing truncation of MIPP to remove the ER localisation results in a significant increase in intracellular concentrations of $I(1,4,5)P_3$ and Ca^{2+} (Yu et al. 2003). Alternatively it has been reported that MIPP is both internally and externally localised to the plasma membrane in erythroblast and fibroblast cells respectively (Carpenter et al. 1989; Estrada-Garcia et al. 1991; Craxton et al. 1995). However, a role for the ER localised MIPP is evident as altered levels of IP_5 and IP_6 observed in fibroblasts from a MIPP knockout mouse, were reversed by introduction of MIPP to the ER (Chi et al. 2000).

1.4.1.3.3 Inositol pyrophosphates

IP_5 may be further phosphorylated via IP_6 and Diphosphoinositol pentaphosphate ([PP]-IP_5) to Bis-Diphosphoinositol tetrakisphosphate ($[PP]_2$-IP_4) by $I(1,3,4,5,6)P_5$ 2-kinase (IPK1), IP_6 kinase and [PP]-IP_5 kinase respectively (Voglmaier et al. 1996; Huang et al. 1998; Verbsky et al. 2002). Alternatively IP_5 may undergo slow phosphorylation by IP_6 kinase to Diphosphoinositol tetrakisphosphate ([PP]-IP_4) and subsequently bis-diphosphoinositol trisphosphate ($[PP]_2$-IP_3) (Saiardi et al. 2000). IP_6Kinase activity is mediated by three enzymes, IP_6K1, 2 and 3 all of which are enriched in the brain and perform a similar function, they do however, vary in their intracellular localisation. IP_6K1 is present throughout the nucleus and cytoplasm

while IP_6K2 shows a nuclear localisation and IP_6K3 is mainly cytosolic (Saiardi et al. 1999; Saiardi et al. 2001).

MIPP is also able to hydrolyse the 5-β-phosphate of [PP]-IP_5 or $[PP]_2$-IP_4, however, this reaction is not thought to occur *in vivo* (Shears et al. 1995; Craxton et al. 1997). Dephosphorylation of [PP]-IP_5, $[PP]_2$-IP_4 and [PP]-IP_4 *in vivo* is catalysed by Diphosphoinositol Polyphosphate Diphosphatase (DIPP), which removes the β-phosphate from these molecules (Safrany et al. 1998) whereas MIPP is responsible for dephosphorylation of IP_6 to IP_5. [PP]-IP_5 and $[PP]_2$-IP_4 undergo a sustained PLC independent metabolic turnover that may be inhibited by fluoride ions in both *Dictyostelium* and mammalian cells (Glennon and Shears 1993; Stephens et al. 1993).

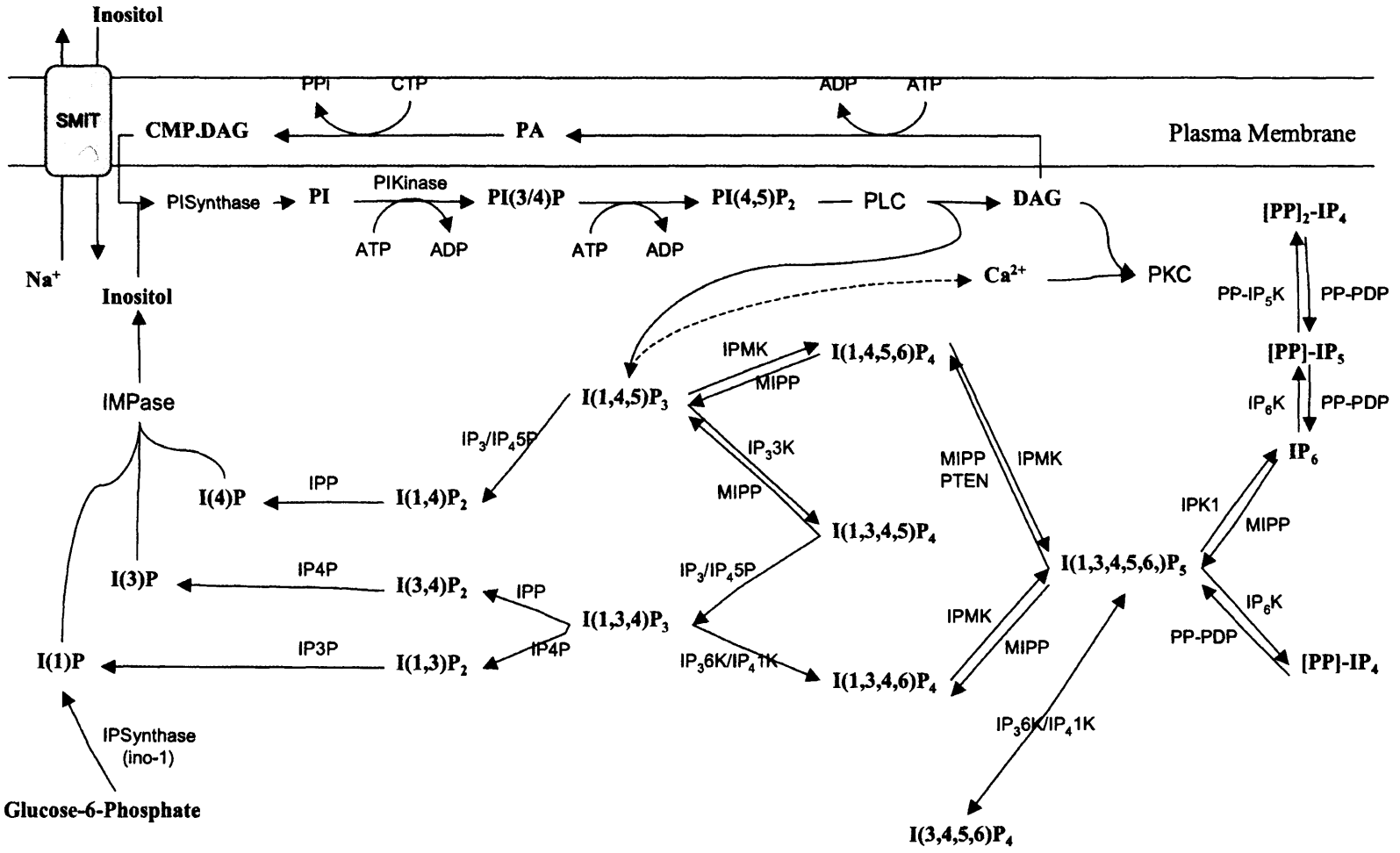


Figure 1-5. Inositol Signalling Pathways Observed Within the Cell.

Intracellular inositol is maintained by transport mediated import across the plasma membrane, de novo synthesis from glucose-6-phosphate and recycling of the second messenger IP_3. IP_3 levels are also affected by interaction with a complex network of higher order inositol phosphates. The entire inositol phosphate signalling network is tightly regulated by a number of specific enzymes. Abbreviations; PA, Phosphatidic acid, IP_3/IP_45P, $I(1,4,5)P_3/I(1,3,4,5)P_45$-phosphatase, IP_33K, $I(1,4,5)P_33$-kinase, IP_36K/IP_41K, $I(1,3,4)P_36$-kinase/$I(3,4,5,6)P_41$-kinase, PP-PDP, Diphosphoinositol-polyphosphate diphosphatase.

1.4.2 Cell Metabolism

1.4.2.1 Calcium Signalling

Undeniably the best known and most reported inositol phosphate cellular function is that of IP_3 mobilisation of Ca^{2+} from intracellular stores (Berridge and Irvine 1984). A study using Xenopus oocytes found IP_3 treatment of cells can also trigger the Ca^{2+} dependent oscillations in membrane potential. They suggest the presence in the cell of an IP_3 senstive and IP_3 in-sensitive pool of calcium with release of the IP_3 in-sensitive pool of calcium dependent on intracellular Ca^{2+} and responsible for the oscillations in membrane potential (Berridge 1988). As well as a central role in Ca^{2+} signalling IP_3 and the other cellular inositol phosphates, especially the higher order IPs, function in numerous and diverse pathways.

1.4.2.2 Neurological Functions

Alterations in inositol phosphate levels in the brain have been linked to a number of different neurological disorders including Bipolar Disease (Berridge et al. 1989), Schizophrenia (Shimon et al. 1998) and Down Syndrome (Berry et al. 1995; Beacher et al. 2005) implying an important role in the function and development of neurons. Both IP_5 and IP_6 are present in the mammalian brain, at different levels in the different brain regions. It is thought that these IPs may function as an extracellular signal as microinjection into a control region within the medulla oblongata of Sprague-Dawley rats resulted in a decrease in blood pressure and heart rate (Vallejo et al. 1987). Inositol phosphates also play a role in synaptic regulation and neuroplasticity (Nahorski et al. 2003).

1.4.2.3 Vesicle Trafficking

Several IPs have been linked to endocytosis and vesicle trafficking within the cell. Mice deficient in the polyphosphoinositide phosphatase synaptojanin 1 show increased levels of $PI(4,5)P_2$ and decreased synaptic vesicle recycling (Cremona et al. 1999). Both IP_4 and IP_6 disrupt interaction of visual arrestins with rhodopsin (Palczewski et al. 1991) and IP_6 binding sites identified on non-visual arrestins suggest it may sequester the arrestins in the cytoplasm preventing their receptor binding to form clathrin coated pits, essential for endocytosis (Milano et al. 2006). A similar role in cytosolic binding of the clathrin adaptor protein AP-2 has been seen (Gaidarov et al. 1996). IP_6 has also been linked to prevention of Cl^- channel desensitisation, a precursor to channel internalisation by endocytosis (Lee et al. 2004b).

1.4.2.4 Nuclear Roles

Inositol phosphates have been assigned several nuclear roles; the ratio of the higher order inositol phosphates affects chromatin remodelling and gene expression by regulating ATP dependant SWI/SNF chromatin remodelling complexes. IP_4 and IP_5 both act as positive regulators while IP_6 acts to negatively regulate and suppress gene expression. Expression of the *ino1* gene in yeast encoding inositol synthase is stimulated by increased IP_4/IP_5 and inhibited by increased IP_6 (Shen et al. 2003; Steger et al. 2003). Inositol phosphates also play a role in DNA stability. Increased levels of inositol pyrophosphates result in shortened telomeres caused by inhibition of the yeast PI3K related kinase (PIKK), Tel1 or an upstream activator, the reverse is also true with a decrease in inositol pyrophosphates causing lengthened telomeres (Saiardi et al. 2005; York et al. 2005).

A yeast screen for mutants showing mRNA nuclear export defects identified yeast homologues for PLC and two IP kinases responsible for IP_3 phosphorylation to IP_6. Studies of these mutants has revealed an essential role for IP_6 in efficient export of mRNA from the nucleus, probably by interaction with factors associated with the nuclear pore complex (York et al. 1999). The majority of IP effects are mediated by IP binding to specific proteins however, [PP]-IP_5 appears to act as a phosphate donor to a number of yeast and mammalian nucleolar proteins (Saiardi et al. 2004; York and Hunter 2004).

1.4.2.5 In Times Of Cell Stress

Along with general cellular functions, inositol phosphates also play a central role during cell differentiation and at times of cell stress. It has been suggested that inositol signalling may play a role in bone formation following a reported up-regulation of MIPP expression during rat chondrocyte hypertrophy, a similar phenomenon to that observed with the structurally similar histidine acid phosphatase in chick chondrocytes (Caffrey et al. 1999).

Levels of $[PP]_2$-IP_4 (IP_8) reportedly increase in response to heat stress and hyperosmotic stress in mammalian cells, though not observed in the yeast *S.cerevisiae* (Pesesse et al. 2004; Choi et al. 2005). Changes in the higher order inositol phosphates have also been related to cell death and apoptosis. Several reports have recorded an increase in IP_6K activity following exposure to apoptotic stimuli as well as a protective effect of IP_6K deletion against cell-death stimuli (Morrison et al. 2001; Nagata et al. 2005).

It is clear that inositol phosphate signalling is part of a complex network, which plays a role in numerous processes within the cell. It is to be expected that such a network must be highly regulated to enable normal cell function

1.4.3 Species Differences

1.4.3.1 Dictyostelium

Dictyostelium have similar inositol phosphate signalling networks to mammalian cells however there are several notable differences. The *Dictyostelium* enzyme equivalent to MIPP, MIPP A, is also able to dephosphorylate I(1,3,4,5,6)P_5 to I(1,4,5)P_3 via both I(1,4,5,6)P_4 and I(1,3,4,5)P_4 however, unlike its mammalian equivalent, the *Dictyostelium* enzyme is reportedly a peripheral membrane protein localised at the inner face of the plasma membrane rather than the endoplasmic reticulum (Van Dijken et al. 1997). The activity of MIPP A is Ca^{2+} sensitive unlike its mammalian counterpart (Van Dijken et al. 1997). However, this localisation and Ca^{2+} dependency is reminiscent of the situation reported in human erythrocytes (Doughney et al. 1988; Estrada-Garcia et al. 1991).

Dictyostelium cells contain much higher concentrations of higher order inositol phosphates than that observed in mammalian cells, in particular the intracellular concentration of IP_6 is estimated at 0.7mM in *Dictyostelium* compared to 5-15μM in mammalian cells (Martin et al. 1987; Szwergold et al. 1987). *Dictyostelium* are able to produce higher order inositol phosphates without PIP_2 hydrolysis by sequential phosphorylation of inositol (Stephens and Irvine 1990). This pathway may be of importance in the *Dictyostelium plc* null mutant, which is able to maintain IP_3 levels in the absence of any PLC activity (Drayer et al. 1994; Van Dijken et al. 1995).

Further analysis has revealed high concentrations of extracellular cAMP signal stimulate IP_5 dephosphorylation to I(1,4,5)P_3 by MIPP at the plasma membrane in a Ca^{2+} dependant manner (Van Dijken et al. 1997).

1.4.3.2 Plants And Yeast

Inositol signalling in plants and yeast remains closely related to that of mammalian organisms. *Arabidopsis* and brewers yeast show evidence of putative homologues to the majority of enzymes involved in inositol phosphate metabolism. Some noticeable differences have been reported however, including a distinct absence of the signalling molecule PIP_3 despite genes encoding putative PTEN protein whose preferred substrate is PIP_3. They also lack inositol-4-phosphatases and PI-3-kinases but show an abundance of inositol 5-phosphatases (reviewed in Mueller-Roeber and Pical 2002). Significantly elevated levels of IP_6 are observed in plant cells compared to mammalian cells, especially seeds, where it may act as a phosphate store.

1.4.4 Effect Of Li^+ And VPA On Inositol Signalling

In 1989 Berridge et al. put forward the inositol depletion hypothesis suggesting the pertinent therapeutic action of lithium in treatment of mood disorders was its depletion of the intracellular inositol pool by inhibition of both *de novo* synthesis and recycling of inositol (Berridge et al. 1989). A similar decrease in intracellular inositol has been observed following treatment of both rat brain and yeast with VPA (O'Donnell et al. 2000; Vaden et al. 2001).

It would now appear that this may be a common mechanism of therapeutic action for multiple mood stabilising drugs. The spreading effect of lithium, VPA and CBZ on growth cones of dorsal root ganglia neurons may be reversed by addition of either extracellular inositol or a PO specific inhibitor, which results in derepression of the IP_5 to IP_3 pathway (Williams et al. 2002). The inositol pathway effects are caused by interactions of the drugs at a number of steps in the inositol phosphate pathway.

Lithium affects inositol signaling by inhibition of both IMPase (inositol monophosphatase) (Berridge et al. 1982) and IPP (inositol polyphosphatase) (Inhorn and Majerus 1987). These enzymes catalyze the dephosphorylation of IP to inositol and IP_2 to IP respectively thus resulting in a decreased intracellular pool of inositol (Figure 1-6). VPA depletes intracellular inositol by inhibition of myo-inositol-1-phosphate synthase, the enzyme which catalyses the conversion of glucose-6-phosphate to inositol-1-phosphate in *de novo* inositol synthesis, in human brain homogenates (Shaltiel et al. 2004). This inhibition was not observed in vitro using the purified enzyme suggesting inhibition by VPA may be an indirect effect (Ju et al. 2004) (Figure 1-6).

The cellular interactions of CBZ that result in inositol depletion remain to be identified. However, CBZ as well as Li^+ and VPA treatment of astrocytes at therapeutic levels results in a decrease in the SMIT inositol transporter activity and mRNA resulting in decreased inositol uptake (Lubrich and van Calker 1999).

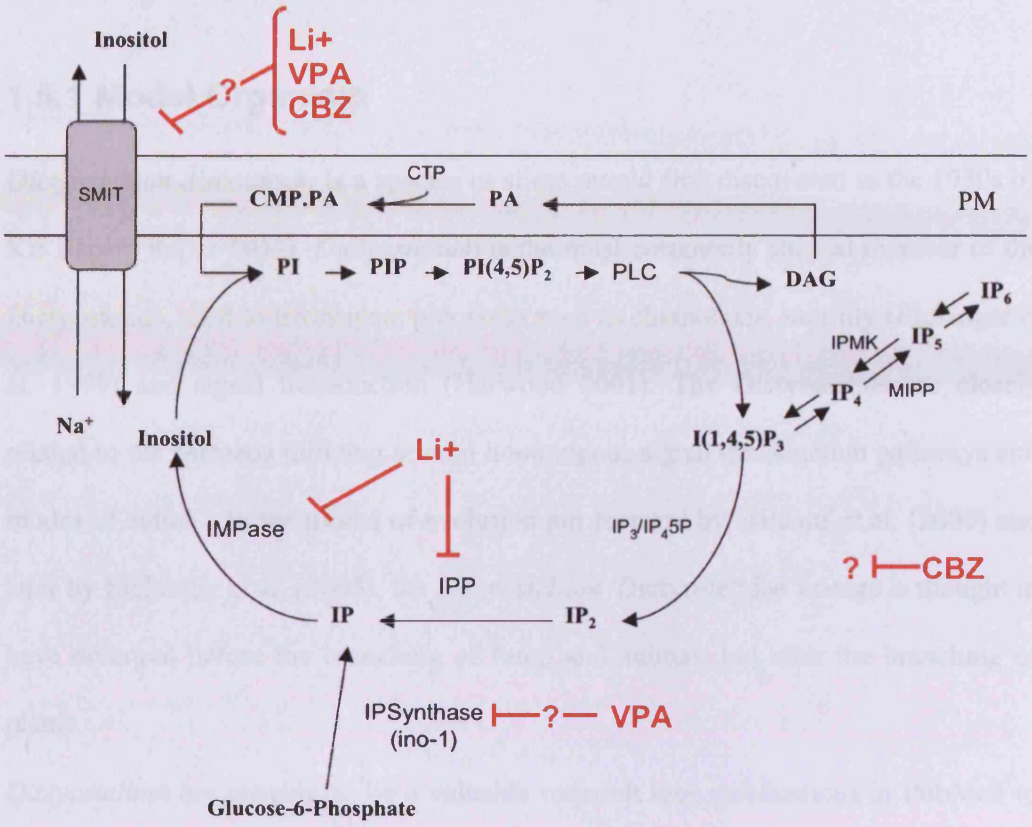


Figure 1-6 Drug Interactions with Inositol Phosphate Pathway

A simplified version of the inositol phosphate pathway showing points of inhibition by the mood stabilising drugs Li^+, VPA and CBZ.

1.5 Dictyostelium Discoideum

1.5.1 Model Organism

Dictyostelium discoideum is a species of slime mould first discovered in the 1930s by KB Raper (Raper 1935). *Dictyostelium* is the most commonly studied member of the Dictyostelids, used to investigate processes such as chemotaxis, motility (Eichinger et al. 1999) and signal transduction (Harwood 2001). The Dictyostelids are closely related to the Metazoa utilizing several homologous signal transduction pathways and modes of action. In the model of evolution put forward by Baldauf et al. (2000) and later by Eichinger et al. (2005), the *Dictyostelium* Dictyostelidae lineage is thought to have diverged before the branching of fungi and animals but after the branching of plants.

Dictyostelium are proving to be a valuable research tool, publications in PubMed to date number over 6,000 and are rapidly increasing. *Dictyostelium* is a highly tractable organism well suited to laboratory conditions and ideal for biochemical studies. It is also a good molecular genetics model for four main reasons; a haploid genome makes knockout models relatively straightforward, it is very amenable to transformation and overexpression studies, thirdly its complex life cycle allows separation of effects on growth and development and finally sequencing of the *Dictyostelium discoideum* genome has been completed providing a wealth of information (Eichinger et al. 2005).

Completion of the genome has enabled identification of a number of *Dictyostelium* genes homologous to human disease genes revealing *Dictyostelium* to have a higher number of human disease orthologues than observed in the yeast *S.cerevisae* and *S.pombe*, though fewer than *D.melanogastor* or *C.elegans*. The *Dictyostelium* genome

is approximately 34Mb in size which is just over one hundredth the size of the human genome or one sixth that of *D.melanogastor.* In this small space it fits half as many genes as the human genome equalling the number observed in *D.melanogastor* (Eichinger et al. 2005).

The *Dictyostelium* life cycle of growth, aggregation and differentiation into a multicellular organism enables observation of distinct aggregation or developmental phenotypes following gene knockouts, protein over-expression, drug treatments or varying growth conditions. It also makes possible large scale screening of mutants for distinctive phenotypes. The signal transduction pathways involved in this process in many cases are homologous to those of mammalian systems including GSK signaling (via the *Dictyostelium* GSK-3 homologue GSKA) (Harwood et al. 1995) and inositol signalling as well as signalling via a number of G-proteins.

1.5.2 Amoeboid Cell Growth

Dictyostelium is a social amoeba whose natural habitat is the soil of forest floors. *Dictyostelium* grow and divide as single celled amoeba feeding on bacteria, the strains generally used for research (Ax2, 3 or 4) are also able to grow on plates or in suspension in nutrient rich media (Axenic Media) in the laboratory.

During amoeboid cell growth cells undergo growth and division independently of one another. Cells are able to sense and move towards bacteria by detecting folate levels using a G-protein coupled cell surface receptor. The folate receptor signal is transduced by the Gα4 subunit (*Dictyostelium* contain 11 G-protein α-subunits but just one β and one γ subunit) (Hadwiger et al. 1994). Folate signalling through the same receptor also acts to repress expression of the developmental marker *discoidin1* and other early developmental genes (Blusch and Nellen 1994; Salger and Wetterauer 2000).

Growing cells also secrete and sense a 68kDa glycoprotein called Pre-Starvation Factor (PSF). This factor builds up in the environment allowing cells to detect the cell density of their surrounding environment (Clarke et al. 1988). When PSF levels reach a threshold cell growth is arrested and cells begin to express *carA* and *acaA*, both of these are developmental genes encoding cAR-1, extracellular cAMP receptor 1, and adenylyl cyclase A (ACA), the enzyme which synthesizes cAMP, respectively (Soderbom and Loomis 1998).

The response to PSF is mediated by a protein kinase Yak-A, expression of the gene *yakA* increases throughout cell growth reaching a peak at the start of starvation in response to build up of extracellular factors. Yak-A activity is responsible for suppression of growth related proteins such as the cysteine protease CprD and expression of developmental genes *acaA*, *carA* and *pka-C* encoding the active subunit of PKA (Souza et al. 1998). YakA induced expression of *acaA* and *carA* is dependent on PKA activity (Soderbom and Loomis 1998).

Thus growth arrest and entry to starvation phase may be initiated by a decrease in folate and increase in PSF levels in the extracellular environment.

1.5.3 Starvation And Aggregation

The most striking feature of *Dictyostelium* is their ability to survive starvation by developing into a multicellular fruiting body by aggregation of single amoeboid cells. This aggregation is stimulated by waves of cAMP that induce the amoeboid cells to migrate towards a central point forming a mound of approximately $1X10^5$ cells.

1.5.3.1 cAMP Signalling

As cells enter starvation both cAMP synthesis (ACA) and detection (cAR-1) are upregulated however, build up of another glycoprotein, conditioned medium factor

(CMF), is also required to facilitate cAMP signalling (Mehdy and Firtel 1985). Secretion of this factor begins as cells enter starvation and builds up in the presence of several starving cells, thus allowing cells to wait for a sufficiently large population of cells in starvation before beginning aggregation, avoiding formation of small ineffective fruiting bodies (Gomer et al. 1991). CMF facilitates cAMP signalling by inhibition of Gα2-GTP hydrolysis by a mechanism involving receptor mediated activation of Gα1, release of Gβγ and subsequent activation of phospholipase C (Brazill et al. 1998).

The process of aggregation is mediated by a cAMP gradient set up by a single cell at the centre of aggregation and relayed by surrounding cells. Single cells are able to sense the pulse of cAMP and move towards it as well as releasing cAMP themselves to signal to subsequent cells. The pertinent receptor in this process is the cAMP receptor cAR1; extracellular cAMP is detected at the plasma membrane by cAR1, a G-protein coupled 7-transmembrane receptor (Klein et al. 1988; Saxe et al. 1988). *Dictyostelium* have four such receptors (cAR1-4), which are expressed at different stages throughout aggregation and development (Kim et al. 1998). cAR1 is the first cAMP receptor to be expressed and is expressed throughout aggregation.

Waves of cAMP emanating outwards from the centre of aggregation are maintained by a self-regulating pathway of cAMP sensing, synthesis and degradation resulting in pulses of cAMP. cAR1 is coupled to the Gα2 subunit (Kumagai et al. 1991), cAMP binding results in release of Gα2-GTP and Gβγ. This leads to activation of ACA and synthesis of intracellular cAMP (Wu et al. 1995), in addition the MAP Kinase, ERK2, is also activated by cAMP binding to cAR1 (Knetsch et al. 1996). One of the actions of ERK2 is to inhibit the cytoplasmic phosphodiesterase RegA. RegA degrades cAMP, thus this facilitates a build up of intracellular cAMP. Increased levels of

intracellular cAMP result in activation of the cAMP-dependent protein kinase PKA. This enzyme, responsible for inducing expression of early developmental genes, completes a feedback loop by inhibiting both ACA and ERK, repressing cAMP synthesis and removing inhibition of its breakdown (Laub and Loomis 1998; Maeda et al. 2004).

1.5.3.2 Chemotaxis

cAR1 signalling also brings about a number of other downstream effects including, actin polymerisation, cyclic GMP accumulation and activation of PLC the enzyme responsible for PIP_2 hydrolysis to IP_3 and DAG. All of these may play an essential role in chemotactic movement (McRobbie and Newell 1984; Europe-Finner and Newell 1987).

Dictyostelium are able to sense a gradient of just 2% difference between the front and back of the cell and move towards the source of the cAMP. cAR1 is distributed evenly around the cell, not localised to the leading edge (Xiao et al. 1997), instead polarisation and directional sensing is mediated by recruitment of the two PI3K homologues PI3K1 and PI3K2 to the leading edge in response to cAMP signalling (Funamoto et al. 2001).

PIP_2 is phosphorylated to PIP_3 (phosphatidylinositol (3,4,5)-trisphosphate) by PI3K, the reverse reaction resulting in dephosphorylation to PIP_2 is controlled by the PTEN (phosphatase and tensin homologue) enzyme. Recruitment of PI3K to the leading edge results in an increase in PIP_3 in this region resulting in recruitment of PH domain proteins including; CRAC, an activator of adenylyl cyclase, PKB, a mammalian protein kinase B homologue and PhdA, pleckstrin homology domain containing protein A (Parent et al. 1998; Funamoto et al. 2001). Both PKB and PhdA are essential for normal chemotaxis to occur (Meili et al. 1999). PhdA is thought to be an

adaptor protein that plays a role in F-actin localisation. Pseudopod formation at the leading edge also requires activity of the Arp2/3 protein complex responsible for actin polymerisation and branching. Arp 2/3 is controlled by activity of the proteins SCAR and WASP (Machesky and Insall 1998), whose activity at the leading edge is controlled by the Rho GTPase Rac1b (Chung et al. 2000).

In addition pseudopod formation at the sides and back of the cell is repressed by the continued presence of PTEN and an absence of PI3K leading to increased PIP_2 and a lack of PIP_3, as well as a cGMP induced regulation of myosin II filaments (Bosgraaf et al. 2002).

1.5.3.2.1 Role of inositol signalling

PLC activity is increased over the first 10 hours of starvation when the cells are undergoing aggregation and mound formation (Bominaar et al. 1994; Bominaar and Van Haastert 1994). Unlike mammals, *Dictyostelium* has just one PLC enzyme, which is similar to the mammalian PLC-δ isoform (Drayer et al. 1994). In the *plc*- mutant strain IP_3 levels are maintained by an alternative pathway whereby the higher order inositol phosphate IP_5 is dephosphorylated by MIPP at the plasma membrane in a Ca^{2+} dependent manner (Van Dijken et al. 1995). Decreased levels of IP_5 are observed in this mutant though the higher order inositol phosphates IP_6-IP_8 appear unaffected (Van Dijken et al. 1997). This pathway is inhibited by PO which blocks the dephosphorylation of IP_5 to IP_3 (Williams et al. 1999).

The use of cAMP derivatives which inhibit PLC activity have shown a clear correlation between IP_3 levels and chemotaxis (Bominaar and Van Haastert 1993), revealing $I(1,4,5)P_3$ plays an integral role in translating this chemical signal within the cell. In the presence of cAMP derivatives IP_3 levels were not maintained in contrast to the *plc*- mutant. Van Dijken et al. (1997) reported that IP_5 dephosphorylation to

I(1,4,5)P_3 by MIPP at the plasma membrane was stimulated by extracellular cAMP. Disruption of this stimulation may explain the discrepancies in IP_3 levels and chemotaxis between cells treated with cAMP derivatives and the *plc*- mutants.

Following cAMP binding a rapid accumulation of IP_3 has been observed in *Dictyostelium* (Europe-Finner and Newell 1987; Van Haastert et al. 1989). IP_3 releases Ca^{2+} from intracellular stores and a number of studies report that introduction of IP_3 or Ca^{2+} to permeablised cells is able to replicate actin polymerisation and cGMP accumulation without signalling through cAR1, suggesting these steps are downstream of IP_3 release (Europe-Finner and Newell 1985, 1986).

cAMP is also able to stimulate receptor mediated Ca^{2+} influx via cAR-1, cAR-2 or cAR-3 independently of G-protein activation (Milne and Devreotes 1993).

1.5.4 Development Of Multicellular Organism

Following aggregation the mound of cells undergo various stages of differentiation and morphogenesis during formation of a multicellular fruiting body (Harwood 2001) (Figure 1-7). The *Dictyostelium* fruiting body consists of spore cells suspended on a thin column of highly vacuolised stalk cells, in the wild these spores would be dispersed to form new colonies. Differentiation of cells into the different subtypes required for a functional fruiting body involves control of differential gene expression mediated by cAMP, differentiation inducing factors (DIFs) and intracellular enzymes (Berks and Kay 1990; Meima and Schaap 1999).

1.5.4.1 Early Differentiation

Cells begin to differentiate into prespore and three types of prestalk cells (pstA, B and O) very early following mound formation. After the mound has formed a tip is generated at the top which appears to co-ordinate differentiation of the other cells

(Williams et al. 1989). The mound elongates behind the tip to form a first finger structure that may topple over to form a slug. Differentiation occurs throughout the slug and clear regions of pre-spore and pre-stalk cells are defined. The tip of the slug contains pstA cells with a small core of pstB cells, these are followed by a layer of pstO cells. The final four fifths of the slug consists mainly of prespore cells but may include a few isolated pstB cells. In the first finger structure these pstB cells form a layer at the base, as the slug moves they are lost but are regenerated before further development and go on to form the basal disk (Williams et al. 1989; Harwood 2001).

1.5.4.2 cAMP And DIF Signalling

Cells secrete cAMP throughout development as well as aggregation and this signal plays a key role in differentiation. cAMP signalling via cAR-3 and cAR-4 controls activity of the protein kinase GSKA, the *Dictyostelium* homologue of the human enzyme GSK-3. At moderate levels of cAMP cAR-3 stimulation results in activation of the protein kinase, Zaphod kinase (ZAK1), which in turn activates GSKA (Kim et al. 1999; Plyte et al. 1999). However, at high concentrations of cAMP, cAR-4 is stimulated resulting in activation of a protein phosphatase (PTP), which acts to downregulate GSKA (Ginsburg and Kimmel 1997; Kim et al. 2002). GSKA activity regulates the ratio of prespore cells to pstB cells by suppression of pstB and induction of the prespore gene *pspA;* in the GSKA null mutant a mis-shapen fruiting body with an enlarged basal disk and small fruiting body is produced (Harwood et al. 1995).

Other key molecules in cell fate determination are the differentiation inducing factors, DIF1-DIF5. These factors isolated from *Dictyostelium* are able to induce prestalk cell differentiation and repress differentiation of prespore cells (Kay and Jermyn 1983). Of the five DIF factors, DIF-1 is the most abundant and is related in structure to DIF-2 and DIF-3, synthesis of all three requires the enzyme des-methyl DIF-1 transferase

(*dmtA*) (Kay et al. 1983; Morris et al. 1988; Thompson and Kay 2000). DIF-1 is synthesised by prespore cells, which show increased levels of *dmtA* mRNA and activity (Kay and Thompson 2001). In a self regulating mechanism DIF-1 acts to suppress the prespore cells which produce it, while inducing prestalk cells which in turn produce DIF-1 degrading enzymes (Kay 2002). Loss of *dmtA* expression results in a mutant lacking pstO cells but not pstA, which must be induced by an alternative signal, DIF-4 and DIF-5 are potential candidates for this role (Thompson and Kay 2000).

1.5.4.3 Terminal Differentiation

The final step in formation of the mature fruiting body is termed culmination and involves terminal differentiation including sporulation of prespore cells, this process is controlled by PKA controlled gene expression. cAMP continues to be produced throughout development however at later stages ACA is downregulated and an alternative adenylyl cyclase ACR is active (Soderbom et al. 1999). Increased PKA activity is achieved by downregulation of RegA resulting in increased cAMP due to decreased degradation. RegA inhibition is brought about by a decrease in ammonia levels and secretion of the sporulation peptide factor SDF-2.

Ammonia activates the histidine kinase DhkC which potentially activates the histidine kinase cascade, RdeA and RegA (Singleton et al. 1998; Harwood 2001). SDF-2 signals via alternative histidine kinases, DhkA and DhkB whose downstream effect is inhibition rather than activation of RegA (Wang et al. 1999). Expression of SDF-2 is controlled by PKA activity, therefore, PKA activity is further increased by an auto-stimulatory loop (Anjard et al. 1997). PKA controlled genes involved in terminal differentiation include the coat protein SpiA, *spiA* and the transcription factor StalkyA, *stkA* (Thomason et al. 1999).

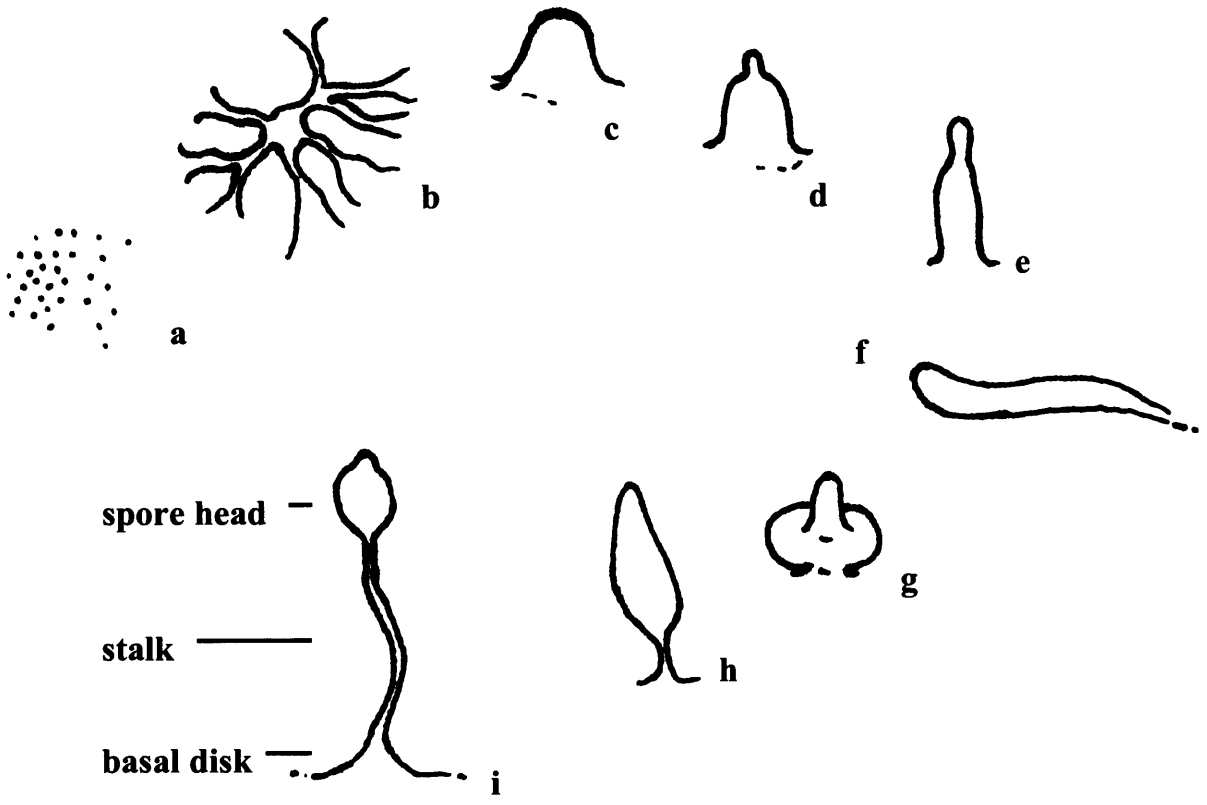


Figure 1-7 Stages of *Dictyostelium* Development

Diagram depicting the morphological stages of development; a, single cells, b, aggregation, c, mound, d, tipped mound, e, first finger, f, slug, g, Mexican hat, h, preculminant and i, mature fruiting body. Not to scale.

1.5.5 Effect Of Li^+ Treatment

Cell differentiation and morphogenesis may be affected by external factors. For example, lithium treatment of wild type cells results in a fruiting body with a large base and a very small spore head, or a complete lack of aggregation when administered at increased concentrations (Maeda 1970). Peters et al. found that Li^+ treatment during starvation resulted in an induction of prestalk associated gene expression and an inhibition of prespore gene expression, in agreement with the previous observations of a small spore head and large base (Peters et al. 1989). This effect is mediated by Li^+ inhibition of GSKA, the *Dictyostelium* homologue of GSK-3, the same spore head to stalk ratio of patterning is observed in the GSKA null mutants as seen with a low dose of Li^+ (Harwood et al. 1995). However, the *gskA-* strain remain sensitive to increased concentrations of Li^+ revealing an additional target of Li^+ inhibition.

Loss of PO results in increased intracellular IP_3 and enables aggregation to proceed in the presence of 10mM Li^+ though the *gskA-* phenotype is maintained (Williams et al. 1999). This clearly points to inositol signalling as the lithium target at increased Li^+ concentrations and provides a clear and efficient model with which to study this pathway.

1.6 Aims

The aim of this study is to elucidate the intracellular role of the enzyme prolyl oligopeptidase using the model organism *Dictyosteleum dicoideum*. Special emphasis is placed on the role of PO in the context of its ability to confer resistance to a number of mood stabilising drugs. This will be approached from a number of directions.

1. To determine the similarity of DpoA to the mammalian enzyme and characterise the enzyme activity in order to ascertain its relevance as a model for the mammalian PO.
2. To discover if the mood stabilising drugs, in particular lithium, valproic acid and carbamazepine elicit any effect directly on PO activity.
3. To identify the localisation of PO within the cell in order to understand its intracellular role, this will also give information on potential protein-protein interactions.
4. To elucidate the inhibitory action of PO on MIPP activity, loss of PO results in increased $IP_{5/6}$ to IP_3 dephosphorylation, a reaction catalysed in both *Dictyostelium* and mammals by this multiple inositol polyphosphate phosphatase.

2 Materials And Methods

2.1 Solutions

2.1.1 Chemicals

All standard chemicals were purchased from Sigma Chemical Company unless stated otherwise.

2.1.2 Molecular Biology

STET

5% Triton X-100, 50mM Tris-HCl (pH8.0), 50mM EDTA (pH8.0), 8% Sucrose

TE

10mM Tris-HCl 1mM EDTA, pH7.5

0.5X TBE

45mM Tris-HCl, 45mM Boric acid, 1mM EDTA, pH 8.3

Loading Dye (6X)

0.25% Bromophenol blue, 1M Sucrose, filter sterilised, stored at 4°C

2.1.3 Bacterial Growth

LB-(Luria Bertani) Broth Miller, ForMedium™, UK

(10% Tryptone , 5% Yeast Extract, 10% NaCl, pH 7.0) filter sterile

LB-Agar

1X LB Broth, 15% Agar

LB-Glycerol

50% LB Broth, 50% Glycerol

LB-Ampicillin Broth/Agar Plates

LB Broth/Agar Plates + 0.1 mg ml^{-1} Ampicillin

NZY+ Broth

10% Casein Hydrolysate, 5% Yeast Extract (Oxoid), 85mM NaCl, 2M $MgCl_2$, 2M $MgSO_4$, 0.15% Glucose, filter sterile, pH 7.5

2.1.4 Northern Blot Solutions

MOPS Buffer

20mM MOPS (pH 7.0), 5mM Sodium Acetate, 1mM EDTA

RNA Loading Solution

1mM EDTA, 0.4% Bromophenol Blue, 0.4% Xylene Cyanol, 50% Glycerol

SSC

150mM NaCl, 15mM Na_3Citrate

Hybridisation Buffer

43% Formamide, 5X SSC, 10X Denhardt's Solution, 200mM Sodium Phosphate buffer (pH 6.8), 200μg ml^{-1} Salmon sperm DNA (boiled immediately prior to use), 0.1% SDS

50X Denhardt's Solution

1% BSA, 1% Ficoll, 1% Polyvinylpyrollidine

2.1.5 Protein Gels

Laemmli Buffer (6X)

75mM Tris-HCl (pH 6.8), 2% SDS, 100mM DTT, 10% Glycerol, 0.1% Bromophenol Blue

Resolving Buffer

1.5M Tris-HCl (pH8.8), 0.4% SDS, filter sterile

Running Buffer

25mM Tris-HCl, 192mM Glycine, 0.1% SDS

Transfer Buffer

25mM Tris-HCl, 192mM Glycine, 20% Methanol

Fixing Solution

0.09% Acetic Acid, 45% Methanol

2.1.6 PO Assays

Homogenisation Buffer

50mM Tris-HCl (pH 7.4), 1mM EDTA, 1mM DTT, 5μg ml^{-1} Aprotinin, 1mM Benzamidine, 10μg ml^{-1} Leupeptin

Bacterial Lysis Buffer

50mM Potassium Phosphate (pH 7.8), 400mM NaCl, 100mM KCl, 10% Glycerol, 0.5% TritonX-100, pH 7.8, store at 4°C

PO Assay Buffer

50mM HEPES (pH 7.8), 1mM EDTA, 1mM DTT, 0.25% DMSO, 141μM Z-Gly-Pro-pNA.

PO Assay Buffer II

100mM HEPES (pH 7.8), 1mM EDTA, 1mM DTT, 0.25% DMSO, 35.25μM Z-Gly-Pro-pNA, (8.8, 17.6, 35.25, 70.5, 141μM Z-Gly-Pro-pNA also used during enzyme kinetics and inhibitor studies), KCl to final ionic strength of 155mM (Beynon 2006).

PO Assay Buffer II (pH adjustments)

100mM Propionate (pH 5-5.5)/MES (pH 5.5-6.5)/HEPES (pH 6.6-8.5)/Tris-HCl (pH 8.5-9), 1mM EDTA, 1mM DTT, 0.25% DMSO, 141μM Z-Gly-Pro-pNA, KCl to final ionic strength of 155mM (Beynon 2006), pH adjusted using HCl or KCl,

pH Elution Buffer

50mM Sodium Acetate, 300mM NaCl, 10% Glycerol, pH 5.0 (pH- glacial acetic acid)

2.1.7 MIPP Assays

TEE Buffer

20mM Triethanolamine (pH 6.5), 5.9mM EGTA, 0.5mM EDTA

MIPP Assay Buffer

1X TEE Buffer, 20mM $CaCl_2$, 50mM LiCl, 200μM IP_6

2.1.8 *Dictyostelium* Cell Culture

Axenic Medium

HL5 Medium with Glucose, Formedium™, UK (14% Peptone, 7% Yeast Extract, 0.5% Na_2HPO_4, 0.5% KH_2PO_4, 13.5% Glucose), 0.5mg ml^{-1} Vitamin B12, 0.2mg l^{-1} Folic Acid, pH 6.4

***Dictyostelium* Freezing Medium**

95% Horse Serum, 5% DMSO

SM (Sussmans Medium) Agar, Formedium™, UK

(10% Glucose, 10% Peptone, 1% Yeast Extract, 17% Agar, 4mM $MgSO_4$, 4mM KH_2PO_4, 6mM K_2HPO_4)

SM Black Agar

1X SM Agar, 5% Charcoal

KK_2 (KK2-UCL), Formedium™, UK

(15.5mM KH_2PO_4, 3.8mM K_2HPO_4, pH 6.2)

2.1.9 Transformation Solutions

MES-HL5 Medium

0.5% Yeast Extract (Oxoid), 1.0% Bactopeptone (Oxoid), 1.0% Glucose, 6.6mM MES, pH 7.0-7.1

Hepes Buffered Saline (HBS)

270mM NaCl, 10mM KCl, 1.2mM Sodium phosphate, 40mM Hepes, 0.2% D-glucose, pH 7.05 (use NaOH)

2.1.10 Immunofluorescence Solutions

PBS (Phosphate Buffered Saline)-A, Formedium™, UK

(160mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 1mM KH_2PO_4, pH 7.4)

PBST

99.95% PBS, 0.05% Tween20

PBS/G/S

PBS, 1% Gelatin, 0.2% Saponin

2.2 Sequence Analysis

2.2.1 Phylogenetic Analysis

Phylogenetic analysis was completed using MacVector® software (MacVector Inc, Cambridge, UK). Sequences were aligned using the ClustalW method (Thompson et al. 1994). Alignment parameters; Matrix = BLOSUM Series, Deletion penalty (open gap penalty) = 10, Gap penalty (extension gap penalty) = 0.1, Residue Specific Penalties used and Hydrophilic penalties used (hydrophilic residues; GPSNDQEKR) (Henikoff and Henikoff 1992). Phylogenetic reconstruction was completed using the following parameters; Tree building method = 'Neighbour joining', Evolutionary distance = Uncorrected 'p' method (this represents the proportion of differences between the two sequences) (Saitou and Nei 1987).

2.2.2 Definition Of Prolyl Oligopeptidase (PO) Domains

PO sequences were defined as those containing both the PO catalytic domain and β-propeller domain.

The catalytic domain was identified by the presence of either of the following protein subsequences, underlined amino acids represent residues essential for a positive match;

PO catalytic triad

NGGSNGGLLVAA (gap 70-110aa) ADHDDRVVP (gap 25-55aa) AGHGAGK (allowed mismatch 8/12, 8/9, 6/7)

PO conserved domain- pfam00326

SFTPNFSVSVASWLNRGGIYAVVNGRGGGEYGQKWHSAGTRRLKKNEFNDF IAAAEYLGKLGYTSPKRIAIFGGSNGGLL (allowed mismatch 60/80)

The β-propeller domain was identified following an alignment of the query sequence with the β-propeller conserved domain using the MacVector® ClustalW alignment function. Alignment parameters; Matrix = BLOSUM Series, Deletion penalty (open gap penalty) = 10, Gap penalty (extension gap penalty) = 0.1, Residue Specific Penalties used and Hydrophilic penalties used (hydrophilic residues; GPSNDQEKR) (Henikoff and Henikoff 1992). Alignments were analysed manually to identify the presence of the β-propeller domain at the N-terminus of the protein. Sequences with a similarity score of less than 20% were discarded unless a clear alignment could be identified manually, a small number of sequences with similarity scores above 20% were also discarded where it was clear by manual inspection that the propeller domain was not aligned.

β-propeller conserved domain- pfam02897

PPTRRDETVVDELHGDVVADPYRWLEDDDSPEVLAWVEAENKYTEDFLAQL
KPLREKIFEELTKLINYDRISAPFRRGGYYYYFRNVGGKNYSVLCRRPALSTE
GPTEEVLLDPNTLSEDGDFTVAGGGFAFSPDGRLLAYSLSLGGSDWYTIRFRD
IETGEDLPDVLEGVKFSGIVWAPDNTGFFYTRYDEPQRGSTNLPQKVWRHRL
GTPQSQDELVFEEPDDPFYLGAERSRDGKYLFISSGSGTDVNELYVLDLGSEV
PHLPLRKVVPREDGVYYYVEHWGDRFYFLTNDGAPNFRLVRVDLNDPSPAD
WKDVIVEHREDVLLESVAVFGNYLVVSYRRDVLSRVRVFDLGSGGVLFEEFL
PGVGSVSSASGEYDSDELFYSFSSFLTPSTLYDLDLDTGERELLKDR

The peptidase domain was taken as sequence from the start of the PO conserved domain through to the C-terminus. This sequence fragment was approximately 200 aa in size and included the majority of the α/β hydrolase fold domain. The β-propeller

domain was taken as the aa region aligned to the β-propeller conserved domain sequence.

2.3 Molecular Biology

2.3.1 Polymerase Chain Reaction (PCR)

PCR Reaction Mix; 100ng Mini-Prep DNA, 1µl 5'-primer (100pmol μl^{-1}), 1µl 3'-primer (100pmol μl^{-1}) (for primer details see section 2.3.13), 2.5µl dNTPs (200µM dATP, dTTP, dCTP, dGTP; Amersham Pharmacia Biotech), 5µl 10X Accubuffer™ (Bioline, UK), 1µl Accuzyme™ enzyme (2.5U μl^{-1}; Bioline) and ddH_2O to 50µl final volume. Reactions were set up in 0.2ml thin walled tubes (Anachem, UK).

PCR Program; 95°C for 45seconds- denaturation, followed by 30 cycles of 95°C for 45seconds, ~56°C (dependent on primer Tm) for 1minute and 68°C for 5minutes. Followed by a final extension step of 68°C for 10minutes and storage at 4°C.

Addition of 3'-Deoxyadenosine (A) Overhang; PCR reaction was incubated at 68°C for 10-15minutes following addition of 1µl Taq polymerase (2.5U μl^{-1}; Bioline), then returned to 4°C.

PCR Equipment; Perkin Elmer PTC-100 thermal cycler.

2.3.2 Restriction Enzyme Digestions

Plasmids were analysed using both single and double restriction digests, fragments produced were visualised by agarose gel electrophoresis.

Restriction Digest Mix; 5µl Mini-prep or 0.1-1µg DNA, 1µl each restriction endonuclease (10 U μl^{-1}), 2µl 10X manufacturers' buffer, 0.2µl BSA (10mg ml^{-1}) if required according to manufacturers' instructions and ddH_2O to a final volume of 20µl. Digests were incubated at 37°C for 2hours. All restriction enzymes and buffers were obtained from New England Biolabs.

2.3.3 Agarose Gel Electrophoresis

DNA fragments were visualised by running on a 0.8% agarose gel prepared in 0.5X TBE (section 2.1.2). Ethidium bromide was added to 0.5μg ml^{-1} to enable visualisation of DNA under UV light. DNA in solution was added to loading dye (section 2.1.2) at a ratio of 5:1 prior to gel loading. The size marker added was 1kb DNA Ladder (New England Biolabs).

2.3.4 TOPO Cloning

The *dpoA* PCR product was cloned into the pBAD TOPO vector according to the manufacturer's instructions then transformed into the chemically competent TOP 10 *E.coli* cells, see below. The pBAD TOPO TA Cloning® kit (Invitrogen) includes the pBAD TOPO vector linearised complete with 3' thymidine overhangs and bound to topoisomerase enzyme. In the presence of a PCR product with 3' deoxyadenosine overhangs, as added in a template independent manner by Taq polymerase, topoisomerase activity results in efficient ligation of the PCR product into the linearised vector.

2.3.5 Plasmids

The following plasmid constructs were used in this thesis:

2.3.5.1 pBAD TOPO:DpoA

The *Dictyostelium* prolyl oligopeptidase gene *dpoA* was obtained by PCR amplification from the cDNA clone DDC47a24 provided by the National Institute of Genetics, Mishima 411-8540, Japan (Morio et al. 1998) using the primer pair DpoA-NcoI-for and DpoA-XhoI-rev (section 2.3.13). It was cloned into the pBAD TOPO TA plasmid (Invitrogen) in frame with C-terminal V5 and His epitope tags. N-

terminal leader sequence was removed by NcoI digestion and re-ligation. The entire length of the gene was sequenced using primers; pBAD Forward, H172-2BREV, H172-2B, 172-3-REV, 172-4-REV, 172-5-REV, LisA4forB, 172-5-FOR and LisA 6 For (section 2.3.13) to confirm the fidelity of the sequence.

2.3.5.2 pTrcHis:Rpo

The pTrcHis plasmid expressing an N-terminal His-tagged rat prolyl oligopeptidase (*rpo*) was a kind gift from Prof T.Takahashi, Hokkaido University, Japan. Expression of the tagged Rpo was under the control of the trc promoter (Kimura and Takahashi 2000).

2.3.5.3 pDXA GFP2

The pDXA GFP2 (Accession No. AF269235) vector contains coding sequence for the GFPmut2 variant of the Green fluorescent protein flanked by multiple cloning sites allowing incorporation of additional coding sequence to produce either N or C-terminal fusion proteins. GFP expression is under the control of the constitutive actin promoter *act15* (Manstein et al. 1995). This vector provides G418 resistance and contains Ddp2, a cis-acting extrachromosomal replication element. To enable extrachromosomal replication pDXA GFP2 must be co-transfected with the pREP vector, in the absence of pREP this vector will be maintained via chromosomal integration (Levi et al. 2000).

2.3.5.4 pDXA GFP2:DpoA

The promoter and coding regions of *dpoA* were previously cloned into pDXA GFP2 by R.Williams in the Harwood lab, such that control of the recombinant DpoA:GFP

expression is under the *dpoA* endogenous promoter. For sequence and vector diagram see appendices I.III.I and I.III.II.

2.3.5.5 pREP

The pREP vector contains the trans acting Dpd2 open reading frame (ORF) enabling extrachromosomal replication of vectors carrying the Dpd2 origin of replication (*ori*) element.

2.3.6 Site-Directed Mutagenesis Of PO Active Site

Active site variants were achieved by site directed mutagenesis of the DNA encoding the catalytic triad in both pBAD TOPO:DpoA and pDXA GFP2:DpoA vectors. The QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) was used according to manufacturer's instructions.

For the QuikChange® site directed mutagenesis protocol; Primers were designed containing point mutations resulting in single amino acid changes at the catalytic triad. These primers were then used for amplification of the entire *dpoA*-containing vector using the non-variant mini-prep as a template. The reaction mix was as follows; 5µl 10X QuikChange® reaction buffer, 0.2µl pBAD TOPO:DpoA or pDXA GFP2:DpoA mini-prep, 1.25µl top-strand primer (100ng μl^{-1}), 1.25µl bottom-strand primer 100ng μl^{-1}), 1µl QuikChange® dNTP mix, 1µl *Pfu*Ultra DNA Polymerase (2.5U μl^{-1}) and ddH_2O to a final volume of 50µl, set up in a 500µl thin-walled PCR-tube. The following PCR programme was used; denaturation of the template vector at 95°C for 30 seconds then 15 cycles of primer annealing and extension, 95°C for 30 seconds, 55°C for 90 seconds and 68°C for 20minutes, followed by storage at 4°C.

Primers used;

Serine (AGU) 609 to Alanine (GCU) variation; DpoA Ser rev and DpoA Ser for.

Aspartic acid (GAU) 693 to Glycine (GGU) variation; DpoA Asp rev and DpoA Asp for.

Histidine (CAU) 730 to Alanine (GCU) variation; DpoA His rev and DpoA His for (section 2.2.13).

1µl of DpnI restriction enzyme (10U μl^{-1}) was added to the reaction and incubated at 37°C for 1hour. DpnI selectively cleaves methylated DNA and therefore will digest the template DNA leaving the newly amplified DNA containing the point mutation. The DpnI treated DNA was transformed into the XL1-Blue supercompetent cells supplied. 1µl DNA was added to 50µl of the supercompetent cells in a chilled 14ml Falcon tube and incubated on ice for 30minutes. Cells were heat shocked in a 42°C water bath for 45 seconds and returned to ice for 2minutes.

NZY+ broth (section 2.1.3) (0.5ml) heated to 45°C was added to the cells and incubated for 1hour at 37°C and 250rpm before being split between two LB-ampicillin agar plates (section 2.1.3) and grown over night. Individual colonies were picked and analysed by mini-prep and sequencing to confirm vector identity and presence of the point mutation.

2.3.7 Transformation Of Bacteria

TOP10™ chemically competent *Escherichia coli* (Invitrogen) were transformed as per the manufacturer's instructions. 10µl and 50µl of transformed cells were then spread onto LB agar (section 2.1.3) plates containing 100µg ml^{-1} ampicillin. Single transformed colonies were picked using a sterile pipette tip and inoculated into 5ml LB (section 2.1.3) also containing 100µg ml^{-1} ampicillin. Cultures were shaken at 250rpm and 37°C in a 20ml tube, then analysed by mini-prep and restriction digests.

Following positive identification, bacterial stocks of correct clones were produced by addition of 0.75ml of overnight culture to 0.75ml LB-Glycerol (section 2.1.3). Bacterial Stocks were stored at -80°C.

2.3.8 Mini-Prep Of Bacterial Plasmids

Two different methods were used for small-scale plasmid preparations; The Rapid Boiling Method (Harwood 1996) was used for screening transformed colonies to identify correct clones while the Ultraclean™ 6 Minute Mini Plasmid Prep Kit™, Mo Bio Laboratories Inc kit was used for preparations used for DNA sequencing and other applications.

2.3.8.1 Rapid Boiling Method

1.5ml of plasmid-containing *E.coli* were centrifuged at 12,000g for 1minute in a benchtop centrifuge and the medium removed. The pellet was vortexed and the cells lysed in 200µl STET (section 2.1.2) containing 1mg ml^{-1} lysozyme then placed in boiling water for exactly 45seconds. The lysate was then centrifuged at 12,000g for 10minutes resulting in formation of a loose white pellet of bacterial genomic DNA and protein precipitate, this pellet was removed from the supernatant using a sterile loop. 200µl of isopropanol was added to the supernatant in order to precipitate the remaining plasmid DNA, which was pelleted by centrifugation at 12,000g for 5minutes. The pellet was washed in 100µl of 70% ethanol, air-dried for 10minutes and resuspended in 100µl TE (section 2.1.2). This method results in yields of 1-5µg of plasmid DNA.

2.3.8.2 Ultraclean™ 6 Minute Mini Plasmid Prep Kit™

The Mo Bio mini-prep kit uses the alkaline lysis method and a silica spin filter to bind plasmid DNA.

Ultraclean™ 6 Minute Mini Plasmid Prep Kit™ Mo Bio Laboratories, Inc. method summary; 2ml of Plasmid-containing *E.coli* were centrifuged at 12,000g for 1minute and the medium removed, all spin steps were carried out using a benchtop centrifuge. The cell pellet was resuspended in 50µl solution 1 (Tris-HCl, EDTA, RNase A) by vortexing. Cells were lysed by addition of 100µl solution 2 (SDS, NaOH, pH 12) followed by gentle inversion. 325µl of solution 3 (Potassium Acetate, binding salt) was added resulting in co-precipitation of proteins, chromosomal DNA and other cell debris with SDS and potassium acetate. Cell debris was removed by centrifugation at 10,000g for 1minute. Supernatant containing plasmid DNA was decanted into a spin filter and centrifuged for 1minute. Plasmid DNA was bound to the white silica membrane, any other impurities passed through the membrane into the collection tube and were discarded. Bound DNA was washed with 300µl solution 4 (Tris-HCl, NaCl, +50% ethanol) before being eluted with 50µl of solution 5 (10mM Tris-HCl), DNA was released from the silica membrane due to the absence of any salt in this final solution. This method resulted in rescue of 5-10µg of plasmid DNA.

2.3.9 Maxi-Prep Of Bacterial Plasmids

Plasmid maxi-preps were carried out when large-scale amounts of DNA plasmid were required, e.g. for *Dictyostelium* transformations. Plasmid maxi preps were carried out using the QIAGEN HiSpeed Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions. This kit is also based on the alkaline lysis method. In brief: 250ml overnight cultures of plasmid containing *E.coli* were harvested by centrifugation at

4,000g for 20minutes at 4°C in a Rotanta 460R centrifuge (Hettich Zentrifugen). The cell pellet was resuspended in 10ml buffer P1 (50mM Tris-HCl, pH 8.0, 10mM EDTA, 100μg ml^{-1} RNAseA), cells were lysed by addition of 10ml solution P2 (200nM NaOH, 1% SDS) and cell debris, genomic DNA and proteins precipitated by addition of 10ml chilled buffer P3 (3M Potassium Acetate, pH 5.5). Precipitate was removed by passing the solution through a Qiafilter Maxi Cartridge into a 50ml falcon tube. A Qiagen-tip 500 containing anion-exchange resin was equilibriated by application of 10ml buffer QBT (750mM NaCl, 50mM MOPS, pH7.0, 15% isopropanol, 0.15% Triton-X100), then lysate was passed through the resin by gravity-flow. The resin was washed twice with 30ml buffer QC (1M NaCl, 50mM MOPS, pH 7.0, 50% isopropanol) to remove any impurities then DNA was eluted from the resin in 15ml buffer QF (1.25M NaCl, 50mM Tris-HCl, pH 8.5, 15% isopropanol). DNA was precipitated by addition of 10.5ml isopropanol and collected by centrifugation at 10,000g for 30minutes at 4°C. The DNA pellet was washed with 5ml 70% ethanol, re-centrifuged and left to air-dry before being dissolved in 350μl TE (section 2.1.2).

2.3.10 Sequencing Of Plasmid DNA

DNA was sequenced by MWG Biotech UK Ltd. or The Functional Genomics Laboratory, Birmingham University. Both were provided with DNA template and primers according to the service provider's requirements. Raw sequence data were returned in a format compatible with analysis using Lasergene, DNAStar™ software or MacVactor® software.

2.3.11 Expression Of PO In Bacteria

2.3.11.1 Rpo Expression

Expression of the tagged Rpo from the pTrcHis:Rpo vector is under the control of the *trc* promoter. Expression of tagged Rpo was induced by addition of 0.5mM IPTG to exponentially growing bacterial cultures for 20hours.

2.3.11.2 DpoA Expression

Expression of the tagged DpoA from the pBAD TOPO:DpoA vector is under the control of the *ara*BAD promoter. Expression of tagged DpoA was induced by addition of 0.05% L-arabinose to exponentially growing bacterial cultures for 4 hours. Following induction 1-1.5ml cell pellets were collected by centrifugation at maximum speed in a microcentrifuge. Cell pellets were resuspended in Bacterial Lysis Buffer (section 2.1.6) for PO activity assays and PO purification or in Laemmli Buffer (section 2.1.5) for western blot analysis to confirm presence of recombinant protein (see sections 2.6.5 and 2.6.2).

2.3.12 Northern Blot Detection Of PO Expression

2.3.12.1 RNA Preparation

Total RNA was isolated from cells using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. $1X10^6$ cells in 200µl PBS (section 2.1.10) were lysed with 400µl lysis buffer (4.5M guanidine hydrochloride, 50mM Tris-HCl, 30% Triton-X100, pH 6.6). The sample was then applied to a glass fibre fleece, which binds nucleic acids in the presence of chaotropic salts, under binding conditions optimised for RNA rather than DNA. The glass fibre fleece was then

incubated at room temperature with 90µl DNase I in incubation buffer (1M NaCl, 20mM Tris-HCl, 10mM $MnCl_2$, pH 7.0), then washed with wash buffers I (5M guanidin hydrochloride, 20mM Tris-HCl, 38% ethanol, pH 6.6) and II (20mM NaCl, 2mM Tris-HCl, 50% ethanol, pH 7.5). RNA was eluted from the filter in 50µl nuclease free, sterile, H_2O.

2.3.12.2 Probe Preparation

Probes were prepared from PCR products for both PO (primers 172-4-REV and H172-2C) and the control gene Ig7 (primers RT-Ig7-F2 AND RT-Ig7-R2) (section 2.3.13). PCR products were labelled with [α-^{32}P]dATP using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). The 5µl of DNA was boiled for 5minutes together with 5µl primer mix (random nonamer primers in aqueous solution) and 16µl ddH_2O then 4µl each of dCTP, dGTP and dTTP, 5µl radiolabelled [α-^{32}P]dATP, 5µl of 10X Reaction Buffer and 2µl of enzyme (1U μl^{-1} DNA Polymerase I Klenow fragment) were added and the reaction incubated at 37°C for 30minutes. The probe was then purified on a Microspin™ S-400 HR column (Amersham Pharmacia Biotech), to remove any unincorporated nucleotides.

2.3.12.3 Northern Blot

RNA Samples were prepared in 1X MOPS Buffer (section 2.1.4), 200mM formaldehyde, 50% formamide and 10% RNA Loading Buffer (section 2.1.4), denatured for 15minutes at 60°C and cooled on ice. RNA (5µg $lane^{-1}$) was run on a 1% agarose gel containing 1X MOPS Buffer, 650mM formaldehyde and 0.17 µg ml^{-1} ethidium bromide. The gel was run in 1X MOPS Buffer for 3.5hours at 105volts. RNA was visualised briefly under UV light before transferring to ddH_2O for 10minutes. RNA was transferred onto Hybond N nylon membrane (Amersham

Pharmacia Biotech) by capillary transfer in 10X SSC then UV cross-linked to the membrane using a Stratalinker (Stratagene).

The filter was then incubated in 30ml of Hybridisation Buffer (section 2.1.4) for 2 hours at 42°C. The radioactive probe was denatured by boiling for 5minutes and cooled immediately on ice before being added to 10ml Hybridisation Buffer, the Hybridisation Buffer on the filter was replaced by Hybridisation Buffer with probe and incubated at 42°C overnight. The filter was washed to remove unbound probe at 65°C for 15minutes twice with 2X SSC +0.1% SDS and twice with 0.5X SSC+0.1% SDS. After washing the filter was sealed in plastic and exposed to a phosphorimaging screen (K-Screen, Kodak) then real time images were obtained using a BioRad phosphorimager.

To re-probe filters with a second probe the filter was stripped by washing in boiling 0.1% SDS twice for 5minutes each. The filter was then incubated for a further 2hours in Hybridisation Buffer at 42°C, probed and visualised as before.

2.3.13 Oligonucleotides

All oligonucleotides used in this thesis were ordered from MWG or Sigma-Genosys. Oligonucleotides were supplied lypholised and resuspended in ddH_2O at 100μM.

Name	Sequence (5'-3')	Tm (°C)
DpoA-NcoI-for	AGATAAAATACCATGGAATTTAATTACCCAG	60.2
DpoA-XhoI-rev	TTAATTTAAAAACTCGAGAAAATTTAATTTAAC	55.8
pBAD Forward	ATGCCATAGCATTTTTATCC	
H172-2BREV	AATGATTTTAAACTCCATGTACC	43.0
H172-2B	AAATCATTTGTAATCTCAAAGAG	63.0

H172-2C	ATTTAGAAGATCAACAATCACCAG	55.9
172-3-REV	GATTAATCTATTGAATGGTGCAG	55.3
172-4-REV	ATATAATAAGTTACCGATGGTG	52.8
172-5-REV	TGCCAAGCTTTACCATACTCACC	60.6
LisA4forB	ACCATCGGTAACTTATTATATGG	55.3
172-5-FOR	GCCGCTGAATATTTGATAAAGG	56.5
LisA 6 For	GACCATGATGATCGTGTCATTCC	60.6
DpoA Ser rev	GATCAGGACGTTGATTTGAAATTGCACCC ATTAACAAACCACCATTAGCACCACC	65
DpoA Ser for	GGTGGTGCTAATGGTGGTTTGTTAATGGG TGCAATTTCAAATCAACGTCCTGATC	65
DpoA Asp rev	GCAGGAATGACACGATCACCATGGTCAC CAGTACAAAGC	68
DpoA Asp for	GCTTTGTACTGGTGACCATGGTGATCGTG TCATTCCTGC	68
DpoA His rev	GCACCAGCACCAGAATCTTTATCAACTCT AATTAAAAGTGGAGTATCAAC	65
DpoA His for	GTTGATACTCCACTTTTAATTAGAGTTGA TAAAGATTCTGGTGCTGGTGC	65
RT-Ig7-F2	GTACTTAAACCGACACTGGTTAATTG	57.1
RT-Ig7-R2	CGCTACCTTAGGACCGTCATAGTTAC	64.8

2.4 Cell Biology

2.4.1 *Dictyostelium* Cell Culture

Dictyostelium were grown vegetatively at 22°C in Axenic Medium (section 2.1.8) with 0.1mg ml^{-1} streptomycin sulphate additional selection was added as appropriate to transformed and knockout cell lines. All transformed cell lines were grown in the presence of 20-80μg ml^{-1} Geneticin (G418). All knockout cell lines, such as the PO null LisA cells (Williams et al. 1999), were grown in the presence of 10μg ml^{-1} Blasticidin.

Cells were grown in 9cm cell culture dishes (Falcon) or in shaking suspension in glass flasks. Cultures were maintained between 2 X 10^4 and 2 X 10^6 cells ml^{-1}. For long-term storage of cell lines, cells were resuspended in *Dictyostelium* Freezing Medium (section 2.1.8) at 10^7 cells ml^{-1} then frozen at -80°C in 500μl aliquots in cryovials (Nunc), which were subsequently transferred to liquid nitrogen storage. Cells were restored from long-term storage by scraping a small amount of frozen cell suspension onto a SM Agar plate (section 2.1.8) already spread with the bacteria *Klebsiella pneumoniae*. These plates were grown at 22°C until there were sufficient numbers of *Dictyostelium* to inoculate an axenic medium culture.

2.4.2 *Dictyostelium* Transformation Using The Calcium Phosphate Method

Cells were plated into a 9cm cell culture dish at 1 X 10^6 cells ml^{-1} and media replaced by 10ml MES-HL5 Medium (section 2.1.9). Cells were left in MES-HL5 Medium for 2-3hours before addition of DNA in HBS (section 2.1.9). 12μg plasmid DNA was prepared in 0.6ml HBS with 2M $CaCl_2$ per plate to be transformed and left for

25minutes at room temperature before addition to cells. DNA/HBS/$CaCl_2$ was added to the cells and left for 4hours. Medium was then replaced with 2ml HBS-Glycerol (HBS + 15% glycerol) for 2minutes at room temperature then subsequently replaced with 10ml Axenic Medium (section 2.1.8) + streptomycin sulphate (Strep). Cells were left overnight then the medium was replaced with fresh Axenic Medium supplemented with 20μg ml^{-1} G418 and 1% heat killed *E.coli.* Medium was then replaced every 2-3 days with fresh Axenic Medium containing Strep and G418 only until appearance of colonies. Colonies were picked using a pipette into a 24 well plate and the G418 concentration was gradually increased to 80μg ml^{-1}. Transformed cell lines were maintained and stored as described in section 2.4.1.

pDXA GFP2:DpoA was transformed in the absence of the trans acting extrachromosomal replication vector pREP in order to create stable colonies. Large variations within populations were observed in the presence of pREP due to variable plasmid replication or partitioning (Levi et al. 2000). However, in studies of active site variants, *Dictyostelium* were co-transformed with pREP and pDXA GFP2:DpoA (variant or un-mutated) in order to produce larger amounts of enzyme for activity assay and an improved transformation efficiency.

2.4.3 Timecourse Of *In Vivo* PO Inhibition

Dictyostelium cells were grown overnight in shaking suspension in FM Minimal Medium (Formedium™, UK). Cells were then set up at 3 $X10^6$ cells ml^{-1} and 9.9ml $flask^{-1}$ in fresh FM Minimal Medium. 100μl of Z-Pro-Prolinal (Bachem) (130mM stock resulting in 1.3mM final concentration) or 100μl of DMSO carrier control were added to the flasks of cells. Two 500μl aliquots were taken from the cell cultures immediately after addition of the inhibitor or control and then at 30minutes, 1hour and

3hours after treatment. One aliquot was immediately centrifuged to harvest cells for PO activity assay as described in section 2.6.5, the other aliquot was immediately added to 100µl of ice cold trichloroacetic acid (TCA) and assayed for IP_3 content as described in section 2.6.10.

2.4.4 *Dictyostelium* Development

96 well plates were set up with 180µl SM Black Agar (section 2.1.8) per well. For observing the effects of drugs on *Dictyostelium* development, 5µl of 38X drug stock or carrier control was added to the well prior to the molten agar. Cells growing at approximately log phase (1-2 X 10^6 cells ml^{-1}) in shaking suspension were centrifuged at 2000rpm for 2minutes in a CentraMP4 centrifuge (International Equipment Company) and resuspended in an overnight culture of *Klebsiella pneumoniae* at 1 X 10^6 *Dictyostelium* cells ml^{-1}. 5µl of the *K.pneumoniae/Dictyostelium* combination was added per well. Plates were observed regularly until the appearance of fruiting bodies in control wells (carrier control, wild type (Ax2) cells). Wells were photographed using a Canon PC1089 digital camera attached to a Zeiss Stemi SV6 stereo zoom dissecting microscope.

2.4.5 Mammalian Cell Culture

The human lymphoblastoid cell line, GC12139, is of human B-cell origin immortalised with Epstein Barr Virus (ECACC). Cells were maintained between $3X10^5$ and $2X10^6$ cells per ml in RPMI 1640 medium (2mM Glutamine, 10% FCS, 1% Pen-Strep-Neo*) (PerBio Science) at 37°C and 5% CO_2 in T25 Nunc cell culture flasks. Cells were prepared for long-term storage in RPMI 1640, 20% FBS, 10% DMSO at 2-$4X10^6$ cells ml^{-1}, 750µl aliquots were added to 2ml cryovials (Nunc). Vials were frozen slowly to -80°C before transfer to liquid nitrogen storage. Cells

were revived from frozen by rapid defrosting at 37°C followed by addition to 10ml of RPMI 1640 medium. The cell suspension was then centrifuged at 2500rpm at 4°C for 10minutes, the cell pellet was resuspended in 10ml pre-warmed fresh medium resulting in removal of DMSO from freezing media. (*=100X Penicillin (5000Uml^{-1})-Streptomycin (5mg ml^{-1})-Neomycin (10mg ml^{-1}) solution, Sigma)

2.4.6 *In Vivo* Effect Of VPA And Analogues

1ml of lymphoblastoid cells (GC12139) at 1X10^{6} cells ml^{-1} grown in RPMI 1640 were added per well of a 24 well cell culture plate (Falcon). Wells were treated overnight by addition of 10µl of 100X Stock of VPA, Valpromide or Octanoic acid to final concentrations of 0.1mM, 0.5mM, 1mM, 3mM and 10mM or 10µl of Ethanol carrier control. Each well was also treated overnight with 1µl of 1000X stock of S17092 to final concentrations of 1.3nM, 13nM and 130nM or 1µl of DMSO carrier control. Following drug treatment, cells were transferred to Eppendorf tubes and assayed for PO activity as described in section 2.6.5.

2.4.7 Cell Viability Assay

50µl of lymphoblastoid cell suspension was combined with 50µl of 0.4% Trypan Blue (Sigma). Viable (bright cells) and non-viable cells (stained blue) were counted using a NeuBauer haemocytometer; 100-150 cells were counted per sample. Trypan blue is a vital dye, the chromophore is negatively charged and does not enter the cell unless the membrane is damaged. Therefore, cells that exclude the dye are viable (Freshney 1987).

2.5 *Immunofluorescence*

2.5.1 Fluorescence Of Live Cells

A greased small rubber O-ring was placed on a coverslip. Cells from a confluent plate (~2 X 10^6 cells ml^{-1}) were diluted 1 in 3 in KK_2 (section 2.1.8) and plated onto coverslips in a large (100µl) droplet within the rubber ring. Cells were left to adhere for approximately 20minutes. Medium was removed and replaced with KK_2 3 times for 15minutes each. Excess KK_2 was removed leaving the cells covered by a small droplet of KK_2. The coverslip and rubber ring were then inverted onto a glass slide carefully to avoid any disruption of the KK_2 meniscus. Cells were observed immediately using a Zeiss Axioskop Compound Microscope with a 40X objective; both bright field and fluorescent images were captured.

2.5.2 Fixing And Staining *Dictyostelium* Cells

Glass coverslips were treated with 10% poly-L-lysine for at least 2hours then rinsed twice in distilled water before adding cells. Cells from a confluent plate (~2 X 10^6 cells ml^{-1}) were diluted 1 in 2 in Axenic Medium (section 2.1.8) and plated onto coverslips. Cells were left to adhere for at least 2hours. Medium was removed and replaced with KK_2 (section 2.1.8) 3 times for 15minutes each. KK_2 was replaced with 3.5% paraformaldehyde in PBS (section 2.1.10) and left for 20minutes. Cells were washed 3 times with PBS each wash was left on for 5 minutes. Cells were then treated with 15mM glycine in PBS twice for 10minutes each then washed again in PBS for 3 times 5minutes. PBS was replaced with PBS 1% gelatin 0.2% saponin (PBS/G/S) 3 times, the final PBS/G/S wash was left on for 20minutes. The primary antibody was diluted in PBS/G/S, the coverslip was inverted onto the antibody (approx. 400µl) on

parafilm and left for 1hour at room temperature. The primary antibody was followed with five 2minute washes in PBS/G/S before the coverslip was incubated in the secondary antibody (also diluted in 400µl PBS/G/S per slide) for 30minutes at room temperature. Slides were washed 4 times for 2minutes in PBS/G/S followed by a final 20minute wash. Finally DAPI stain (1µg ml^{-1} in PBS) was added for 15minutes. Slides were washed 3 times for 5minutes in PBS before being rinsed in distilled water and mounted onto glass slides using Vectashield® liquid mountant (Vectorlabs). Slides were sealed using nail varnish before viewing.

2.5.3 Immunofluorescence Of Inhibitor Treated Cells

Cells were plated at 5 X 10^5 cells ml^{-1} in a 9cm dish lined with poly-L-lysine treated cover slips. Cells were treated with 1.3mM Z-Pro-Prolinal (Bachem) overnight then fixed and stained as described above.

2.5.4 Immunofluorescence Of Polarised Cells

Cells were polarised using the Lab-Tek® Chamber Slide™ System chamber slides (Nunc). Slides were treated with 10% poly-L-lysine for at least 2hours then rinsed with ddH_2O. One end of the chamber was filled with 0.75ml of 1% LM (Low Melting point) agarose in KK_2 (section 2.1.8) containing 20nM cAMP.

Cells were grown in shaking suspension in Axenic Medium (section 2.1.8). Cells in log-phase growth were harvested, washed twice in KK_2 then resuspended in KK_2 at 5 X 10^6 cells ml^{-1}. 10ml of cells in KK_2 was added to a 50ml flask and returned to the shaker. Cells were sensitised to cAMP signalling by being pulsed with 100nM cAMP (50µl of a 2mM stock) at 6minute intervals using a Watson Marlow 505 Di Pump for five hours. After five hours, an aliquot of the pulsed cells was taken and diluted 1:4 with vigorous pipetting to break up any cell clumps. 100µl of diluted cells were

pipetted in to the slide chamber in 2 thin lines parallel to the end containing the agar block. Cells were left to adhere to the slide for 10minutes. An additional 650µl of KK_2 was added, cAMP from the agar block diffused into the KK_2 in the chamber creating a cAMP gradient at an appropriate concentration for cell motility (Varnum and Soll 1984). Cells were left to polarise for twenty minutes.

Cells were fixed by replacing KK_2 with 6% paraformaldehyde in PBS (section 2.1.10) for 20minutes; the increased paraformaldehyde concentration is necessary to allow for diffusion into the agar block. Cells were then washed three times with PBS. The agar block and the chamber were removed from the slide which was then stained and mounted as described above.

2.5.5 Antibodies Used

Primary Antibodies:

Detection of microtubules; anti-α-Tubulin (Mouse IgG1 isotype, Sigma) was used at a 1:400 dilution.

Detection of endoplasmic reticulum; anti-Dd-PDI antibody 221-64-1 raised in mice against the C-terminus of *Dictyostelium* Protein Disulfide Isomerase, an endoplasmic reticulum marker, (Monnat et al. 1997) (kind gift from Prof. M. Maniak) was used at a dilution of 1:3.

Secondary Antibody:

Texas Red® goat anti-mouse IgG (Invitrogen) was used at a dilution of 1:1000.

2.5.6 Imaging

Fixed and stained cells were visualised using a DM600B or DMIRE2 (inverted) microscope together with a Leica TCS SP2 confocal microscope. Images were recorded using Leica Confocal Software (LCS). A Z-series of confocal images was

taken through each cell using the 63X objective. These cell 'slices' were compiled into 3D images or maximum projections using the LCS software.

For GFP imaging argon ion laser excitation was used at 488nm, emission was recorded at 500-560nm. For DAPI imaging blue diode laser excitation was used at 405nm, emission was recorded at 415-480nm. For Texas Red® imaging He/Ne laser excitation was used at 543nm, emission was recorded at 595-705nm

2.6 Biochemistry

2.6.1 Protein Separation

Prior to western blot or silver stain analysis, proteins in cell extracts or other samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared by addition of Laemmli Buffer (section 2.1.5). Cell pellets were resuspended in 10-30% of the original culture volume, samples from purification or MACs column steps (section 2.6.9) were mixed at a ratio of 1:1 with Laemmli Buffer. 10-15µl of sample was loaded per well, 5µl of the size marker SeeBlue® Pre-Stained Standard (Invitrogen) was loaded alongside samples to measure protein size.

Two SDS-PAGE methods were followed;

1. 7.5% Acrylamide gels (25% Protogel acrylamide (30% acrylamide:0.8% methylene bisacrylamide), 25% Resolving Buffer (section 2.1.5), 0.03% APS, 0.1% TEMED) poured between two glass plates, were run in Running Buffer (section 2.1.5) at 100Volts for 1-2 hours until the leading edge had progressed at least two-thirds the length of the gel.
2. NuPAGE® Novex Bis-Tris Gels (Invitrogen) were run in NuPAGE® MES SDS Running Buffer (Invitrogen) at 200Volts for 40minutes.

After electrophoresis was complete, gels for western blot analysis were equilibrated in Transfer Buffer (section 2.1.5) while gels for silver stain analysis were transferred to Fixing Solution (section 2.1.5).

2.6.2 Western Blot Analysis

Protein was transferred from the gel to a nitrocellulose filter (Hybond C+™ nitrocellulose membrane, Amersham Pharmacia Biotech) using a semi-dry blotting unit (BioRad). The membrane and gel were placed between six layers of 3MM paper soaked in Transfer Buffer (section 2.1.5) and subjected to 20Volts for 48minutes. The membrane was blocked by soaking in PBST+5% Marvel® milk powder (section 2.1.10) at room temperature for 1 hour, then incubated in primary antibody in PBST at room temperature for 1 hour. If required the membrane was washed in PBST five times for five minutes each and incubated in secondary antibody in PBST at room temperature for 1 hour. The membrane was then given five 5minute washes in PBST before a 5minute incubation in chemi-luminescence reagent (Supersignal™, Pierce and Warringer Ltd) to detect the HRP-conjugated antibody (primary or secondary). Bands were visualised by exposure to Biomax film (Kodak), which was developed in an Optimax X-Ray Film Processor (Scientific Laboratory Supplies).

The following antibodies were used:

Primary antibodies;

Anti-V5:HRP (Invitrogen) was used at a 1:5000 dilution. Anti-6XHis:HRP (Clontech) was used at a 1:5000 dilution. Anti-α-Tubulin (Mouse IgG1 isotype, Sigma) was used at a 1:400 dilution. Anti-GFP (Mouse IgG, Roche) was used at a 1:10000 dilution.

Secondary antibodies;

Anti-Mouse IgG:HRP , developed in goat (Sigma) was used at a 1:10000 dilution.

2.6.3 Silver Staining

Gels were taken from Fixing Solution (section 2.1.5) for silver staining using the Sterling™ Rapid Silver Stain Kit (National Diagnostics). Gels were fixed further in Sterling fixative for 5minutes before washing for twice 15minutes in ddH_2O+0.1% Tween20. Gels were then immersed in staining solution and observed for the appearance of black/brown bands revealing the proteins present. When desired intensity was reached the gel was incubated in 5% acetic acid to stop the reaction. A permanent record was taken by photographing the gel before or after gel drying.

2.6.4 PO Purification

150ml *E.coli* containing either pTrcHis:Rpo or pBAD TOPO:DpoA bacterial cultures were grown overnight then induced as described in section 2.3.11. Following induction cells were harvested by centrifugation at 9,000g at 4°C for 5minutes in 50ml aliquots, cell pellets were resuspended in 8ml Bacterial Lysis Buffer (section 2.1.6). Cells were lysed by sonication. Cell suspensions were subjected to three 10second bursts at Amplitude 30 using a vibra-cell™ ultrasonic processor (Sonics and Materials Inc.) and rested on ice for at least 30seconds between bursts. Cell debris was removed by centrifugation at 10,000g at 4°C for 30minutes.

All subsequent steps were carried out at 4°C. Supernatant containing PO was loaded onto a medium width FPLC column packed with 2ml Talon® metal affinity resin slurry (BD Biosciences) (1ml total beads) and equilibrated with Bacterial Lysis Buffer. The FPLC (pump P-500, controller LCC-501 Plus, motor valve MV-7, optical unit UV-1, Pharmacia Biotech) was run at a speed of 0.5ml min^{-1}. Following loading of supernatant onto the His-binding column, the column was washed with Bacterial Lysis Buffer for a further 20minutes. At this point the buffer entering the column was

switched from 100% Bacterial Lysis Buffer to 100% pH Elution Buffer (section 2.1.6) and fraction collection began. 0.5ml fractions were collected every minute for 30minutes. Samples were taken from the supernatant prior to purification, from the flowthrough and from each fraction for analysis by silver stain as described in section 2.6.3. 3µl of each sample was assayed for PO activity and protein concentration was also determined as described in section 2.6.5.

This method resulted in a clear peak of activity eluting from the Talon™ column, all fractions which cleaved more than 3µmoles of Z-Gly-Pro-pNA during the 90minute assay time were pooled to produce a standard stock of purified enzyme. Stock enzyme was stored in 100µl aliquots at -20°C.

2.6.5 PO Activity Assay

2.6.5.1 Cell Extract Preparation

Dictyostelium or lymphoblastoid cells were pelleted and washed in KK_2 (section 2.1.8) or PBS (section 2.1.10) respectively then resuspended in Homogenisation Buffer (section 2.1.6) to give $2X10^8$ cells ml^{-1}. For bacterial cells expressing recombinant PO, 1.5ml of induced overnight bacterial culture was pelleted and resuspended in 500µl of Bacterial Lysis Buffer. Cells were lysed by sonication, cell suspensions were subjected to three 5 second bursts at Amplitude 30 and rested on ice for at least 30 seconds between bursts. Cell debris was removed by centrifugation at 10,000 g at 4°C for 30minutes. Supernatant containing PO was collected and kept at 4°C for up to 24hours. For measurement of PO activity the volume of cell extract containing 2.5µg total protein was added per assay.

2.6.5.2 Measurement Of Protein Concentration

Protein concentration of cell extracts or purification fractions was determined using BioRad Protein Assay Dye Reagent (BioRad Laboratories). This reagent changes colour on contact with protein and is based on the dye binding method of Bradford (Bradford 1976). Assays were set up in 96 well flat-bottomed plates (Nunc), 2µl of sample was added to 198µl BioRad Assay Dye Reagent and absorbance at 595nm was measured on a BioRad microplate reader (model 3550). Protein concentration was calculated from a standard curve produced from BSA samples with concentrations from 0.25 to 1mg ml^{-1}.

2.6.5.3 PO Assay I

All samples were assayed in a final volume of 60µl PO Assay Buffer I (section 2.1.6) in a 96 well plate, the sample and PO Assay Buffer were incubated at 37°C for 90minutes. Samples were set up in duplicate in the presence and absence of enzyme substrate Z-Gly-Pro-pNA (Bachem). PO cleavage of the substrate Z-Gly-Pro-pNA results in the release of p-nitroaniline (pNA). Production of this chemical was observed by measurement of absorbance at 405nm using a BioRad microplate reader (model 3550). The difference between absorbance in the presence and absence of substrate was calculated and converted into moles of pNA produced (or moles of substrate cleaved) using a standard curve of absorbance of 60µl of pNA standards from 5 to 200µM.

2.6.5.4 PO Assay II; Enzyme Kinetics

Activity was measured using the fluorescent substrate Z-Gly-Pro-AMC (Z-Gly-Pro-7-amido-4-methylcoumarin). Cleavage of Z-Gly-Pro-AMC results in release of 7-

amino-4-methylcoumarin (AMC), detected by fluorescence at excitation 351nm, emission 430nm. This substrate is similar to Z-Gly-Pro-pNA however the pNA moiety has been replaced by AMC. Release of 7-amino-4-methylcoumarin was measured using a FLUOstar OPTIMA luminometer (BMG labtechnologies) with excitation and emission filters of 355nm and 460nm respectively. Use of this machine enables a higher accuracy of detection as well as taking readings under temperature-controlled conditions.

A set of standards of known concentrations of AMC were included on the plate to be read. This enabled conversion of fluorescence readings to concentration of reaction product. The standards were also used to set the maximum gain of the luminometer. In each experiment 90% maximum gain was set using a standard concentration of AMC equal to approximately 15% the assay substrate concentration thus increasing sensitivity of readings in the range before significant substrate depletion occurs.

The activity assays were incubated in the luminometer at 25°C and the increase in fluorescence measured at 10minute intervals.

Assays were carried out in 100mM HEPES buffer at pH 7.8 (PO Assay buffer II, section 2.1.6). A pH profile of PO activity between pH 5 and pH 8.5 confirmed pH 7.8 to be in the range of optimal PO activity. Enzyme activity, v, was taken as the initial rate of AMC production observed between 0 and 20minutes. PO activity was assayed across a range of Z-Gly-Pro-AMC concentrations from 2.2 to 141μM. The K_M and V_{max} of DpoA and Rpo were calculated using Michaelis-Menten kinetics by Prism© software (GraphPad Prism 4.0a).

2.6.6 Determination Of Inhibition Constants

PO assays were carried out as described in section 2.6.5.4 above using $1X10^{-5}$U purified recombinant DpoA or Rpo. Specific inhibitors S17092 and Z-Pro-Prolinal or

carrier controls were added to PO Assay Buffer II (section 2.1.6) prior to incubation at 25°C. S17092 (kindly provided by Dr F Lamour, Institut de Recherches Internationales Servier, Courbevoie, France) was dissolved in DMSO and added at a range of final concentrations from 0.1 to 1nM. Z-Pro-Prolinal (Bachem) was dissolved in DMSO and added to a range of final concentrations from 1 to 10μM.

PO activity was measured across a range of substrate concentrations (8.8, 17.6, 35.3 and 70.5μM) at each inhibitor concentration. Inhibition constants were calculated using Prism© software (GraphPad Prism 4.0a) as described in chapter 3 section 3.2.4. Values of K_i for Z-Pro-Prolinal were calculated using Michaelis-Menten kinetics and for S17092 using tight-binding kinetics as described by Henderson (1972), both were calculated using Prism© software.

2.6.7 pH Profile Of PO Activity

To assay PO activity across a range of pH values, 4 different buffers were used (Propionate pH 5-5.5, MES pH 5.5-6.5, HEPES pH 6.6-8.5, Tris-HCl pH 8.5-9, see section 2.1.6). Each buffer was used at an assay concentration of 100mM brought to the required pH using HCl or KOH. The ionic strength of all buffers was maintained at 155mM (physiological ionic strength) using KCl as calculated using the buffer calculator created by Rob Beynon at The University of Liverpool (Beynon 2006). Ionic strength of a solution can be measured using the following equation (Equation 2-1);

Equation 2-1

$I_c = \frac{1}{2} \sum C_B Z_B^2$

I_c = ionic strength, C_B = concentration of salt in buffer, Z_B = valency of salt.

Purified recombinant PO activity ($1X10^{-5}$U $assay^{-1}$) was assayed as described in section 2.6.5.3.

2.6.8 Inhibition Studies Of Mood Stabilising Drugs

PO assays were carried out as described above (section 2.6.5.4) using $1X10^{-5}U$ purified recombinant DpoA or Rpo. LiCl, CBZ, VPA or carrier controls were added to sample and PO Assay Buffer II (section 2.1.6) prior to incubation at 25°C. All three drugs were added at several times their therapeutic concentration, LiCl (therapeutic concentration 1.5mM) was added at 40μM, 200μM, 1mM, 5mM and 25mM, CBZ (therapeutic concentration 75μM) was added at 4μM, 20μM, 100μM, 500μM and 1mM, VPA (therapeutic concentration 0.6mM) was added at 10μM, 48μM, 240μM, 1.2mM and 6mM.

2.6.8.1 Effects Of VPA And Analogues

Further activity assays were set up in assay buffers representing a range of pH as in section 2.6.7 with addition of VPA, Valpromide or Octanoic acid to a final concentration of 6mM, or a carrier control. All compounds were dissolved in MeOH.

2.6.9 Isolation Of DpoA:GFP Using Magnetic Columns

The GFP tagged *Dictyostelium* PO was isolated from cell extracts using the μMACS™ Epitope Tag Protein Isolation Kit (Miltenyi Biotec).

A confluent 10ml plate of *Dictyostelium* cells were pelleted and washed in KK_2 (section 2.1.8) then resuspended in 1ml of Homogenisation Buffer (section 2.1.6). Cells were lysed by sonication, cell suspensions were subjected to three 5second bursts at Amplitude 30 and rested on ice for at least 30seconds between bursts. Cell debris was removed by centrifugation at 10,000 g at 4°C for 30minutes, supernatant was added to 50μl of Anti-GFP MicroBeads and incubated for at least 30minutes at 4°C.

The μ Column was placed into the magnetic field of the μMACS Separator and prepared by addition of 200μl of Homogenisation Buffer +1% Triton-X100. The labelled cell lysate was applied to the column and the flow through collected. The column was then washed four times with 200μl Homogenisation Buffer followed by 100μl μl of wash buffer 2 (20mM Tris-HCl, pH 7.5), all wash buffers were also collected for analysis.

2.6.9.1 Isolation Of DpoA Binding Proteins

The μ Column was prepared, loaded and washed as described above, following the two washes (Homogenisation Buffer and wash buffer 2), the bound GFP-tagged protein along with any specifically-bound proteins were eluted. Bound proteins were incubated with 20μl of pre-warmed (95°C) elution buffer (50mM Tris-HCl pH6.8, 50mM DTT, 1% SDS, 1mM EDTA, 0.005% bromophenol blue, 10% glycerol) for 5minutes followed by elution from the column in 50μl of the warmed elution buffer. The MicroBeads were then eluted by removing the μ Column from the magnetic field and washing with 200μl of wash buffer 2. The elution buffer was applied directly onto an SDS-PAGE gel for western and silver stain analysis as described in section 2.6.1-3. All other samples were combined with Laemmli Buffer (section 2.1.5) at a 3:1 ratio before gel loading.

2.6.9.2 PO Activity Assay

The μ Column was prepared, loaded and washed as described above. Following wash buffer 2 the column was washed again with 100μl of PO Assay Buffer (section 2.1.6). A further 25μl (column capacity) of PO Assay Buffer was added, 1μl of sealing solution was added to prevent evaporation and the column incubated at 37°C for

90minutes. An aliquot of the cell lysate before addition of Anti-GFP MicroBeads and the column flowthrough were also assayed for PO activity as described in section 2.6.5. The PO Assay on the column was eluted directly into a 96 well plate by addition of 60µl of PO Assay Buffer, and activity measured as described above (section 2.6.5.3).

2.6.10 IP_3 Assay

IP_3 concentrations were determined using the Inositol-1,4,5-Trisphosphate [^{3}H] Radioreceptor Assay Kit (PerkinElmer Life Sciences, Inc). This kit contains a membrane preparation containing the IP_3 receptor bound to the radiolabelled ligand [^{3}H]IP_3 (tracer). Sample IP_3 concentration is determined by measurement of the amount of radiolabelled ligand displaced by the unlabelled IP_3 present in the sample.

Samples were prepared by addition of 0.2 volumes of 100% trichloroacetic acid solution. The sample/TCA solution was vortexed vigorously and incubated for 15minutes on ice before being centrifuged at 13,000 g for 1minute in a benchtop microfuge. The supernatant was kept for IP_3 analysis. TCA was removed from the sample by addition of TCTFE (1,1,2-Trichloro-1,2,2-trifluoroethane):trioctylamine (3:1 ratio) at a ratio of 2:1 with the sample/TCA solution. The mixture was vortexed for 15 seconds then left to allow phase separation. The top, aqueous, layer containing the IP_3 was removed and placed on ice.

A working receptor tracer solution was prepared on the day of the assay by dilution of the concentrated receptor preparation/tracer in Assay buffer (0.05% sodium azide, 5mM EDTA, 5mM EGTA, 50mM sodium TAPS buffer, pH 8.6) at a ratio of 1:15. 400µl of working receptor/tracer solution was added to 100µl of sample in a minitube on ice. All samples were assayed in duplicate. 100µl aliquots of a series of IP_3

dilutions made up in ddH_2O (for MIPP assays) or FM Minimal Medium (ForMedium™, UK) (for *Dictyostelium* cell extracts) were also set up in minitubes with receptor/tracer solution as well as two minitubes containing 100µl blanking solution to measure non-specific binding and two counting vials containing receptor/tracer solution alone to measure total counts.

All tubes were vortexed for 3-4seconds then incubated for one hour at 4°C. Tubes were centrifuged in their racks at 3000g for 20minutes at 4°C in a Rotanta 460R centrifuge (Hettich Zentrifugen) and supernatant decanted. The pellets were resuspended in 50µl of 0.15M sodium hydroxide by vortexing for 3seconds, incubating at room temperature for 10minutes and vortexing for a further 5seconds. The membrane suspension was then transferred to a counting vial by pipetting. 4ml of Ecolite (+)™ Liquid Scintillation Fluid (MP Biomedicals) was added to each vial, including the total counts vial, and mixed by inversion and vortexing. Counts per minute (CPM) were measured using a TriCarb 2800TR scintillation counter (PerkinElmer), with Quantasmart software, each vial was counted for 5minutes.

To calculate moles of IP_3 present in each sample, the average non-specific binding CPM was subtracted from the CPM for all other samples, then the percent of radiolabelled ligand bound was calculated for each standard and sample using the total counts CPM. The concentration of IP_3 present in each sample was taken from a standard curve created by plotting percentage of radiolabelled ligand bound against concentration of the IP_3 dilutions.

2.6.11 MIPP Activity Assay

To measure the effects of DpoA on MIPP activity, membrane fractions from a MIPP over-expressor cell line (developed in the Harwood lab by Jason King) were used.

Cells were grown in Axenic Medium (section 2.1.8) in shaking culture, cell pellets were harvested, washed in KK_2 (section 2.1.8) and pellets frozen on dry ice. Pellets were then defrosted and resuspended at $2X10^8$ cells ml^{-1} in TEE Buffer (section 2.1.7). Cells were lysed by being passed through a Nucleopore® filter (5μm pore size) directly into a pre-chilled ultracentrifuge tube (Beckman). The cell suspension was spun at 39,000rpm for 30minutes at 4°C in a Beckmann Optima™ TLX ultracentrifuge, rotor TLS-55. Supernatant was discarded and the pellet resuspended in the original volume of TEE Buffer. Protein concentration was calculated by Bradford assay as described in section 2.6.5.2.

Cell extracts were incubated for 30minutes at room temperature with and without purified recombinant DpoA (see section 2.6.4) and Z-Pro-Prolinal. The incubation mixture contained; 10μl $MIPP^{OEX}$ Pellet fraction, 10μl Purified recombinant DpoA (~3mU) or pH Elution Buffer (section 2.1.6), 29.5μl TEE Buffer, 0.5μl Z-Pro-Prolinal (to final concentration of 1.3mM) or DMSO. The MIPP assay was started by addition of 50μl of MIPP Assay Buffer (section 2.1.7) containing 200μM IP_6. The solution was mixed by pipetting and 50μl was immediately removed then added to 10μl TCA on ice; this was the time 0 sample.

The remaining 50μl of reaction was incubated at room temperature for 15minutes before addition of 10μl ice cold TCA. 440μl ddH_2O was added to all samples, which were then vortexed for 15seconds and centrifuged 13,000g for 1minute in a microcentrifuge. The supernatant was transferred to a fresh tube and TCA extraction was carried out as described above (section 2.6.10). Samples were further diluted 1:2 in ddH_2O before IP_3 was assayed as described (section 2.6.10). MIPP activity was determined by the increase in IP_3 from time 0 to the 15minute timepoint. IP_3 is produced by MIPP mediated dephosphorylation of IP_6 in the assay buffer.

3 Biochemistry Of DpoA

3.1 Introduction

3.1.1 Serine Protease Family

The serine protease family is a large family of proteases whose common feature is a catalytic triad, His-Asp-Ser, in which the Ser residue is responsible for nucleophilic attack. This family can by sub-divided into the chymotrypsin, subtilisin and serine carboxypeptidases (clans SA, SB and SC respectively) (Rawlings and Barrett 1993). The Prolyl Oligopeptidase family (family S9) is a sub-group of the serine carboxypeptidase family and contains prolyl oligopeptidase (the family namesake), dipeptidyl peptidase IV (DPPIV), acylaminoacyl peptidase (AP), oligopeptidase B (OB) and prolyl endopeptidase like A (PREPL A). While these enzymes do not all specifically cleave at a proline residue, all preferentially cleave short oligopeptides and show clear sequence homology around the active site.

Prolyl oligopeptidase is particularly similar at sequence level to oligopeptidase B and the 3D structure of oligopeptidase B was modelled using PO as a template (Gerczei et al. 2000). Both PO and OB are formed from a catalytic domain containing the active site and a β-propeller domain. Other enzymes in the family which also have a β-propeller domain are acylaminoacyl peptidase (7-bladed) and dipeptidyl peptidase IV (8-bladed) (Polgar 2002; Bartlam et al. 2004). In contrast to prolyl oligopeptidase and oligopeptidase B, which both operate as monomers, acylaminoacyl peptidase, dipeptidyl peptidase IV and prolyl endopeptidase like A form dimeric structures.

Because of the similarity within this family it is important to confirm the identity of DpoA, which based on sequence analysis and limited biochemistry has been considered to be a prolyl oligopeptidase homologue, as a prolyl oligopeptidase rather than any other S9 family enzyme.

3.1.2 AIM

This chapter aims to determine the relevance of DpoA as a model for the mammalian enzyme, using a more detailed sequence analysis, enzyme kinetics and mutagenesis.

3.2 Results

3.2.1 DpoA Is A Characteristic Prolyl Oligopeptidase

In order to determine the relationship of DpoA and the mammalian prolyl oligopeptidase, protein sequence comparison of the prolyl oligopeptidase family was carried out.

3.2.1.1 Obtaining Prolyl Oligopeptidase (S9) Family Sequences

The first step was to identify and download all PO family protein sequences. In order to form a reliable picture the maximum number of sequences were included. Initially sequences from *Dictyostelium* and other species were downloaded from the NCBI Entrez database via the MacVector™ sequence analysis program. A search for any sequences with the enzyme name present in the title enabled identification not only of all named proteins but also sequences identified as potential, putative, proteins homologous to each specific enzyme and fragments of each specific enzyme. Searches carried out for 'prolyl oligopeptidase' 'prolyl endopeptidase' 'oligopeptidase B' 'dipeptidyl peptidase' and 'acylaminoacyl peptidase' identified a total of 763 sequences (prolyl oligopeptidase 169, prolyl endopeptidase 127, oligopeptidase B 45, dipeptidyl peptidase 351, acylaminoacyl peptidase 71). The sequences identified in the 'prolyl endopeptidase' search include a very small number of sequences encoding prolyl endopeptidase like A (PREPL A). PREPL A was only recently identified as a novel member of the prolyl oligopeptidase family (Szeltner et al. 2005) and very few protein sequences have been elucidated. Sequences were annotated with species name, enzyme name and accession number (full species names are listed in appendix I.I).

Alignments of multiple sequences belonging to one species were carried out for each enzyme and any duplicate sequences removed from the analysis. All protein sequence fragments (less than 100aa) were also removed from the analysis. This decreased the sequence total to 326 (prolyl oligopeptidase/endopeptidase 111, oligopeptidase B 22, dipeptidyl peptidase 157, acylaminoacyl peptidase 36).

3.2.1.2 Prolyl Oligopeptidase Family Alignment

Analysis of the phylogenetic tree of the entire PO family was carried out by completing a ClustalW alignment followed by neighbour joining method of tree construction using a distance matrix of uncorrected p-values. This analysis shows clear multi-species clusters for each enzyme. This is indicative of the sequence conservation between homologous enzymes from different species and illustrates that there are distinct differences between the enzymes despite the structural similarity throughout the family (See Figure 3-1).

Best tree analysis of the prolyl oligopeptidase family phylogeny revealed a clear grouping of the mammalian sequences within each enzyme cluster. *Dictyostelium* PO did group together with the mammalian PO sequences, however, it showed a greater divergence from the mammalian enzyme than that of the plant *A.thaliana* and the fruit fly *D.melanogaster*. Bacterial PO sequences clustered closely with one another and showed a weaker association with the eukaryote PO sequences (Figure 3-1).

The oligopeptidase B sequences show an association with those for PO as would be expected by their similar structure, a similar relatedness is observed for acylaminoacyl peptidase and dipeptidyl peptidase IV.

PREPL has been identified in humans as well as a number of other mammals it shows closest similarity to the oligopeptidase B enzymes and only a very weak similarity to the mammalian prolyl oligopeptidase (Szeltner et al. 2005). As expected, this

alignment also resulted in clustering of PREPL sequences (just one sequence shown) with OB. Despite conservation of the catalytic triad no substrates have yet been identified for PREPL and it is unable to hydrolyse substrates of PO or OB (Martens et al. 2006). Interest in this potential enzyme is largely due to observation that PREPL is deleted in a population of patients suffering the congenital disorder 'the hypotonia-cystinuria syndrome' (Jaeken et al. 2006).

A very small number of sequences annotated as PO appeared to be displaced as they were distributed throughout the phylogeny; a number of these were placed in groups with dipeptidyl peptidase IV and acylaminoacylpeptidase however the large majority were among those sequences later discarded as not representing true PO (see later) (Figure 3-1).

Dictyostelium also express proteins homologous to oligopeptidase B, 2 acylaminoacyl peptidases and a dipeptidyl peptidase all of which associate with their respective enzyme clusters. No PREPL sequence has been detected to date in *Dictyostelium.*

In this case the best tree diagram appears to represent a reliable picture of the PO phylogeny as it is largely supported by the bootstrap tree. Bootstrap significance is weakest around the AP family suggesting it must contain greatest divergence (See Figure 3-1).

Bootstrap analysis of the phylogenetic tree involves re-sampling of groups of sequences within the complete data set and recording the frequency with which sequences are placed together, the standard bootstrap resampling frequency of 1000X was used. This provides evidence of significance of the clusters identified. In this software any branches with a bootstrap value of below 50 are 'collapsed'.

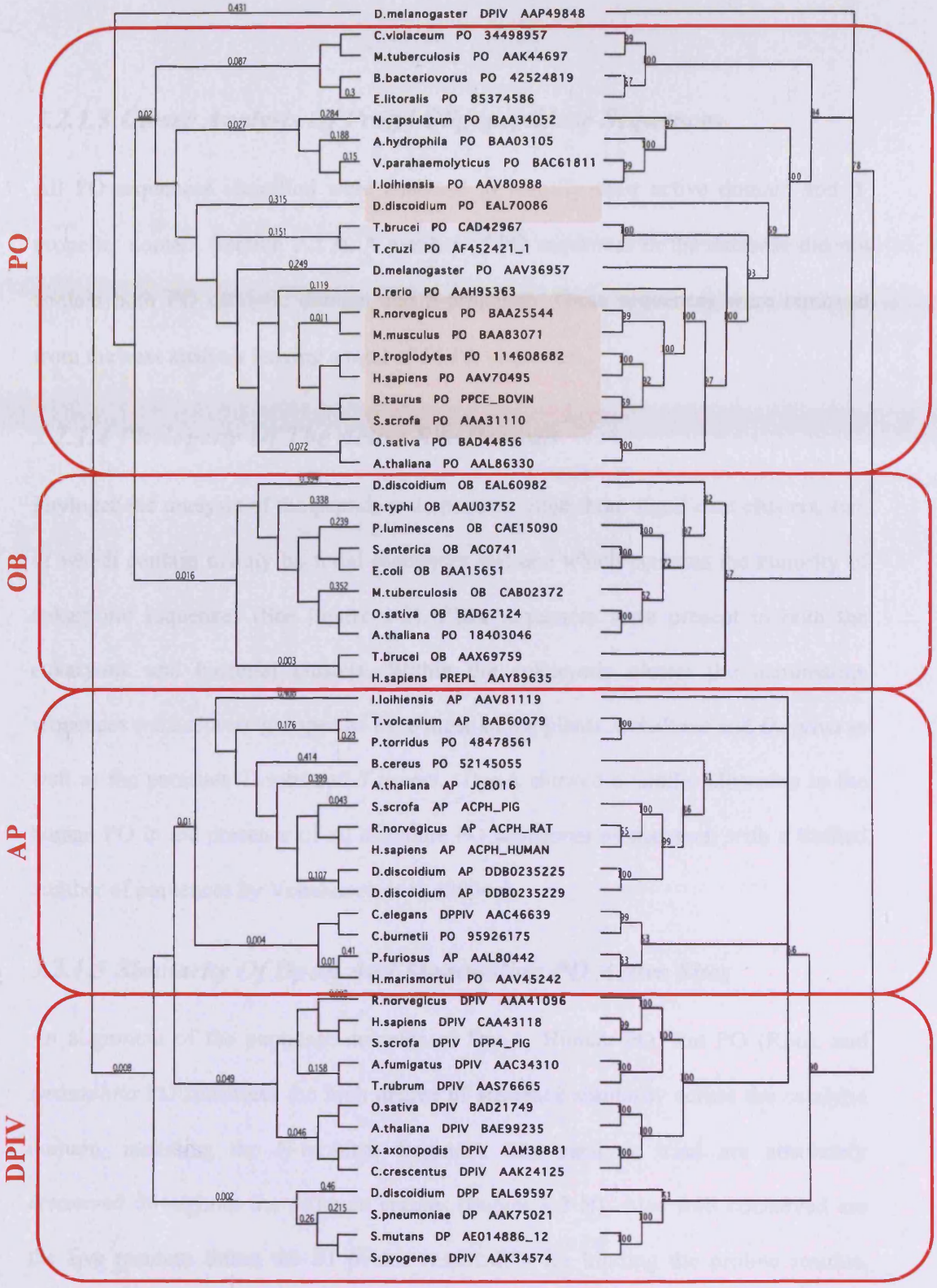


Figure 3-1 Prolyl Oligopeptidase Family Phylogeny.

This tree shows a scaled down phylogenetic tree containing representative sequences from the complete phylogeny. Enzymes are named in the format; species name-enzyme abbreviation-accession number (DP Dipeptidyl peptidase, OB Oligopeptidase B, PO Prolyl Oligopeptidase and AP Acylaminoacylpeptidase). Branches to the left represent Best Tree Analysis, those to the right represent Bootstrap analysis. Mammalian and *Dictyostelium* PO sequences are highlighted in red.

3.2.1.3 Closer Analysis Of Prolyl Oligopeptidase Sequences

All PO sequences identified were analysed to identify their active domain and β-propeller domain (section 2.2.2). A number of PO sequences in the database did not contain both PO catalytic domain and β-propeller. These sequences were removed from the next analysis leaving a total of 54 PO sequences.

3.2.1.4 Phylogeny Of The Active Site Domain

Phylogenetic analysis of the peptidase domain revealed three significant clusters, two of which contain mainly bacterial sequences and one which contains the majority of eukaryotic sequences (See Figure 3-2). Plant sequences were present in both the eukaryotic and bacterial clusters. Within the eukaryotic cluster the mammalian sequences were closely grouped as were those of the plants *A.thaliana* and *O.sativa* as well as the parasites *T.cruzi* and *T.brucei*. DpoA showed a similar closeness to the human PO in the presence of all available PO sequences as that seen with a limited number of sequences by Venalainen et al. (2004a).

3.2.1.5 Similarity Of DpoA And Mammalian PO Active Sites

An alignment of the peptidase domains of DpoA, Human PO, Rat PO (Rpo), and *Drosophila* PO illustrates the high degree of sequence similarity across the catalytic domain, including the N-terminal fragment. The catalytic triad are absolutely conserved throughout the different species (Figure 3-3 B). Also well conserved are the five residues lining the S1 pocket responsible for binding the proline residue, F476, V580, W595, Y599 and V644, and residues Y473 and N555 (numbering for human PO) of the oxyanion binding site.

As well as the surrounding sequence homology, the catalytic triad is positioned at a similar distance from the C-terminus of each of the proteins (Figure 3-3 A and B). One clear difference observed in the *Drosophila* sequence is additional amino acids at the N-terminus. This may affect the active site as the N-terminal fragment is responsible for two α-helices and two strands of the β-sheet structure of the α/β-hydrolase fold, alternatively this region may be cleaved to produce the active enzyme.

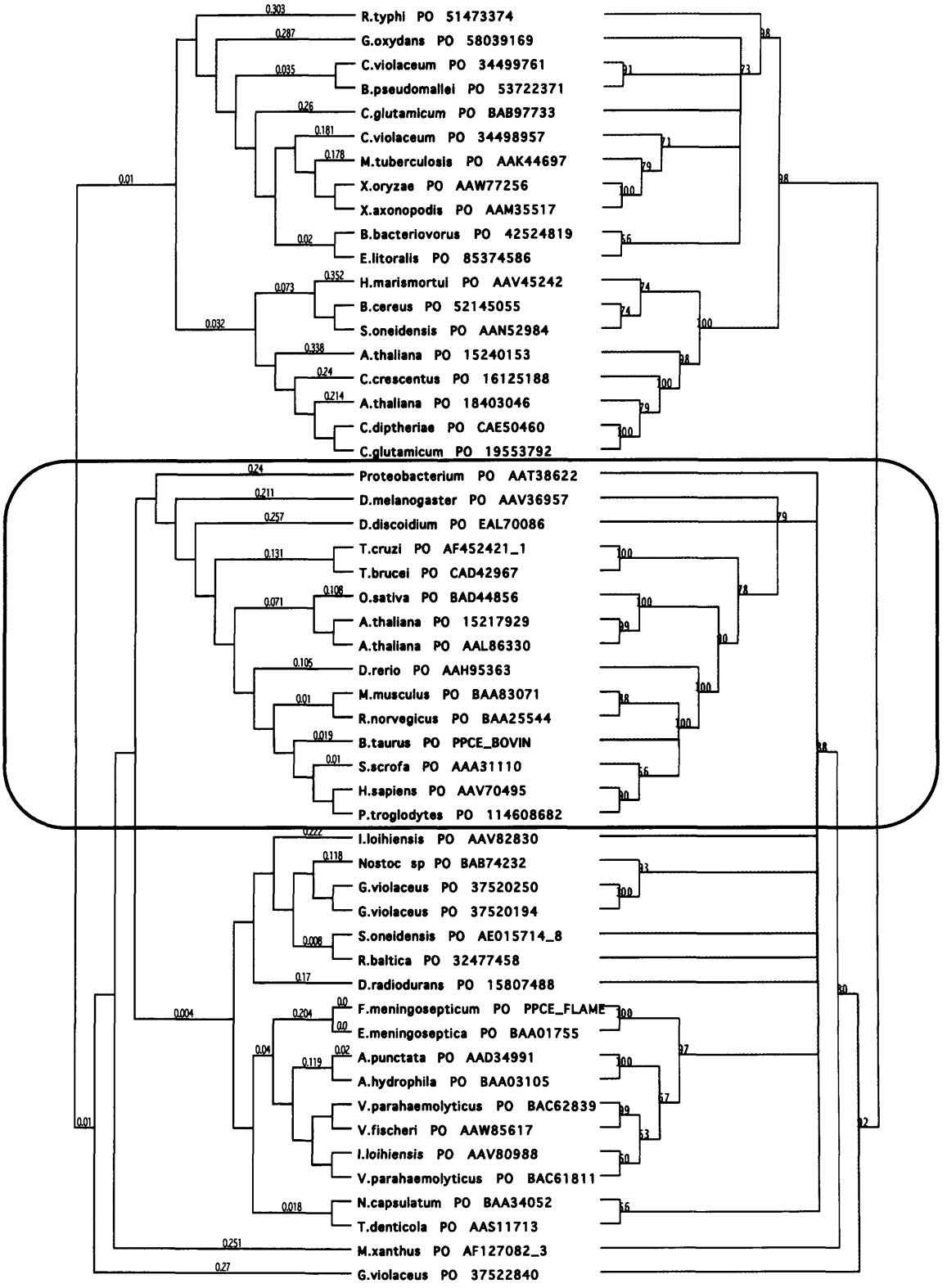


Figure 3-2 PO Active Site Phylogeny.

Phylogenetic analysis of the catalytic domain of all available PO sequences in the Entrez database. Branches to the left represent the best tree analysis of the sequences and show phylogenetic distance (p-values), those to the right represent Bootstrap analysis compiled of 1000 repeats, these branches are labelled with confidence levels. Both human and *Dictyostelium* PO are present in the cluster outlined in red.

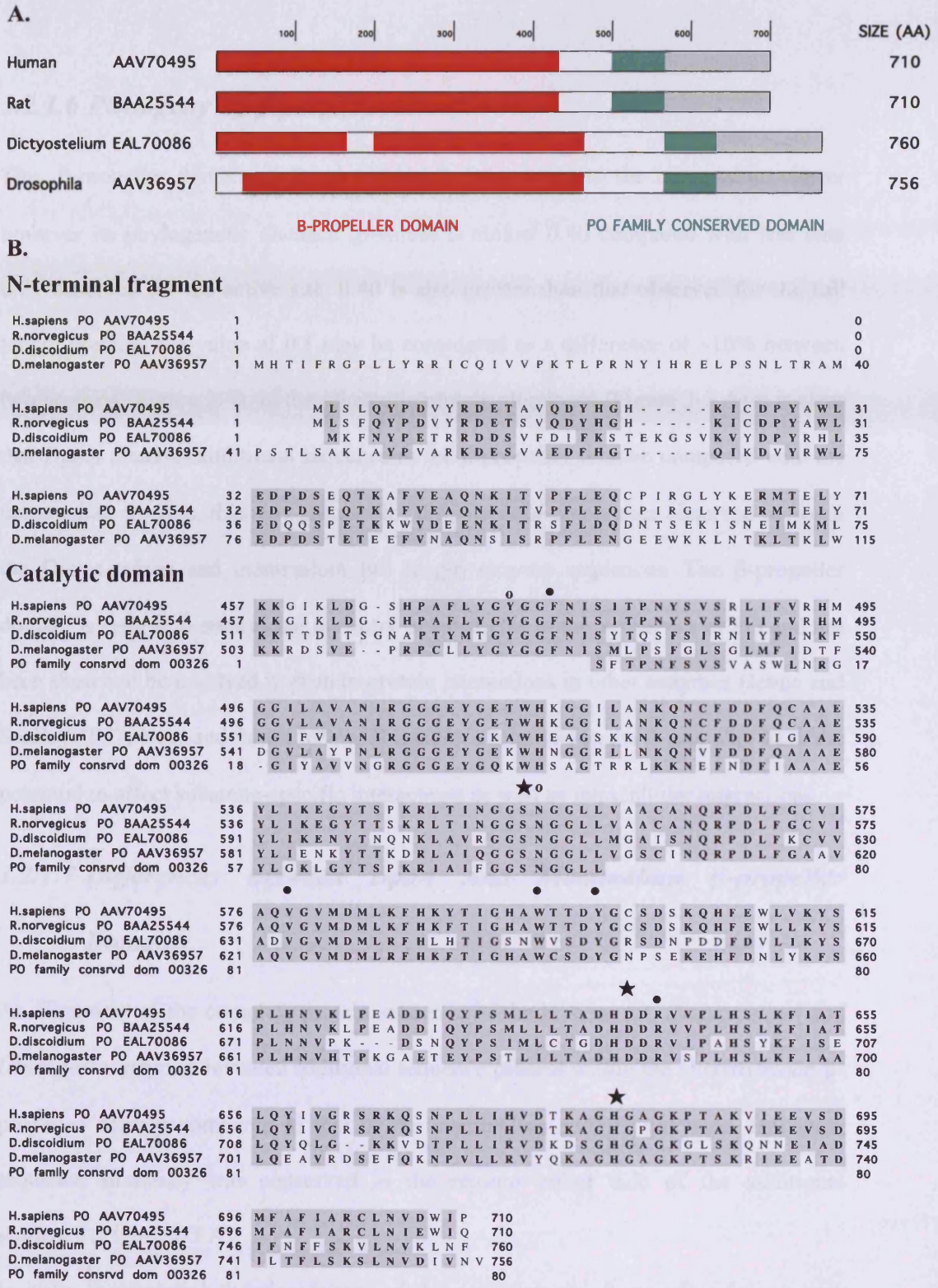


Figure 3-3 Full Length and Active Site Alignment.

A. Schematic of Full Length Protein Alignment of Mammalian, *Dictyostelium* and *Drosophila* PO. Regions shaded in green represent the PO conserved domain, red the β-propeller domain and grey sequence similarity outside of the specific conserved domains. Unshaded regions represent areas of poor sequence similarityy between the proteins. **B. Sequence alignment of the α/β hydrolase fold domain.** Salient residues are marked; ★ = active site residues, o = oxyanion binding site, ● = proline binding pocket. Homologous and similar amino acid residues as defined by MacVector® using the ClustalW grouping method are shaded grey.

3.2.1.6 Phylogeny Of β-propeller Domain

The β-propeller domain of DpoA retains its association to the mammalian cluster however its phylogenetic distance (p-value) is almost 0.40 compared with less than 0.25 observed for the active site. 0.40 is also greater than that observed for the full length protein. A p-value of 0.1 may be considered as a difference of ~10% between two proteins (Figure 3-4). In the schematic protein alignment (Figure 3-3 A) it is clear that DpoA contains additional sequence in the β-propeller domain compared with the mammalian enzyme, this may account for the increased divergence observed between the *Dictyostelium* and mammalian full length enzyme sequences. The β-propeller domain is central to controlling the entry of substrates to the active site of PO and has been shown to be involved in protein-protein interactions in other enzymes (Jenne and Stanley 1987; Neer and Smith 1996). Therefore, alterations to the β-propeller have the potential to affect substrate-specific interactions as well as intracellular interactions.

3.2.1.7 Differences Between DpoA And Mammalian β-propeller Domains

An alignment of the complete protein sequence of the human, rat, *Dictyostelium* and *Drosophila* enzymes revealed additional sequence present within the *Dictyostelium* β-propeller domain compared to that of the mammalian and fly enzymes. However, sequence similarity was conserved in the regions either side of the additional sequence (Figure 3-3 A).

In order to determine the significance of this insertion, the β-propeller domain was considered in terms of the seven individual blades; each of which is a four stranded β-sheet. An insertion in the loop between two blades would be expected to have a much

smaller impact on the shape of the domain than one in the centre of a β-sheet. The *Dictyostelium* β-propeller domain was modelled on the structure described by Fulop et al. using a secondary structure prediction program and crystal structure analysis (Fulop et al. 1998). According to this model, the *Dictyostelium* enzyme has additional amino acids in between the 3rd and 4th strands of both the 1st and 2nd blades (Figure 3-5 and Figure 1-2). It is difficult to predict the effect of these amino acids, however, their position towards the outside of the propeller domain may allow them to form loops without affecting the β-sheet.

The hydrophilic loop between the 2nd and 3rd strands of blade 3, which interacts with the catalytic domain by hydrogen bonding, is also conserved in the *Dictyostelium* enzyme (Figure 3-5).

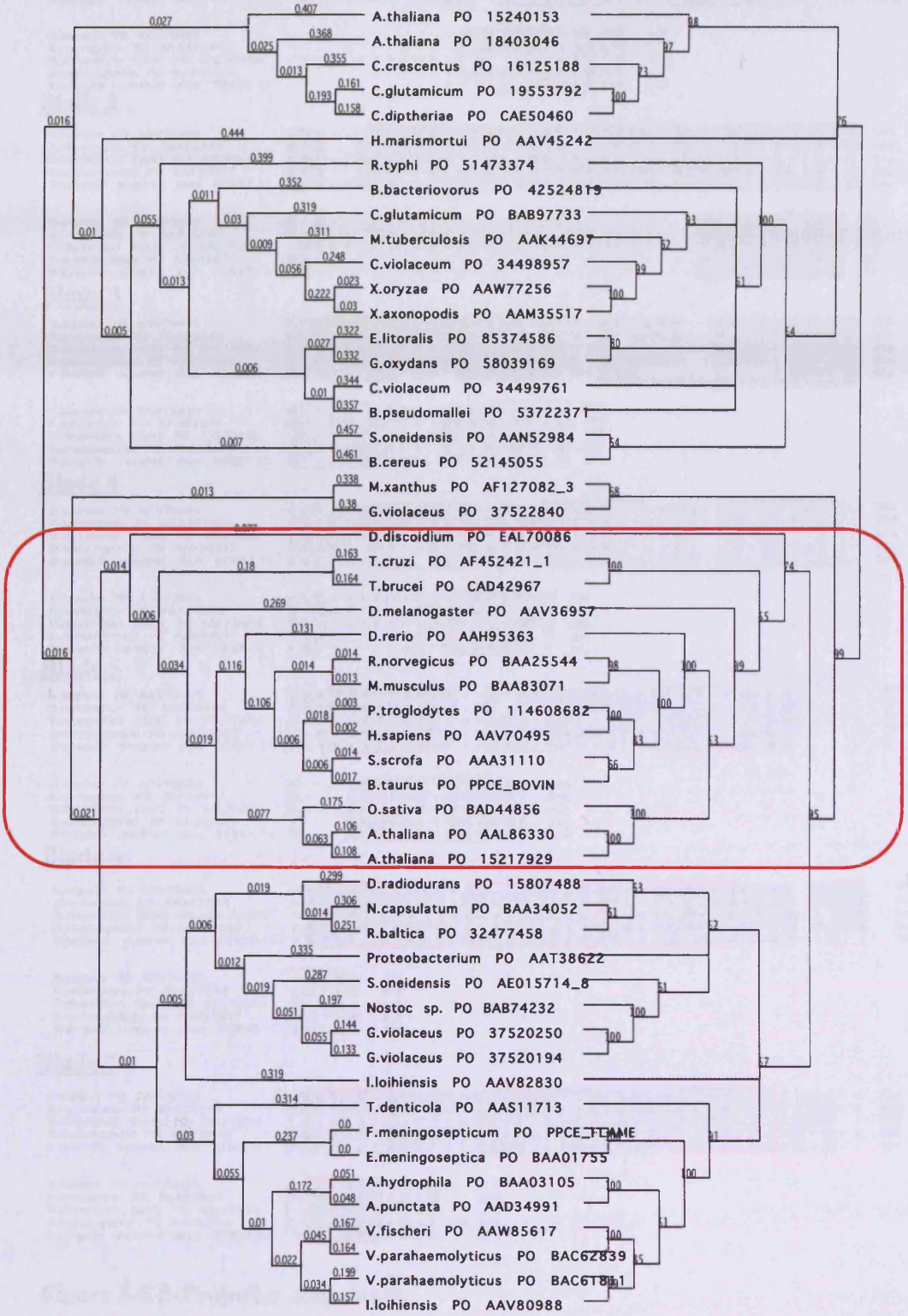


Figure 3-4 β-Propeller Phylogeny.

Phylogenetic analysis of the β-propeller domain of all available PO sequences in the Entrez database. Branches to the left represent the best tree analysis of the sequences and show phylogenetic distance (p-values), those to the right represent Bootstrap analysis compiled of 1000 repeats, these branches are labelled with confidence levels. Both human and *Dictyostelium* PO are present in the cluster outlined in red.

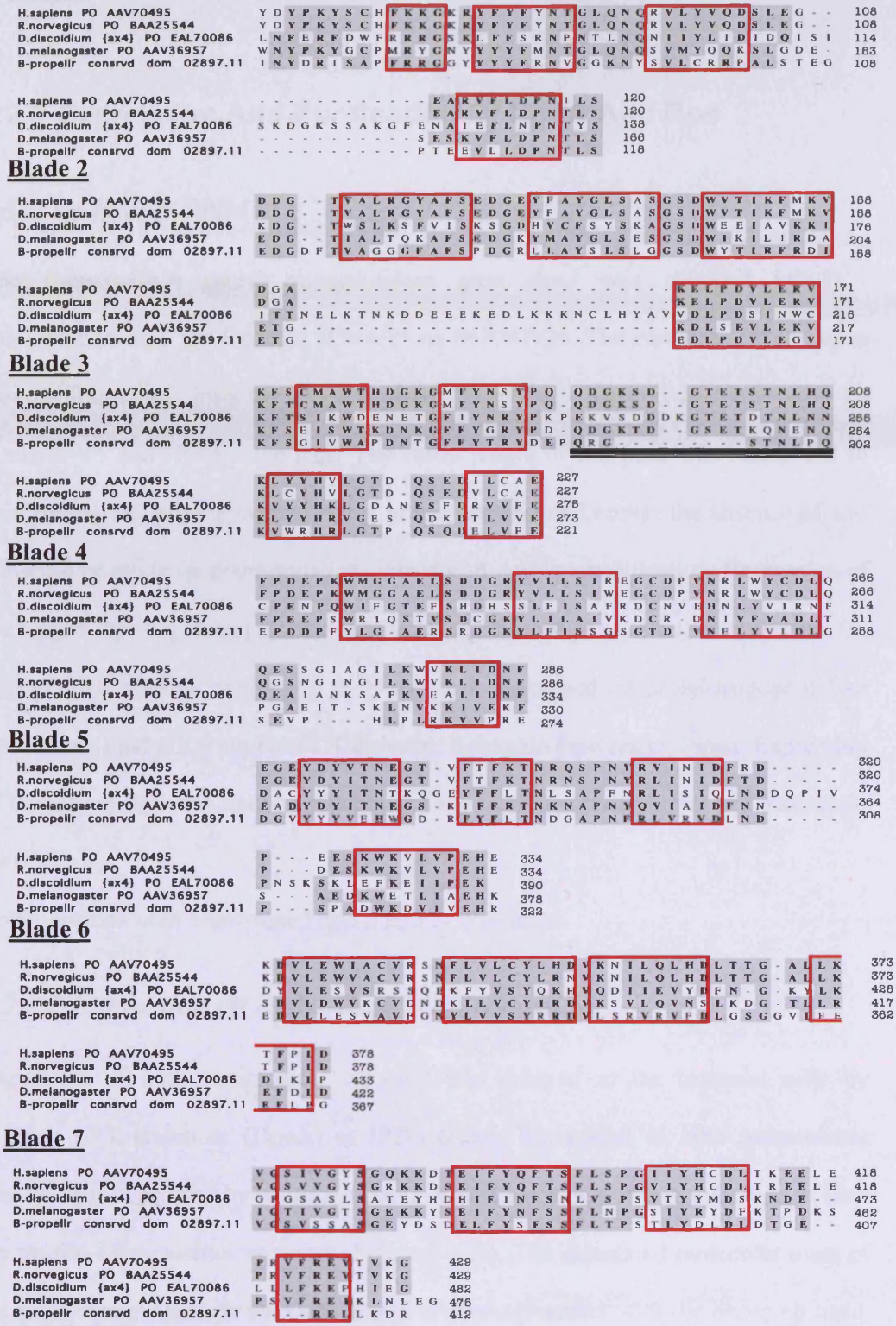


Figure 3-5 β-Propeller Alignment.

Alignment of the amino acid sequences of the β-propeller domains of the mammalian, *Dictyostelium* and *Drosophila* enzymes. The sequence is divided into the seven individual β-sheets forming the seven blades. Each β-strand is outlined in red, the hydrophilic loop involved in interdomain interaction is underlined by a double black line. Homologous and similar amino acid residues as defined by MacVector® using the ClustalW grouping method are shaded grey.

3.2.2 Expression And Purification Of DpoA And Rpo

3.2.2.1 Cloning DpoA

The *Dictyostelium* prolyl oligopeptidase gene *dpoA* was obtained by PCR amplification from the Japanese cDNA clone DDC47a24. The complete 2283bp open reading frame was inserted into the pBAD TOPO TA plasmid in frame with C-terminal V5 and His epitope tags. The entire length of the gene was sequenced to confirm the sequence agreed with that in the database and ensure the absence of any nonsense or missense point mutations introduced during amplification. Expression of the tagged DpoA was under the control of the arabinose promoter (See Appendix 0). The pTrcHis plasmid expressing an N-terminal His-tagged rat prolyl oligopeptidase (*rpo*) was a kind gift from Prof T.Takahashi, Hokkaido University, Japan. Expression of the tagged Rpo was under the control of the trc promoter (Kimura and Takahashi 2000).

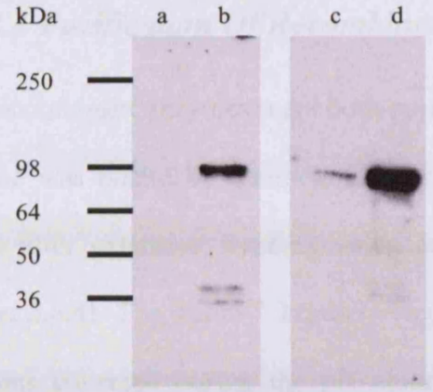
Both plasmids were transformed into *E.coli* TOP10 cells.

3.2.2.2 Expression Of Active Prolyl Oligopeptidase

Expression of both recombinant enzymes was induced in the bacterial cells by addition of L-arabinose (DpoA) or IPTG (Rpo). Expression of both recombinant proteins was confirmed by western blotting, which revealed a strong induction of both the rat and *Dictyostelium* enzymes (Figure 3-6 A). The calculated molecular mass of the recombinant DpoA is 90kDa which is in clear agreement with the observed band. The band observed for the recombinant Rpo also correlates with the molecular mass of 75kDa reported by Kimura and Takahashi (Kimura and Takahashi 2000).

Enzyme activity assays of total cell extract following induction of cells transformed with plasmid encoding the recombinant protein or empty plasmid confirmed the activity of the recombinant protein. Enzyme activity was measured by the appearance of the yellow-coloured p-nitroaniline, which is released from the substrate Z-Gly-Pro-pNA following cleavage after the proline residue (Figure 3-6 B). Activity is recorded in enzyme units (U); one enzyme unit (1 U) is defined as the amount of enzyme required to produce 1μmole of product per minute.

A.



B.

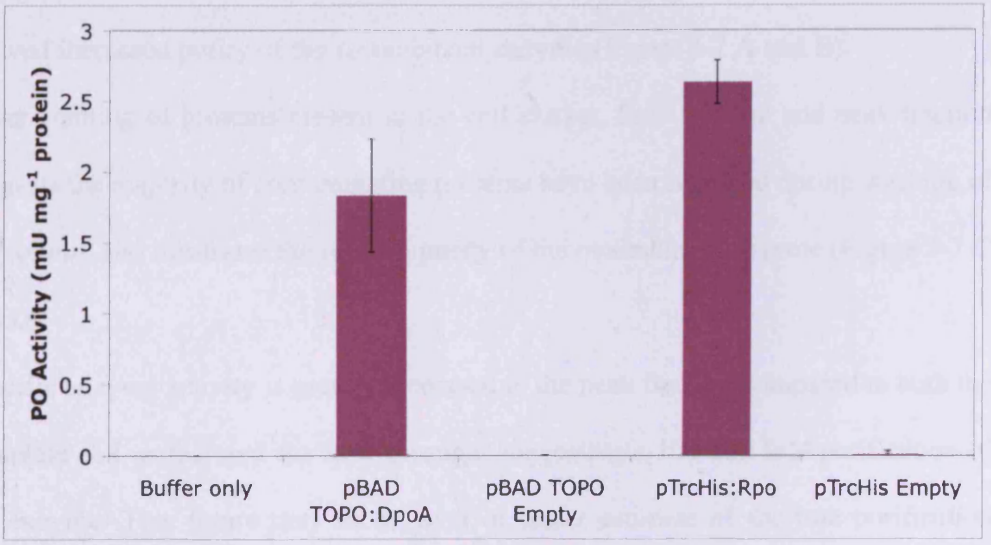


Figure 3-6 Expression of Active Tagged DpoA and Rpo

A. Induction of Active Tagged DpoA and Rpo. Western blot of 10µl cell extract from bacteria transformed with pBAD TOPO;DpoA uninduced, a, or induced with L-arabinose, b, and pTrcHis;Rpo uninduced, c, or induced with IPTG, d. Western blot probed using anti-V5:HRP antibody, a&b or anti-6X:HRP antibody, c&d. **B. Activity of Expressed enzyme.** PO activity in cell extracts of bacterial cells transformed with plasmid expressing the tagged enzyme compared with bacterial cells transformed with empty plasmid. pBAD TOPO plasmid containing cells were induced with L-arabinose, pTrcHis plasmid containing cells were induced with IPTG. PO activity was assayed in HEPES buffer using the substrate Z-Gly-Pro-pNA. Values are mean of two repeats done in duplicate ± SEM.

3.2.2.3 Purification Of Recombinant Prolyl Oligopeptidase

The recombinant enzymes were both purified using Talon™ metal affinity resin. The enzyme was bound by interaction between the His tag and the cobalt beads then eluted after extensive washing using a pH gradient (see Materials and Methods section 2.6.4). The Talon™ beads were packed into a column set up for FPLC. The fractions collected during the pH elution were tested for the presence of PO by assaying the enzyme activity. Analysis of the fraction showing the maximum activity (the peak fraction) alongside the total extract and flow through (i.e. unbound extract) showed increased purity of the recombinant enzyme (Figure 3-7 A and B).

Silver staining of proteins present in the cell extract, flow through and peak fraction suggests the majority of contaminating proteins have been removed during washing of the column and illustrates the relative purity of the recombinant enzyme (Figure 3-7 C and D).

Specific enzyme activity is greatly increased in the peak fraction compared to both the complete cell extract and the flow through, suggesting a 100-200 fold purification of the enzyme. This figure may be an over or under estimate of the true purification factor if any inhibitory or stimulatory factors have been removed.

Presence of a significant amount of enzyme activity in the flow through indicates that the Talon™ column was saturated with enzyme enabling some to pass through without binding (Figure 3-7 & Table 3-1). This helps to minimise non-specific binding and binding of naturally His-rich proteins, which should be present at much lower concentrations than the induced recombinant protein.

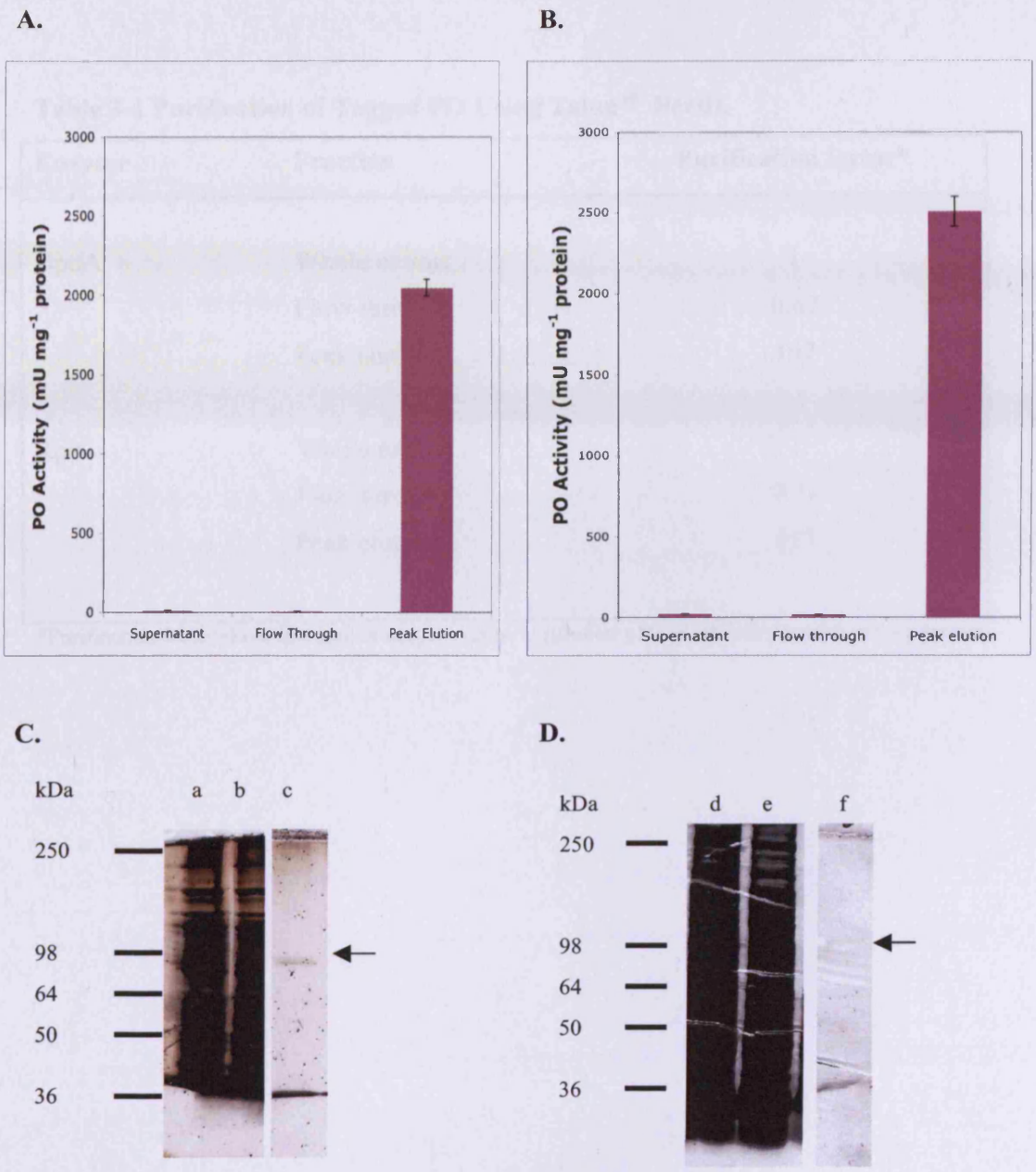


Figure 3-7 Purification of Tagged Enzyme

A & B. Activity of Purified Enzyme. *E.coli* expressing tagged DpoA, A and Rpo, B. PO activity of bacterial cell extracts before loading onto Talon™ beads, flowthrough following binding to Talon™ beads and peak fraction eluted from beads. PO activity was measured in HEPES buffer (pH 7.8) using the substrate Z-Gly-Pro-pNA. **C & D. Purification of the Tagged Enzyme.** Silver stain of purified DpoA, C and Rpo, D. Silver stain of protein content in bacterial cell extract expressing tagged PO columns a and d, flow through following binding of enzyme to Talon™ beads columns b and e and eluted fraction containing maximum PO activity columns c and f. Arrow indicates the position of the tagged enzyme.

Table 3-1 Purification of Tagged PO Using Talon™ Beads.

Enzyme	Fraction	Purification factor*
DpoA	Whole extract	1
	Flow through	0.67
	Peak elution	197
Rpo	Whole extract	1
	Flow through	0.78
	Peak elution	117

*Purification factor=Fold increase in enzyme units, U (μmoles pNA produced per min) per mg protein.

3.2.3 Activity Of DpoA

3.2.3.1 PO Enzyme Assay

All further enzyme assays were completed using a stock of purified recombinant enzyme produced by pooling the eluted fractions containing significant enzyme activity (see Materials and Methods section 2.5.4).

Initial assays were carried out using DpoA across a pH range from 5.0 to 9.0, using 100mM Propionate, MES, HEPES and Tris-HCl buffers all at an ionic strength of 154mM (physiological ionic strength). Activity was measured by cleavage of the substrate Z-Gly-Pro-AMC at a temperature of 25°C. Appearance of the fluorescent product, 7-amino-4-methylcoumarin (AMC), was monitored at 10 minute intervals (Figure **3-8**).

The pH profile revealed a clear peak of activity around pH 8 and very little variation in enzyme activity when measured in the different buffers at the same pH. PO activity assays were subsequently carried out in HEPES buffer at pH 7.8. A pH of 7.8 was used in PO activity assays when DpoA activity was first reported (Williams et al. 1999), this pH is within the range of optimal PO activity and close to physiological pH (7.4) of both mammalian and *Dictyostelium* cells (Martin et al. 1987).

A curve of best fit was added to the pH profile data produced using the equation, pH optimum (Equation 3-1) (Rob John, personal communication).

Equation 3-1

$$v = V_{max}/(((1+10^{-pKa1})/10^{-pH})+(10^{-pH}/10^{-pKa2}))$$

A curve of best fit was achieved with the parameters V_{max} 0.238μM min^{-1}, pKa_1 8.93 and pKa_2 7.68.

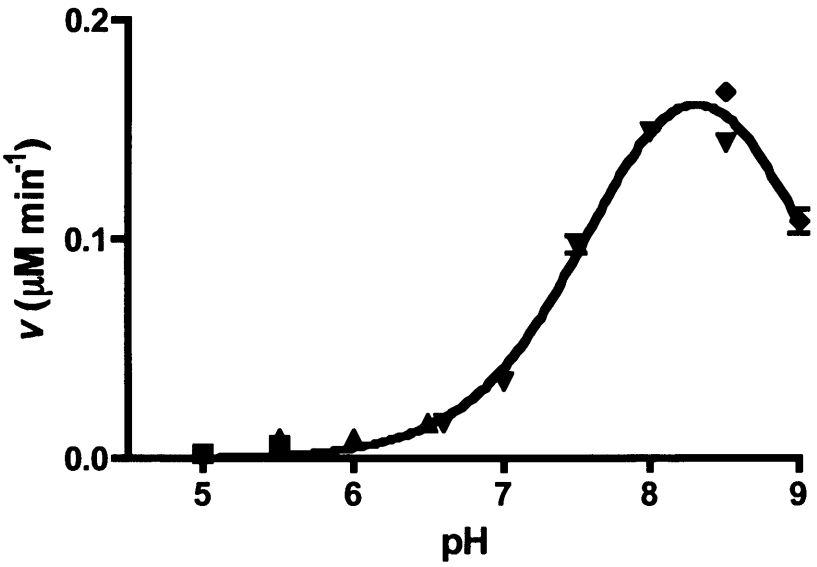


Figure 3-8 Effect of pH on DpoA Activity.

DpoA activity was measured at pH 5.0 to 9.0 in 100mM Propionate ■, MES ▲, HEPES ▼ or Tris-HCl ◆ buffer at 25°C. All buffers were maintained at an ionic strength of 154mM and contained 141μM PO substrate, Z-Gly-Pro-AMC. *v* represents the initial rate measured between 0 and 20 minutes. Points represent an average of three repeats ± standard deviation. Curve of best fit produced using equation; pH optimum.

DpoA activity was assayed across a range of substrate concentrations from 2.2 to 140μM. These activity assays were incubated in the luminometer at 25°C and the increase in fluorescence measured at 10 minute intervals (Figure 3-9 A & B)

Enzyme velocity, v (μM/min), was calculated for each substrate concentration at every time point where appearance of product was in a linear range and product represented less than 20% of the substrate. Values plotted represent average v at each substrate concentration ± standard deviation, [S] = substrate concentration, (Figure 3-9 C).

Values for K_M and V_{max} were calculated by Prism© software using the Michaelis-Menten equation (Equation 3-2).

Equation 3-2

$v = V_{max} * [S] / (K_M + [S])$

Results are given plus and minus standard error.

$K_M = 31 \pm 1.5$ μM

$V_{max} = 0.175 \pm 0.003$ μM/min.

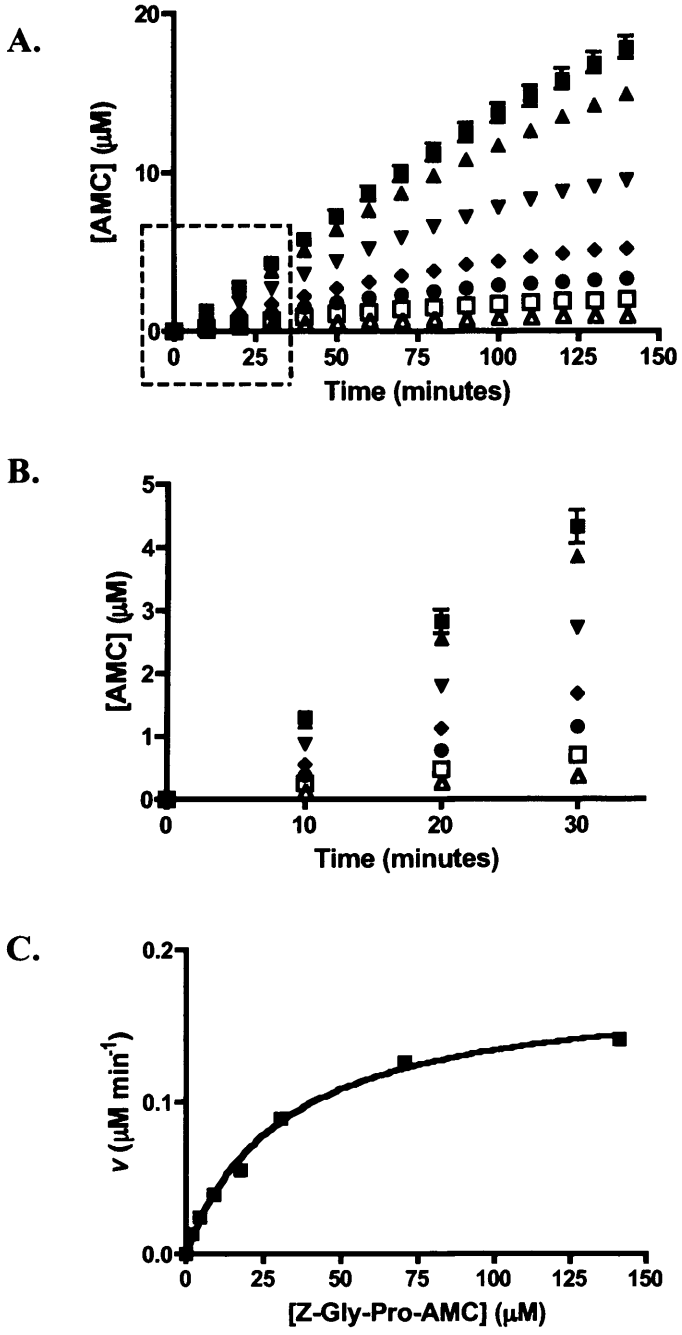


Figure 3-9 DpoA Enzyme Activity.

A & B, DpoA activity was assayed in 100mM HEPES buffer (pH 7.8) at 25°C across a range of substrate (Z-Gly-Pro-AMC) concentrations 141μM ■, 70.5μM ▲, 35.3μM ▼, 17.6μM ◆, 8.8μM ●, 4.4μM □, 2.2μM △. Appearance of the fluorescent product AMC was measured at 10 minute intervals. Points represent an average of three repeats ± standard deviation. Graph B shows the points on graph A indicated by a dashed box at an increased magnification.

C, v (μM/min) was calculated for each substrate concentration at every time point where appearance of product was in a linear range and product represented less than 20% of the substrate. Values plotted represent average v at each substrate concentration ± standard deviation.

3.2.3.2 Mammalian PO Shows Similar Kinetics To DpoA

A pH profile of purified recombinant Rat PO (Rpo) activity from pH 5.0 to 9.0 was measured as described above. The mammalian enzyme showed a very similar peak in activity around pH 8.0 to that seen with the *Dictyostelium* enzyme (Figure **3-10**). A curve of best fit was produced using the equation pH optimum (Equation 3-1), with the parameters V_{max} 0.251μM min^{-1}, pKa_1 9.54 and pKa_2 7.59.

Rpo activity was also assayed across a range of substrate concentrations from 2.2 to 140μM as described for DpoA (Figure 3-11 A & B).

Again the enzyme velocity, v (μM/min), was calculated for each substrate concentration at every time point where appearance of product was in a linear range and product represented less than 20% of the substrate. The data points plotted represent average v at each substrate concentration ± standard deviation (Figure 3-11 C).

Values of K_M and V_{max} for Rpo calculated by Prism© software using the Michaelis-Menten equation (Equation 3-2) were very similar to the K_M and V_{max} of DpoA.

Results are given plus and minus standard error.

$K_M = 36 \pm 3.3$ μM

$V_{max} = 0.210 \pm 0.007$ μM/min.

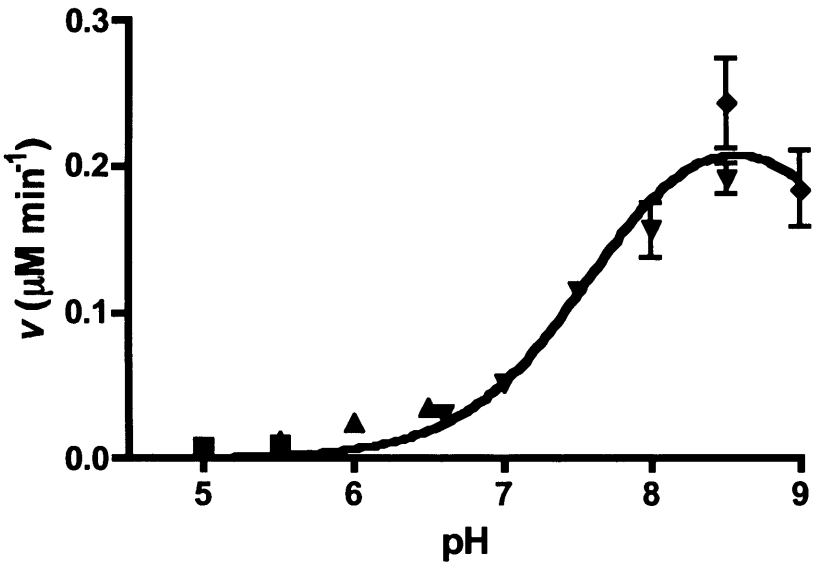


Figure 3-10 Effect of pH on Rpo Activity.

Rpo activity was measured at pH 5.0 to 9.0 in 100mM Propionate ■, MES ▲, HEPES ▼ or Tris-HCl ◆ buffer at 25°C. All buffers were maintained at an ionic strength of 154mM and contained 141μM PO substrate, Z-Gly-Pro-AMC. *v* represents the initial rate measured between 0 and 20 minutes. Points represent an average of three repeats ± standard deviation. Curve of best fit produced using equation pH optimum.

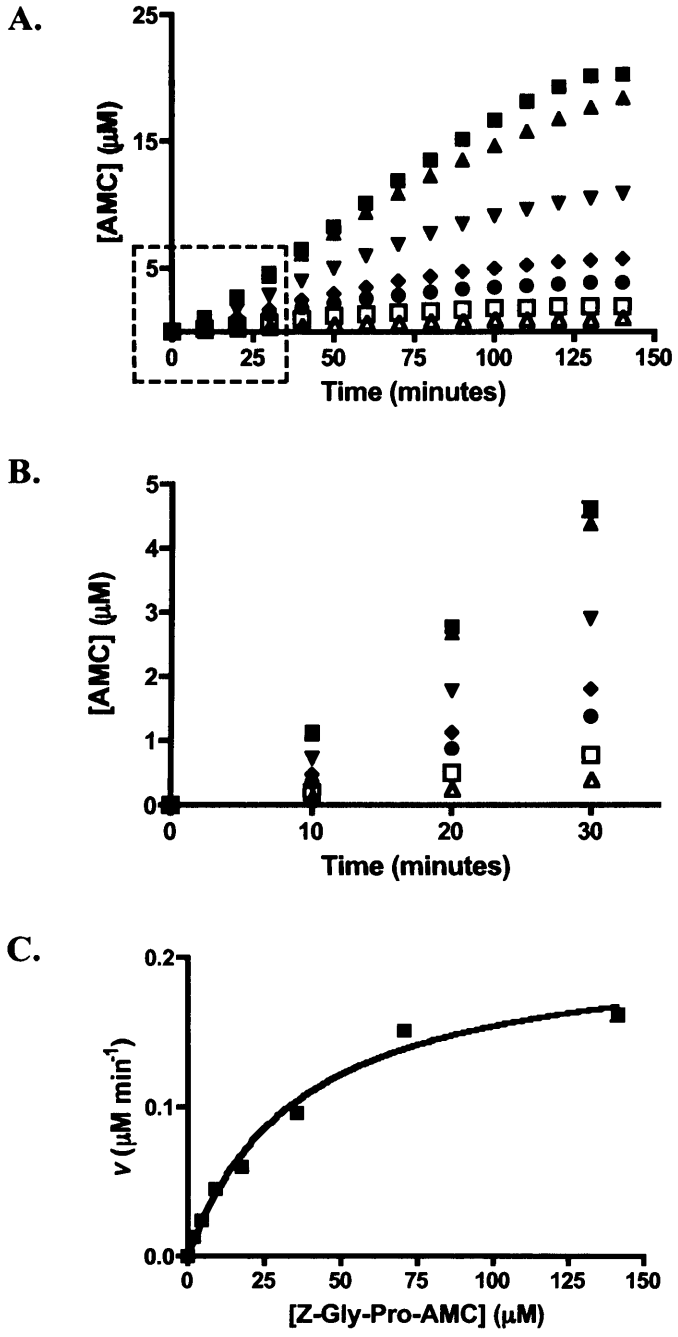


Figure 3-11 Rpo Enzyme Activity.

A & B, Rpo activity was assayed in 100mM HEPES buffer (pH 7.8) at 25°C across a range of substrate (Z-Gly-Pro-AMC) concentrations 141μM ■, 70.5μM ▲, 35.3μM ▼, 17.6μM ◆, 8.8μM ●, 4.4μM □, 2.2μM △. Appearance of the fluorescent product AMC was measured at 10 minute intervals. Points represent an average of three repeats ± standard deviation. Graph B shows the points on graph A indicated by a dashed box at an increased scale.

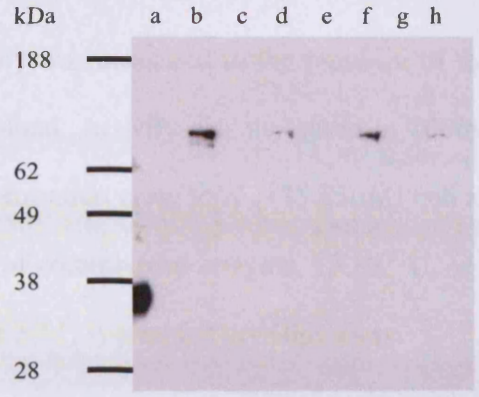
C, v (μM/min) was calculated for each substrate concentration at every time point where appearance of product was in a linear range and product represented less than 20% of the substrate. Values plotted represent average v at each substrate concentration ± standard deviation.

3.2.3.3 The Active Site Triad Are Required For DpoA Activity

The identity of the active triad identified by sequence alignment in the *Dictyostelium* PO was tested by observation of enzyme activity following mutation of these three amino acids. Site-directed mutagenesis was used on the pBAD TOPO;DpoA vector to produce plasmids encoding 3 mutant versions of the recombinant DpoA; S609A, D693G and H730A each of which lacks one member of the putative catalytic Ser-Asp-His triad respectively. The mutagenesis was confirmed by sequencing and mutant DpoA was expressed in TOP10 cells as before. Expression of the mutant protein was induced using L-arabinose and presence of the tagged protein was confirmed by western blotting (Figure 3-12 A).

Enzyme assay results using the total cell extract revealed a total loss of Z-Gly-Pro-pNA hydrolysing activity in the absence of any one of the catalytic triad amino acids, supporting a role for these amino acids (Figure 3-12 B). It suggests that all three residues are required for enzyme activity however it is also possible that the loss of enzyme activity was a result of protein mis-folding caused by the mutations,

A.



B.

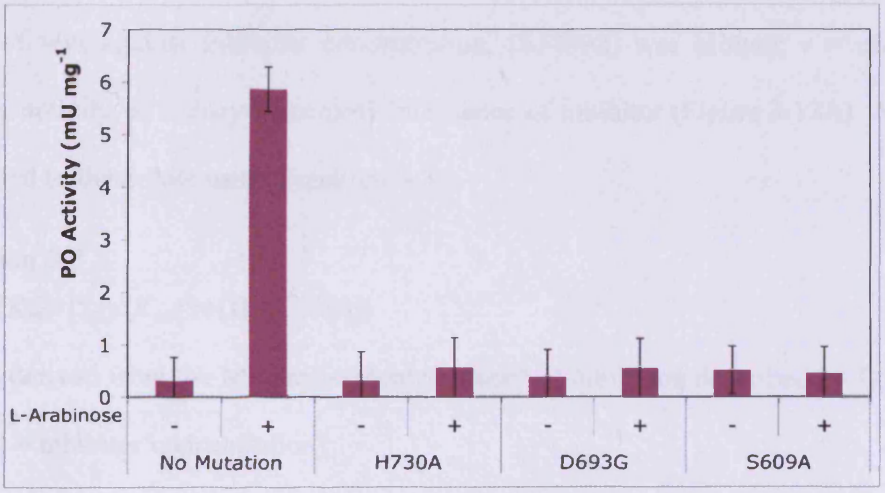


Figure 3-12 Induction and Activity of Mutant DpoA

A. Induction of Mutant, Tagged DpoA. Western blot of bacterial cells expressing tagged DpoA mutated at the catalytic triad; no mutation, lanes a&b, H730A, lanes c&d, D693G, lanes e&f and S609A, lanes g&h. Bacterial extracts were taken before, a,c,e&g and after, b,d,f&h, induction of protein expression with L-arabinose. Western probed using anti-V5:HRP Ab. **B. Activity of Mutant Protein.** PO activity assays of bacterial cell extracts expressing tagged DpoA containing mutations in the catalytic triad. Cell extracts were prepared with and without L-arabinose induction. PO activity was assayed in HEPES buffer using substrate Z-Gly-Pro-pNA. Results are average of two repeats done in duplicate ± standard deviation

3.2.4 Inhibition Of DpoA By Two Specific Inhibitors

Enzyme activity was measured in the presence of the PO specific inhibitors S17092 and Z-Pro-Prolinal. Activity was measured in 100mM HEPES buffer (pH 7.8), at a substrate concentration close to K_M (35.25μM) and an assay time of 20 minutes. The same amount of recombinant enzyme, $1X10^{-5}$ U, as used in previous assays (Figure 3-9 and Figure 3-11) was added per 60μl assay.

3.2.4.1 Inhibition By S17092

PO activity was measured in the presence of S17092 from 0.1-1nM. An inhibition curve of v/v_0 against inhibitor concentration, [S17092] was plotted: v = observed enzyme activity, v_0 = enzyme activity in absence of inhibitor (Figure 3-13A). A curve was fitted to these data using Equation 3-3;

Equation 3-3

$v/v_0 = (K_M + [S])/(K_M(1+[I]/K_i) + [S])$

This is derived from the Michaelis-Menten model of inhibition described by Equation 3-4 ([I] = inhibitor concentration);

Equation 3-4

$v = (V_{max}[S]) / (K_M(1+[I]/K_i) + [S])$

The observed data show a systematic diversion from the curve fit using this model. This can be illustrated more clearly by a plot of the residuals i.e. the difference between the experimental points and the theoretical points predicted by the curve fit by Equation 3-3 (Figure 3-13 B). This shows a clear pattern of diversion rather than random deviation from the model.

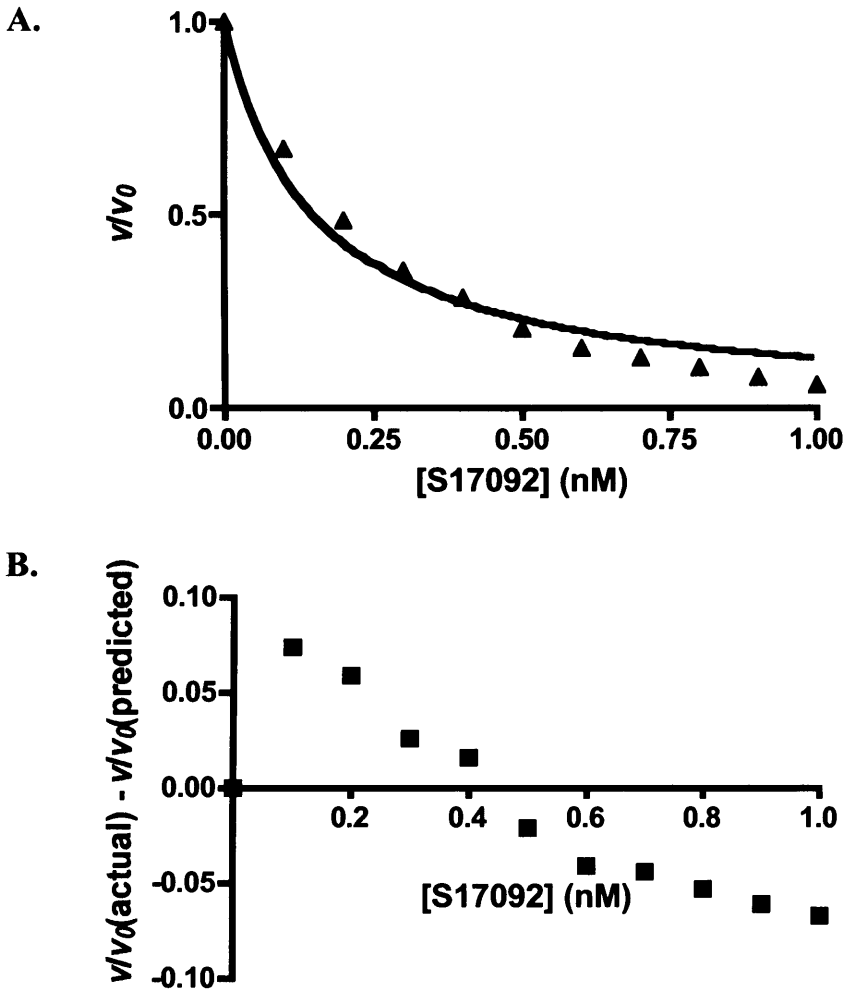


Figure 3-13 Inhibition of DpoA by S17092; Michaelis-Menten Model.

A. $1X10^{-5}$ U DpoA enzyme activity was assayed in a final volume of 60µl HEPES buffer (pH 7.8) at 25°C, in the presence of inhibitor S17092 at concentrations from 0.1 to 1nM. The reaction rate v (µM increase in product (AMC) min^{-1}) was measured at 20 minutes, $v_0 = v$ when [I] = 0. The Michaelis-Menten model of inhibition was used to fit a curve to these data. **B.** A plot of the residuals i.e. difference between the observed data and data points given by the Michaelis-Menten model.

3.2.4.2 Tight Binding Inhibition By S17092

Inhibition as modelled using the Michaelis-Menten model assumes that the free concentration of inhibitor is not significantly reduced by formation of the enzyme-inhibitor complex. This assumption is not true in the case of tight binding inhibition where the enzyme-inhibitor complex may represent a significant amount of the total inhibitor concentration. S17092 has previously been described as a potent and long lasting inhibitor (Portevin et al. 1996). It is shown here that S17092 inhibition of PO is consistent with that of a tight binding inhibitor.

Low concentrations of inhibitor (nanomolar range) were needed to produce the inhibition observed, therefore, an equation derived for tight-binding inhibition was used to fit the same data. Derivation of this more complex equation (Equation 3-5) takes into account the depletion of free inhibitor concentration by formation of the enzyme-inhibitor complex (Henderson 1972). The Henderson equation for competitive inhibition;

Equation 3-5

$$[I]/(1-v/v_0) = E_o+K_i(([S]+K_M)/K_M)v_0/v$$

E_o = total enzyme concentration. The Henderson equation can be rearranged to be given in terms of v/v_0 (Equation 3-6).

Equation 3-6

$$v/v_0=\left(-([I]-E_o+(([S]+K_M)/K_M)*K_i)+\sqrt{(([I]-E_o+(([S]+K_M)/K_M)*K_i)^2+4*E_o*(([S]+K_M)/K_M)*K_i)}\right)/(2*E_o)$$

Known values for [S] (35.25μM) and previously calculated values K_M (30.8μM) were included in the equation before the curve was fitted using Prism© software.

The curve representing tight binding inhibition provides a much better representation of the inhibition data observed in the presence of S17092 (Figure 3-14 A). A plot of the residuals from this model indicates small non-systematic deviation from the tight binding model (Figure 3-14 B).

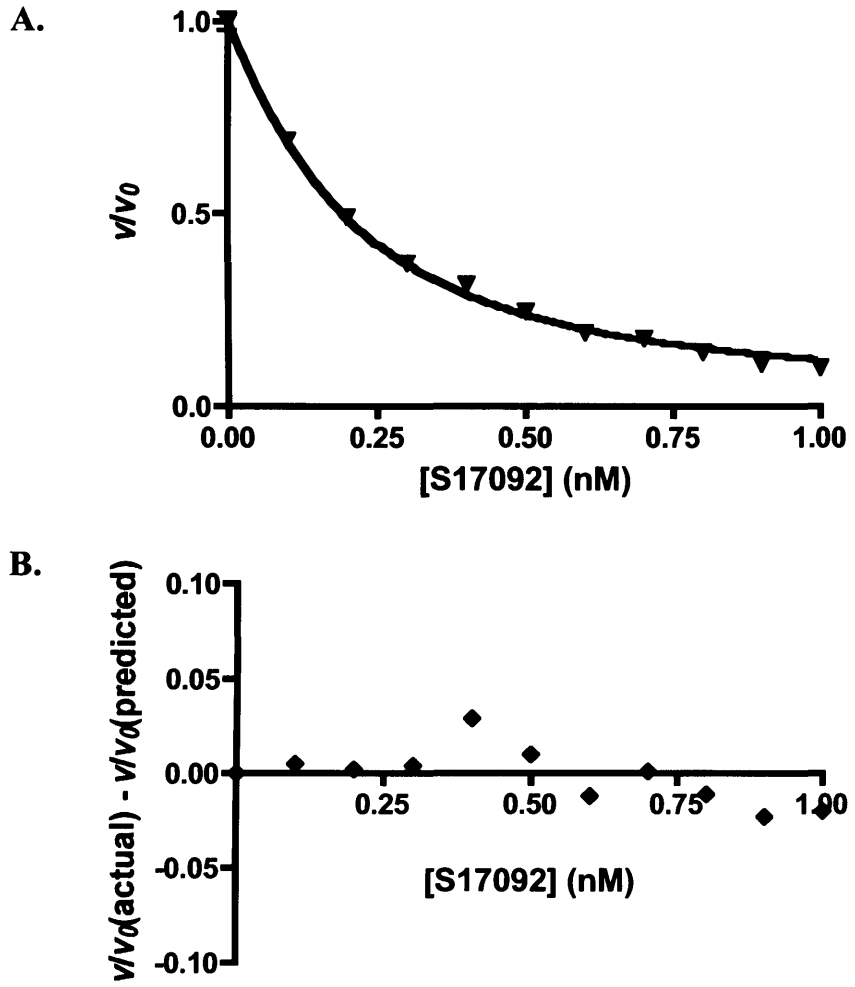


Figure 3-14 Inhibition of DpoA by S17092; Tight Binding Model.

A. $1X10^{-5}$ U DpoA enzyme activity was assayed in a final volume of 60µl HEPES buffer (pH 7.8) at 25°C, in the presence of inhibitor S17092 at concentrations from 0.1 to 1nM. The reaction rate v (µM increase in product (AMC) min^{-1}) was measured between 0 and 20 minutes, $v_0 = v$ when [I] = 0. The tight binding model of inhibition was used to fit a curve to these data. **B.** A plot of the residuals i.e. difference between the observed data and data points given by the tight binding model.

The model of tight binding inhibition also calculates the concentration of enzyme present in the assay. This figure can be more accurately calculated at higher enzyme concentrations. When E_o is significantly greater than the concentration of a tight binding inhibitor, a linear relationship is observed between [I] and v from which the enzyme concentration may be extrapolated (Henderson 1972). This may be illustrated by theoretical tight binding curves showing increasing concentrations of enzyme (Figure 3-15).

Because of assay limitations it was not possible to increase the concentration of enzyme above four times ($4X10^{-5}$ U enzyme activity) that of the normal assay because of the enzyme volume that would need to be added to the assay. At four times enzyme concentration the assay remained in the linear range, product always represented less than 20% of the total substrate concentration. A curve fitted to these data using the tight binding inhibition equation (Equation 3-6) by Prism© software returned a value for the total active enzyme concentration (E_o) of 0.58 ±0.04nM (Figure 3-16 A). From this it is possible to estimate the total active enzyme concentration for all assays containing $1X10^{-5}$U enzyme activity to be 0.15nM

The accuracy of any model is increased by replacing as many variables as possible with known values. The calculated value for [E_o] and K_M were entered into the tight binding equation before values for K_i were calculated, again using Prism© software, at 4 different substrate concentrations (Figure 3-16 B).

The average value returned for the K_i of S17092 was 58pM, indicating S17092 is a very efficient inhibitor of Dpo (Table 3-2).

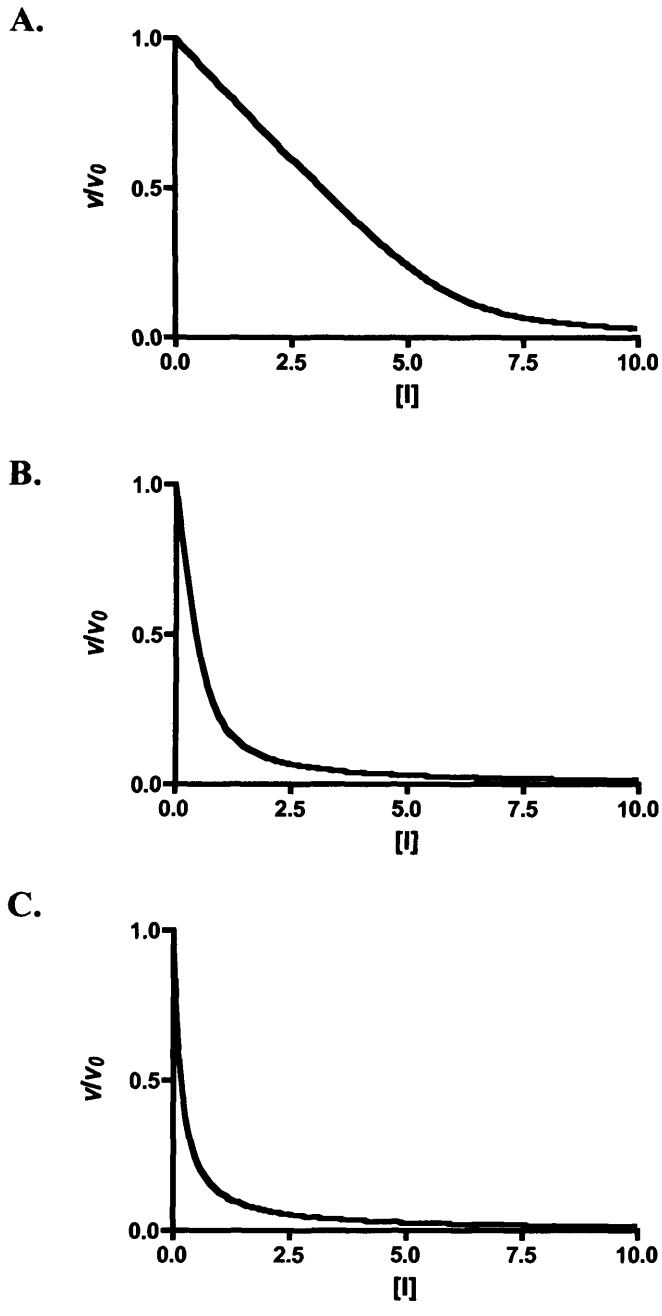


Figure 3-15 Effect Of Enzyme Concentration On Tight Binding Inhibition

Family of theoretical curves illustrating effect of increased enzyme concentration. Curves generated using the tight binding inhibition equation (See Equation). 150 points were calculated at inhibitor concentration [I] values ranging from 0.0 to 10.0. [S] = K_M, K_i =0.07, **A.** E_o=6, **B.** E_o=0.6, **C.** E_o=0.06.

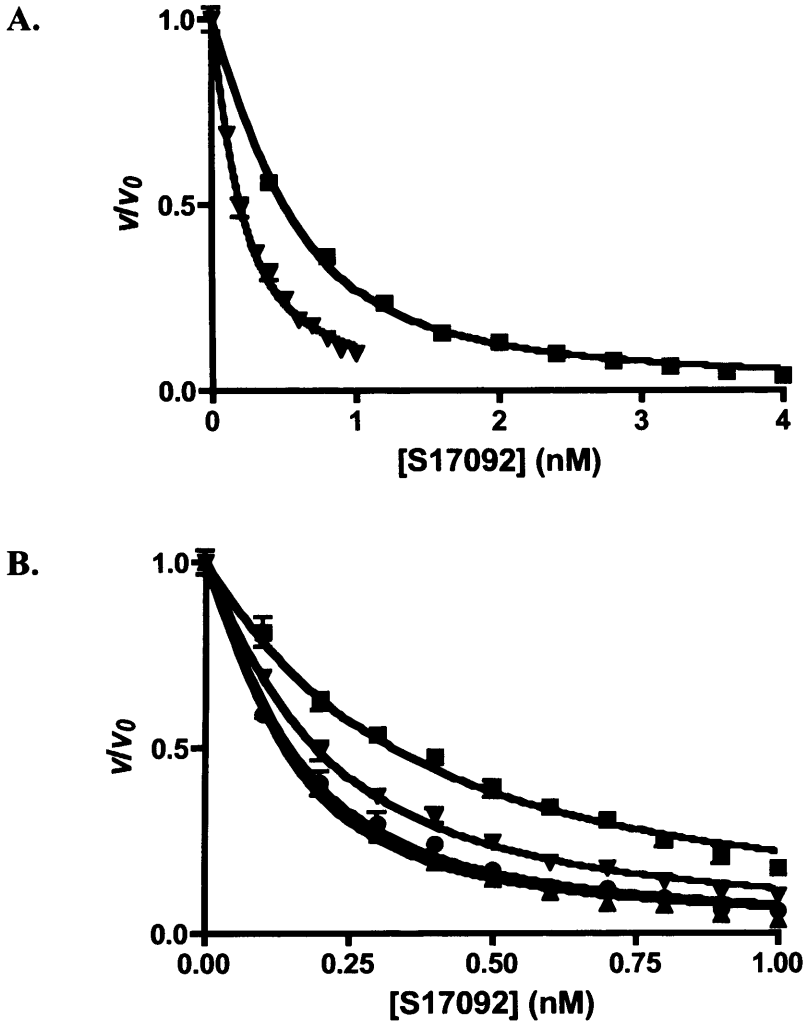


Figure 3-16 Inhibition Of DpoA By S17092.

A. $4X10^{-5}$ U , ■, and $1X10^{-5}$ U, ▼, DpoA enzyme activity were assayed in a final volume of 60µl HEPES buffer (pH 7.8) at 25°C, in the presence of inhibitor S17092 at concentrations ranging from 0.1 to 4nM. The reaction rate v (µM increase in product (AMC) min^{-1}) was measured between 0 and 20 minutes: $v_0 = v$ when [I] = 0. The tight binding model of inhibition was used to fit a curve to these data and estimate the concentration of total active enzyme. **B.** S17092 inhibition was measured across a range of substrate ,Z-Gly-Pro-AMC, concentrations, ■ 70.5µM, ▼ 35.25µM, ● 17.6µM, ▲ 8.8µM. $1X10^{-5}$ U DpoA enxyme activity added per assay. Results represent mean ± standard deviation.

Table 3-2 K_i of S17092 for DpoA

Substrate (Z-Gly-Pro-AMC) Concentration In Assay (μM)	**K_i (pM)**
70.5	77 ±3.0
35.25	56 ±1.4
17.6	48 ±1.8
8.8	50 ±2.0
Average value of K_i	**58**

3.2.4.3 Inhibition By Z-Pro-Prolinal

PO activity was measured in the presence of Z-Pro-Prolinal from 1-10μM. An inhibition curve of v/v_0 against inhibitor concentration, [Z-Pro-Prolinal] was plotted. The inhibition of DpoA by Z-Pro-Prolinal required a concentration of inhibitor in the micromolar range, compared to the nanomolar range observed for S17092, indicating this inhibitor is not exhibiting tight binding inhibition (Figure 3-17 A). A curve was fitted to these data using the Michaelis-Menten model of inhibition (Equation 3-3). The inhibition curve was repeated across a range of substrate concentrations (70.5, 35.25, 17.6 and 8.8μM) and the values for K_i calculated using Prism© (Figure 3-17 B). An average K_i of 1.70μM was calculated for DpoA inhibition by Z-Pro-Prolinal (Table 3-3).

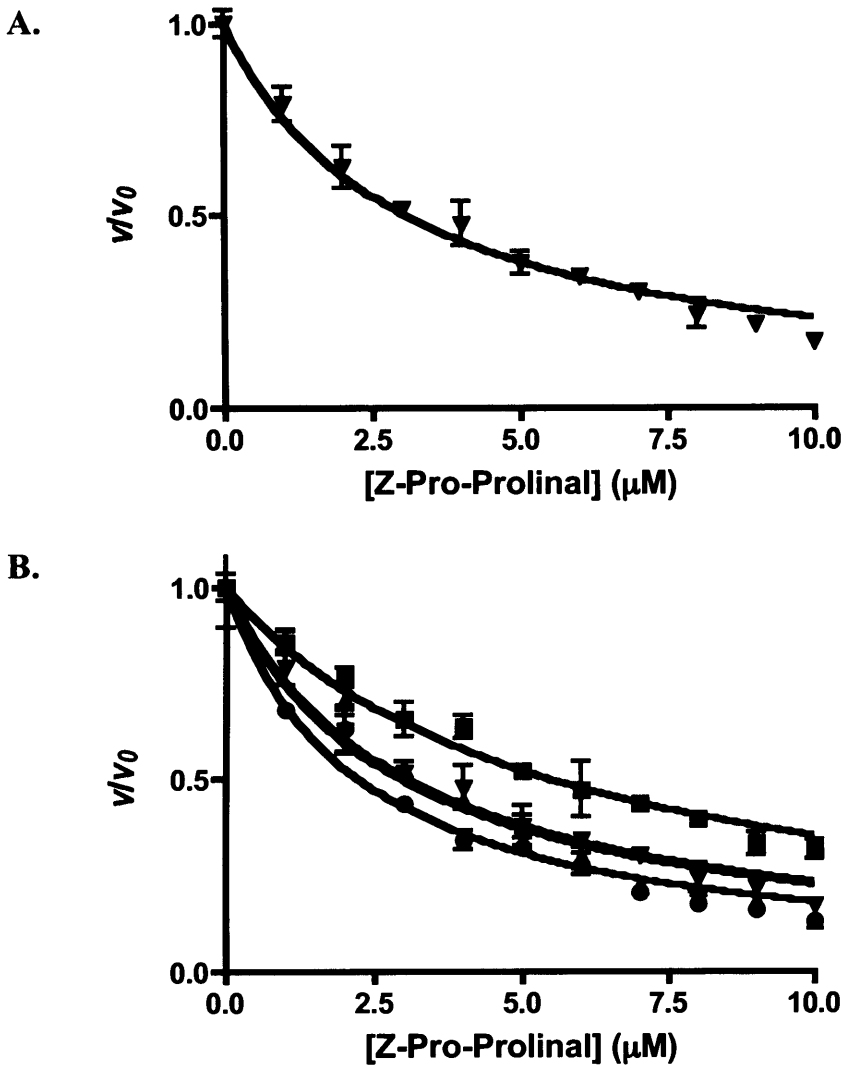


Figure 3-17 Inhibition of DpoA by Z-Pro-Prolinal

A. $1X10^{-5}$ U DpoA enzyme activity was assayed in a final volume of 60μl HEPES buffer (pH 7.8) at 25°C, in the presence of inhibitor Z-Pro-Prolinal at concentrations from 1 to 10μM. The reaction rate v (μM increase in product (AMC) min^{-1}) was measured between 0 and 20 minutes, $v_0 = v$ when [I] = 0. The Michaelis-Menten model of inhibition was used to fit a curve to these data. **B.** Z-Pro-Prolinal inhibition was measured across a range of substrate ,Z-Gly-Pro-AMC, concentrations, ■ 70.5μM, ▼ 35.25μM, ● 17.6μM, ▲ 8.8μM. Data represent mean ± standard deviation.

Table 3-3 K_i Of Z-Pro-Prolinal For DpoA

Substrate (Z-Gly-Pro-AMC) Concentration In Assay (μM)	**K_i (μM)**
70.5	1.66±0.07
35.25	1.42±0.06
17.6	1.43±0.07
8.8	2.28±0.21
Average value of K_i	**1.7**

3.2.5 Inhibition Of Rpo By PO Specific Inhibitors

Enzyme activity of the mammalian enzyme, Rpo, was measured in the presence of the PO specific inhibitors S17092 and Z-Pro-Prolinal under the same conditions as the *Dictyostelium* enzyme (See 3.2.4). Activity was measured in 100mM HEPES buffer (pH 7.8), at a substrate concentration close to K_M (35.25μM). $1X10^{-5}$ U recombinant enzyme was added per 60μl assay.

S17092 inhibition of Rpo again showed deviation from the Michaelis-Menten model as observed previously for the *Dictyostelium* enzyme (section 3.2.4.2) (data not shown). Therefore, tight binding kinetics were also used to calculate the K_i for S17092 with the mammalian enzyme.

The total active enzyme concentration in the assay was calculated from an S17092 inhibition curve in the presence of $4X10^{-5}$ U enzyme activity using the tight binding inhibition model (Equation 3-6) (Figure 3-18 A). This model calculated the concentration of $4X10^{-5}$ U enzyme activity in 60μl to be 0.72 ±0.14nM active enzyme. From this it is estimated that the enzyme concentration, E_o, per assay containing $1X10^{-5}$ U Rpo enzyme activity is 0.18nM. This value along with the previously calculated K_M of 36.1μM were included in the tight binding model equation to calculate the K_i of S17092 for Rpo.

Rpo activity was measured in the presence of S17092 at concentrations from 0.1-1nM at three different substrate concentrations (70.5, 35.25 and 17.6μM) (Figure 3-18 B) (Table 3-4). The values calculated for K_i under these conditions result in an average K_i of 134nM.

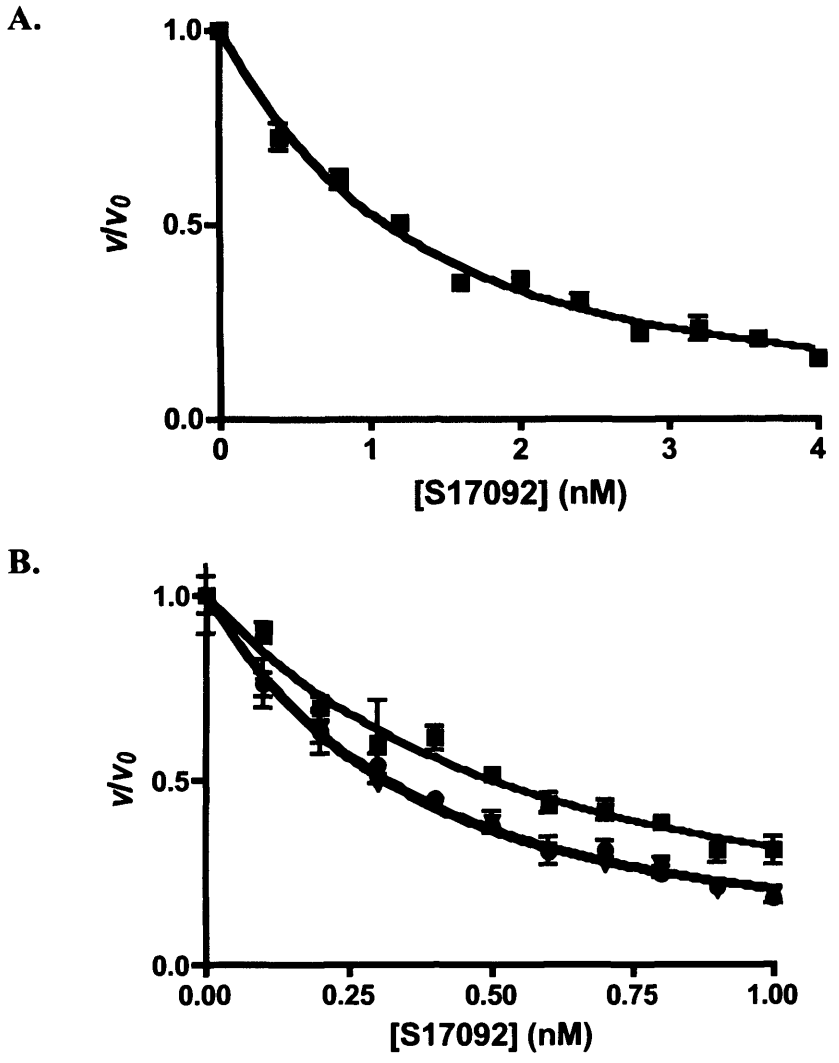


Figure 3-18 Inhibition Of Rpo By S17092.

A. $4X10^{-5}$ U , ■ , Rpo enzyme activity was assayed in a final volume of 60µl HEPES buffer (pH 7.8) at 25°C, in the presence of inhibitor S17092 at concentrations ranging from 0.1 to 4nM. The reaction rate v (µM increase in product (AMC) min^{-1}) was measured between 0 and 20 minutes, $v_0 = v$ when [I] = 0. The tight binding model of inhibition was used to fit a curve to these data and estimate the concentration of total active enzyme. **B.** S17092 inhibition was measured across a range of substrate, Z-Gly-Pro-AMC, concentrations, ■ 70.5µM, ▼ 35.25µM, ● 17.6µM. $1X10^{-5}$ U Rpo enzyme activity added per assay. Results represent mean ± standard deviation.

Table 3-4 K_i of S17092 Calculated For Rpo

Substrate (Z-Gly-Pro-AMC) Concentration In Assay (μM)	**K_i (pM)**
70.5	139 ±7
35.25	110 ±3
17.6	153 ±7
Average value of K_i	**134**

Rpo activity was also measured in the presence of Z-Pro-Prolinal at concentrations of 1-10μM (Figure 3-19). The K_i of Z-Pro-Prolinal for Rpo was calculated using the Michaelis-Menten model of inhibition (Equation 3-3) at four different substrate concentrations ranging from 2 to 0.25 times K_M (70.5, 35.25, 17.6 and 8.8μM). The values for K_i were calculated using Prism©, an average K_i of 3μM was calculated for Rpo inhibition by Z-Pro-Prolinal (Table 3-5).

Table 3-5 K_i Of Z-Pro-Prolinal For Rpo

Substrate (Z-Gly-Pro-AMC) Concentration In Assay (μM)	**K_i (μM)**
70.5	3.6 ±0.15
35.25	3.2 ±0.12
17.6	2.8 ±0.09
8.8	3.3 ±0.18
Average value of K_i	**3**

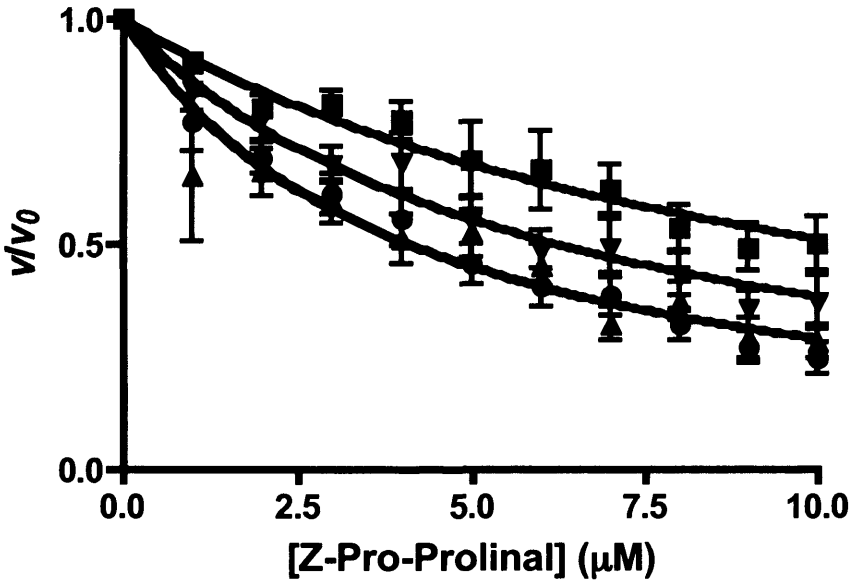


Figure 3-19 Inhibition of Rpo by Z-Pro-Prolinal

$1X10^{-5}$ U Rpo enzyme activity was assayed in a final volume of 60μl HEPES buffer (pH 7.8) at 25°C, in the presence of inhibitor Z-Pro-Prolinal at concentrations from 1 to 10μM. The reaction rate v (μM increase in product (AMC) min^{-1}) was measured at 20 minutes, $v_0 = v$ when [I] = 0. Inhibition was measured across a range of substrate, Z-Gly-Pro-AMC, concentrations, ■ 70.5μM, ▼ 35.25μM, ● 17.6μM, ▲ 8.8μM. The Michaelis-Menten model of inhibition was used to fit a curve to these data. Data represent mean ± standard deviation.

3.2.6 Inhibition Of Rpo Resembles That Of DpoA

S17092 has a picomolar K_i of 58pM and 134pM for the *Dictyostelium* and rat enzymes respectively. These values are lower than those previously reported which include a K_i of 1nM in enzyme purified from human brain and IC50 of 1.3nM for enzyme purified from rat brain (Portevin et al. 1996; Barelli et al. 1999)

K_is in the micromolar range were calculated for Z-Pro-prolinal indicating it is a less efficient inhibitor than S17092. Z-Pro-prolinal shows a K_i of 1.7μM and 3μM for the *Dictyostelium* and rat enzymes. This compares well with the previously reported K_i of 1.15μM for the *Dictyostelium* enzyme in cell extract (Williams et al. 1999) and is within 10-fold of the IC50 of 0.13μM for the purified human platelet PO (Goossens et al. 1997).

The *Dictyostelium* and mammalian enzymes also showed similar specific activity, $1X10^{-5}$ U of enzyme activity in 60μl was calculated to be produced by 0.15nM *Dictyostelium* or 0.18nM Rat enzyme.

The similarity of the *Dictyostelium* and rat enzymes supports the sequence alignment results suggesting similar amino acid composition around the active triad and substrate binding regions. Comparison of these results with those observed by other groups using non-recombinant enzyme purified from animal tissue gives confidence that the V5-His epitope tag is not interfering significantly with the structure of the active site.

3.3 Discussion

This chapter presents a study of the biochemical properties of the *Dictyostelium* PO in comparison to a mammalian enzyme.

Phylogenetic analyses have grouped DpoA with other PO enzymes within the prolyl oligopeptidase family, consisting of PO, OB, DPP and AP, within this group DpoA was placed in the same cluster as the mammalian enzymes. Complete phylogeny of the prolyl oligopeptidase family has illustrated that all family members are distinct despite sharing characteristic structural features and catalytic triad order.

A previous study also showed distinct clustering of the different sequences of a single enzyme following evolutionary analysis of the peptidase domains of a limited selection of the prolyl oligopeptidase family (Venalainen et al. 2004a). In this case a small number of enzyme sequences were selected based on a blast search using the mammalian enzyme. Here it is shown that the specific enzyme clusters are maintained when all available sequences are included.

Subsequence analysis (alignments of PO active site and β-propeller conserved sequences) has enabled separation of true prolyl oligopeptidases from mis-identified sequences. The latter may in fact belong to alternative groups of the prolyl oligopeptidase family. Further phylogenetic analyses clearly illustrate the similarity between functional domains of these enzymes.

A number of tree building methods are available. In this example a ClustalW alignment was carried out using the blosum series of distance matrices. The blosum series represents a range of matrices which are based on amino acid pairs in blocks of aligned protein segments, these matrices compare favourably with previous methods based on accepted mutations in closely related groups (Henikoff and Henikoff 1992). The series of matrices contains a number of matrices whose suitability varies with the

degree of divergence between the sequences to be analysed, the ClustalW allows the correct matrix to be employed at the correct point in the alignment. ClustalW also weights results for each region of sequence allowing alternate weighting of runs of hydrophilic amino acids which often represent more flexible regions (Thompson et al. 1994). A phylogenetic tree of this alignment was constructed using the neighbour joining method, a progressive method, therefore any mistakes introduced early on in the tree construction may be maintained. However, a number of studies have found this an efficient method when compared with alternatives such as maximum likelihood and maximum parsimony (Saitou and Nei 1987; Tateno et al. 1994).

I have been able to establish good confidence in the reliability of this method by calculating the bootstrap tree. Bootstrap analysis is based on multiple resampling of the data to provide confidence levels for identified relationships, the usefulness of this analysis is widely accepted however a number of studies criticise bootstrapping for frequently underestimating confidence values (Sitnikova et al. 1995; Price et al. 2005) While this does not affect its usefulness in confirming identified relationships, more tenuous relationships should not be dismissed on the basis of bootstrapping alone.

In the case of the β-propeller domain, the observed phylogenetic differences are larger than those seen between the active site sequences. The explanation for this may be that the absolute sequence, other than those residues in close proximity to the active site, is less critical as long as the correct structure is maintained. Therefore this domain can accept evolutionary amino acid substitutions, which may represent adaptation to specific environmental conditions, for example the archaea *H.marismortui* the proteins of which tend to contain negatively charged amino acids on the protein surface to allow it to cope with its highly saline environment. The β-propeller domain may also be involved in protein-protein interactions. It is reasonable

to presume that the outer amino-acids may vary depending on the structure of any binding partners.

In contrast, the catalytic domain is involved in substrate binding and activity for which specificity may be essential. In this case, similarity would be expected between sequences encoding enzymes showing the same specificity regardless of the organism.

3.3.1 Active Site Sequence And Structure

The sequence and structure at the active site of prolyl oligopeptidase play an essential role in both substrate specificity and enzyme activity. The Ser-Asp-His order of the catalytic triad is unique to the SC clan of serine proteases, however, throughout the entire serine protease family nucleophilic attack of the substrate is mediated by the serine hydroxyl group and stabilised by the aspartic acid and histidine residues via proton transfer and formation of hydrogen bonds.

A specific binding pocket (the S1 binding pocket) accommodates the substrate proline residue while the backbone amide group of Asn555 (human PO numbering) and the OH group of Tyr 473 (also known as the oxyanion binding hole) forms hydrogen-bonds with the oxyanion of the proline residue, thus providing substrate stereo-specificity. A hydrogen bond is also formed between a nitrogen atom of the catalytic triad histidine and the first amino acid following the proline residue (the P1' position). A number of hydrogen bonds and hydrophobic interactions also occur from positions P4 through to P2' however it is the interactions directly surrounding the proline residue which confer the majority of the observed enzyme specificity.

The essential nature of the catalytic triad is illustrated by an apparent loss or severe decrease of activity following substitution of S609, D693 or H730 for a small neutral amino acid (alanine or glycine). Previously, Szeltner found the effect of Asp variants

D641N and D641A of the pig PO depended greatly on the substrate leaving group, the stronger the bond to be broken the greater the effect of the variant on enzyme activity. They also reported that the Asp variant did not affect binding of substrate to the enzyme implicating a role purely in hydrolysis of the bond to be cleaved (Szeltner et al. 2002).

A similar situation has been observed in other serine proteases. Site-directed mutagenesis of the catalytic triad of trypsin found that while only the Ser mutant resulted in a total loss of enzyme activity, substitution of either His or Asp decreased enzyme activity to between 0.04 and 0.0004% of wild type (Corey and Craik 1992). His substitution also results in severe reduction in subtilisin activity (Carter and Wells 1987).

3.3.2 β-Propeller Domain

Access to the active site of PO is controlled by a 7-bladed β-propeller domain, formed from seven 4-stranded anti-parallel β-sheets arranged around a central tunnel. Whether the substrate enters the active site via the central tunnel or through a gap between the two domains has been a matter of contention: it is currently thought that the latter is true (Fulop et al. 1998; Szeltner et al. 2004; Juhasz et al. 2005).

β-propeller domains are present in a number of proteins, other than the prolyl oligopeptidase family of proteases, where they play a role in protein-protein interactions and enzyme co-factor binding (Jenne and Stanley 1987; Faber et al. 1995; Neer and Smith 1996). The amino acid sequence of different β-propeller domains can be very varied, however, conservation of the propeller structure and residues involved in interactions with surrounding molecules is vital to their function.

This is also the case for PO. Inter-domain hydrophilic interactions and salt-bridge formation between the β-propeller and the catalytic domain play a significant role in enzyme activity (Shan et al. 2005). This interaction is likely to require a degree of flexibility as mutations introducing a disulphide link between the two domains resulted in a loss of enzyme activity (Szeltner et al. 2004; Juhasz et al. 2005).

β-propeller domain alignments have revealed the presence of additional amino acid residues in the DpoA β-propeller domain which can be mapped to the loop between the third and fourth anti-parallel strands of the 1st and 2nd blades. It is relevant to discover the positioning of these additional regions within the entire protein, any protrusion towards the catalytic domain could affect specific interdomain interactions with potential to improve or inhibit substrate access to the active site. According to the crystal structure of PO described by Fulop et al. both of the extended loops are positioned on the outer side of the protein pointing away from the active domain (Fulop et al. 1998). This positioning is very unlikely to interfere with the structure or activity of PO however it is this domain of the β-propeller that is responsible for protein-protein interactions in other β-propeller proteins (Wall et al. 1995; Neer and Smith 1996). This raises the possibility that the additional residues may play a role in any intracellular protein-protein interactions of DpoA. Future studies on any binding partners of the β-propeller domain and the role, if any, of these residues in that interaction may provide a valuable insight into the endogenous function of PO.

3.3.3 PO Activity

Both Rpo and DpoA showed a clear peak in activity around pH 8.0 when assayed across a pH range from 5 to 9. This is consistent with an enzyme whose activity is dependent on the ionisation state of a residue to allow activity.

Previous studies of PO activity across a pH range reported a sigmoidal distribution of activity. This distribution was attributed to activity of two forms of the enzyme, one of which is predominant at low pH (below pH 6.1-6.9) and one which is predominant at higher pH (above pH 6.1-6.9) (Polgar 1991b).

Deuterium oxide studies (a 'heavy' water molecule which slows acylation and deacylation reactions by interfering with acid base catalysis) on enzyme activity have shown an effect only on the low pH form of the enzyme resulting in the conclusion that this relies on an acid/ base catalysis to mediate formation and decomposition of the tetrahedral intermediate. It is thought that the rate-limiting step of hydrolysis by the high pH form relies on a conformational change (Polgar 1991b). In this study the low pH PO activity was not observed, the physiological relevance of a low pH form is questionable as it would not be expected to occur under normal intracellular conditions.

A curve of best fit to the pH data observed, was achieved using values for pKa_2 of around 7.5 and pKa_1 of around 9. This suggests an ionisation event around 7.5 that is required for enzyme activity. This is consistent with the estimated pK of the His residue at the active site of serine proteases of 7.0 (Polgar 1991b) or 6.25 for the His residue in porcine PO (Fulop et al. 2001) and the hypothesis of Polgar (1991a) that this imidazole side chain must be deprotonated for ion transfer, essential for enzyme activity, to occur.

At pH 7.8 both the mammalian and *Dictyostelium* enzymes show similar kinetics for cleavage of the fluorigenic substrate Z-Gly-Pro-AMC. Under these conditions the two enzymes have a very similar K_M, 30μM (DpoA) and 36μM (Rpo), and V_{max}, 0.18μM min^{-1} (DpoA) and 0.21μM min^{-1} (Rpo). These values are in agreement with the

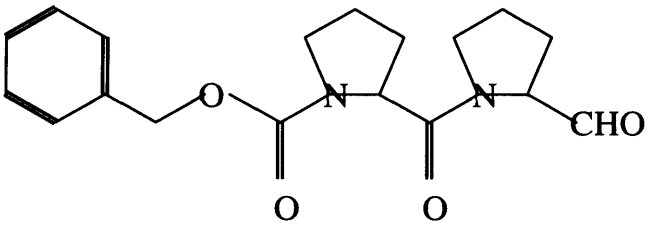
previously reported K_M of Z-Gly-Pro-AMC of 20±3μM and 30±3μM for purified porcine PO and porcine brain homogenate respectively (Venalainen et al. 2002).

3.3.4 PO Specific Inhibition

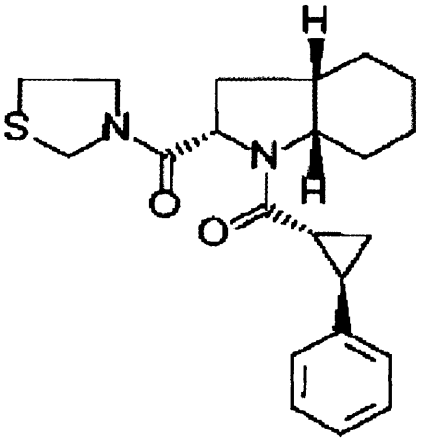
The association of PO with a number of neurological disorders has led to discovery and development of numerous specific and non-specific inhibitors (see introduction Table 1-1). Several have been identified in plant and fungal extracts however a large number have been developed synthetically, largely due to interest in their potential as treatments.

S17092 and Z-Pro-prolinal are both potent, specific inhibitors which interact with prolyl oligopeptidase at the active site and substrate binding sites. Both are reportedly specific for PO in mammalian cells. Z-Pro-prolinal is commonly used in the study of PO activity. It is based on a Pro-Pro dipeptide structure thus mimicking the substrate and binding in the S1 proline binding pocket of the enzyme (Figure 3-20) (Wilk and Orlowski 1983). The aldehyde moiety of the prolinal forms a hemiacetal adduct with the active site serine (Kahyaoglu et al. 1997a). Wilk and Orlowski (1983) found that Z-Pro-prolinal was unable to inhibit a number of other proteases at 150 times the K_i observed for PO.

S17092 also capitalises on the S1 proline-binding pocket and was developed around the same basic structure, of two heterocycles and an N-terminal side chain, as Z-Pro-prolinal (Figure 3-20). In this case the efficiency of inhibition has been improved by substitution of the central proline residue, which binds the S2 specificity pocket, with non-natural amino acid PHI containing a perhydroindole group. It also has a 2-phenylcyclopropyl group N-terminal side chain and a thiazolidine in place of the 2nd heterocycle bound at position S1 (Portevin et al. 1996).



Z-Pro-L-Prolinal



S17092

Figure 3-20 PO Inhibitors.

Chemical structures of the PO specific inhibitors Z-Pro-L-Prolinal and S17092.

A number of studies have examined the best structures at each of these three positions in order to produce an efficient and specific inhibitor.

S17092 shows strong PO specificity with concentrations up to 5000 fold that of the K_i of PO inhibition having no effect on a number of enzymes including; elastase, aminopeptidase B, dipepitidyl peptidase IV, aminopeptidase M and the coagulation/fibrinolysis cascade components thrombin, plasmin, factor Xa, kallikrein, activated protein C and trypsin (Portevin et al. 1996; Barelli et al. 1999).

Both of these inhibitors rely on interaction with the S3 hydrophobic pocket as well as the S2 and S1 specificity pockets. Any changes in the catalytic domain, especially around the substrate binding site have potential to affect the inhibition constants achieved by these compounds.

Despite the differences in sequence between DpoA and the mammalian enzyme the enzyme kinetics appear very similar. DpoA also shows similar K_i values to Rpo for both of the specific inhibitors Z-Pro-prolinal and S17092 supporting their suitability for further *in vivo* and *in vitro* studies of DpoA. Taken together the enzyme kinetics and inhibition constants indicate that the substrate binding site and surrounding environment of DpoA and Rpo are very similar.

S17092 is a potent specific inhibitor of the human PO enzyme (Barelli et al. 1999). A comparison of the fit of the Michaelis-Menton model of non-tight binding competitive inhibition and the model of tight binding inhibition with the DpoA S17092 inhibition curve revealed the tight binding model to be a much more accurate fit to the observed data. Both DpoA and Rpo were inhibited by S17092 at picomolar concentrations with a calculated K_i of 56±1.4pM (DpoA) and 110±3pM (Rpo).

Z-Pro-prolinal only inhibited both enzymes at μmolar concentrations. Following estimates of enzyme concentrations of 0.15nM and 0.18nM respectively for DpoA

and Rpo it may be assumed that Z-Pro-prolinal is not a tight binding inhibitor. For the Michaelis-Menten inhibition model to hold true E_o/K_i must be less than 0.01 (Henderson 1972). This assumption is met for Z-Pro-prolinal inhibition of DpoA and Rpo, therefore this model was used to calculate K_is of 1.42±0.06μM (DpoA) and 3.2±0.12μM (RpoA) for these two enzymes.

Both the tight binding and Michaelis-Menten equation used to model enzyme inhibition are specific to competitive inhibition (Henderson 1972; Cornish-Bowden 2001). Observation that, using these models, inhibition curves carried out at differing substrate concentrations returned a consistent value for the inhibition constant, K_i, is in agreement with the model of competitive rather than un-competitive or non-competitive inhibition.

3.3.5 Summary

- Following sequence analysis PO family enzymes from bacteria, mammals and plants were found to cluster in to distinct enzyme groups.
- DpoA shows close homology to the mammalian enzyme especially in the catalytic domain where the active residues are absolutely conserved.
- The *Dictyostelium* PO homologue also shares similar enzyme kinetics (K_M and V_{max}) and specific inhibition (K_i) by Z-Pro-prolinal and S17092 with the mammalian (rat) enzyme.

4 Effect Of Mood Stabilising Drugs On PO

4.1 Introduction

4.1.1 Bipolar Disorder And Mood Stabilising Drugs

Bipolar Disorder is a mood disorder defined by cycling between periods of depression and mania often interspersed with periods of normal behaviour. This disorder affects up to 1% of the population and carries a high risk of suicide (NIMH 2001). Treatment for Bipolar Disorder can be complex, a number of drugs are currently available which may be given exclusively or in combination, the majority of these carry numerous unwanted side effects.

Lithium, an alkali metal element, has been used to treat Bipolar Disorder for well over a century, however, the therapeutically relevant molecular target of this treatment remains uncertain. Uncertainty also prevails over the relevant targets of other regularly prescribed mood stabilising drugs.

Identification of a common mechanism of action for lithium, valproic acid and carbamazepine (all mood stabilising drugs) in inositol depletion suggests a possible therapeutic mechanism (Williams et al. 2002). Each of these compounds has multiple, varied targets within the cell. However, when used to treat explanted rat dorsal root ganglion neurons all three drugs resulted in increased numbers of spread growth cones and a smaller proportion of collapsed growth cones implying at least one common target. In all three cases this effect was reversed following combined treatment with extracellular myo-inositol. These observations strongly implicated inositol depletion as a common therapeutic mechanism.

4.1.2 Inositol Depletion Hypothesis

Intracellular levels of inositol and the second messenger, $_{IP3}$, are controlled by; recycling of inositol phosphates, de-novo synthesis of inositol, dephosphorylation of higher order inositol phosphates and import of extracellular inositol. Li, VPA and CBZ deplete inositol by acting at different points in inositol synthesis and recycling (Figure 1-6). Lithium is a potent inhibitor of IPP and IMPase; enzymes responsible for dephosphorylation of IP_2 to IP and IP to inositol (Berridge et al. 1982; Inhorn and Majerus 1987). While VPA inhibits Inositol-1-Phosphate Synthase, an enzyme involved in de novo synthesis of inositol by conversion of glucose-6-phosphate to inositol-1-phosphate (Shaltiel et al. 2004). The mechanism of CBZ depletion of inositol is not yet characterised.

4.1.3 Role Of Prolyl Oligopeptidase In Inositol Signalling

Prolyl Oligopeptidase was first identified in *Dictyostelium* following a 10mM lithium screen for resistance mutants. Loss of DpoA conferred lithium resistance on *Dictyostelium* aggregation (Williams et al. 1999).

Following the onset of starvation, single cell amoebae *Dictyostelium* aggregate to form mounds, which eventually undergo cell differentiation and develop into a fruiting body. The process of aggregation is mediated by a cAMP gradient set up by a single cell at the centre of the mound, and relayed by surrounding cells. The receptor involved in this process is the cAMP receptor cAR1, a G-protein coupled 7-transmembrane receptor whose downstream effects include; actin polymerisation, cyclic GMP accumulation and activation of PLC, the enzyme responsible for PIP_2 breakdown to IP_3 and DAG, all of which may play an essential role in chemotactic movement (Parent and Devreotes 1996). Following cAMP binding a rapid

accumulation of IP_3 has been observed in *Dictyostelium* (Europe-Finner and Newell 1987; Van Haastert et al. 1989). IP_3 releases Ca^{2+} from intracellular stores and a number of studies report that introduction of IP_3 or Ca^{2+} to permeabilised cells is able to replicate actin polymerisation and cGMP accumulation without signalling through cAR1 suggesting these steps are downstream of IP_3 release. This implies an essential role for inositol signalling in *Dictyostelium* aggregation (Europe-Finner and Newell 1985, 1986). The essential role of Ca^{2+} in chemotaxis is questioned by a mutant lacking IplA, the IP_3 receptor responsible for rapid Ca^{2+}entry. The absence of cAMP stimulated Ca^{2+} entry to the cell does not appear to affect normal chemotaxis of *Dictyostelium* (Traynor et al. 2000).

In addition, loss of Phospholipase C activity in the PLC null mutant does not affect *Dictyostelium* chemotaxis as might be expected (Drayer et al. 1994). In this mutant IP_3 levels are maintained at an almost normal level by dephosphorylation of higher order inositol phosphates (IP_5 and IP_4) by the enzyme MIPP. This may account for the unaltered chemotaxis and developmental phenotype observed (Van Dijken et al. 1995).

PO activity plays a role in regulation of inositol levels by inhibiting MIPP mediated dephosphorylation of IP_5 to IP_3. *Dictyostelium* mutants lacking PO show increased breakdown of IP_5 to IP_3, treatment of wild type *Dictyostelium* with the specific PO inhibitor Z-Pro-Prolinal also results in an increase of IP_3 (Williams et al. 1999). Levels of IP_3 are also increased in mammalian astroglioma cells by treatment with PO inhibitors or transfection with an antisense vector against PO (Schulz et al. 2002).

Inhibition of PO is able to reverse inositol depletion by derepression of the IP_5 to IP_3 pathway thus replenishing IP_3, and subsequently inositol, levels by dephosphorylation of higher order inositol phosphates. This is evident in the ability of PO inhibition to

reverse effects of Li^+, VPA and CBZ attributed to inositol depletion. Li^+, VPA and CBZ induced spreading of neural growth cones is reversed by addition of PO inhibitors (Williams et al. 2002), similarily addition of PO inhibitors may reverse autophagy induced by any of these three drugs (Sarkar et al. 2005), both of these effects are also reversed by addition of inositol. *Dictyostelium* mutants lacking DpoA are able to aggregate in the presence of Li^+ or VPA, while wild type amoebae are unable to aggregate due to an inability to chemotax towards a cAMP gradient (Williams et al. 1999; Williams et al. 2002).

4.1.4 VPA Interaction With Prolyl Oligopeptidase

A direct interaction between Li^+, VPA or CBZ and PO would not be expected, if one did exist it would be expected that these drugs activate PO. Surprisingly, a recent report suggests that VPA acts as a PO inhibitor. Cheng et al. (2005) reported significant, mixed, inhibition of recombinant porcine and human PO activity at 1mM VPA, close to the therapeutic levels of 0.3-0.7mM.

VPA is a saturated branched short chain fatty acid, it is possible to hypothesise that the carboxylic group and side chain will be integral to any inhibition of PO.

4.1.5 AIM

This section aims to determine the direct effects, if any, of a number of mood stabilising drugs on PO. It will also further investigate the apparent inhibition of PO by VPA and determine any intracellular effect of this inhibition.

4.2 Results

4.2.1 Mood Stabilising Drugs Do Not Act Through PO

Loss of PO activity results in resistance to the mood stabilising drugs LiCl, VPA and CBZ. Therefore it is expected that any direct effects of these compounds on PO would result in an increase in PO activity. Purified recombinant DpoA and Rpo were both assayed in the presence of LiCl, VPA or CBZ at a range of concentrations representing from 0.1 up to at least 10 times the therapeutic concentration of each of the drugs. Activity was measured against purified enzyme with a carrier control (H_2O for VPA and LiCL and EtOH for CBZ, NaCl was also added as a salt control for LiCl,) (Figure 4-1).

None of the mood stabilisers LiCl, VPA and CBZ had any direct effect on PO enzyme activity within the therapeutic range. However, at approximately 10 times therapeutic concentration CBZ treatment resulted in a significant decrease in Rpo but not DpoA activity. A significant decrease in both Rpo and DpoA was also observed at 10 times therapeutic concentration of VPA (6mM).

Inhibition of PO by VPA has previously been described by Cheng et al. (2005) who report an 80% decrease in PO activity at 6mM VPA as well as a significant decrease at therapeutic concentrations. This is a much larger decrease than that observed in this study. At 6mM VPA PO activity is decreased by just 32% (Rpo) and 28% (DpoA) respectively while no significant decrease is observed at therapeutic concentrations.

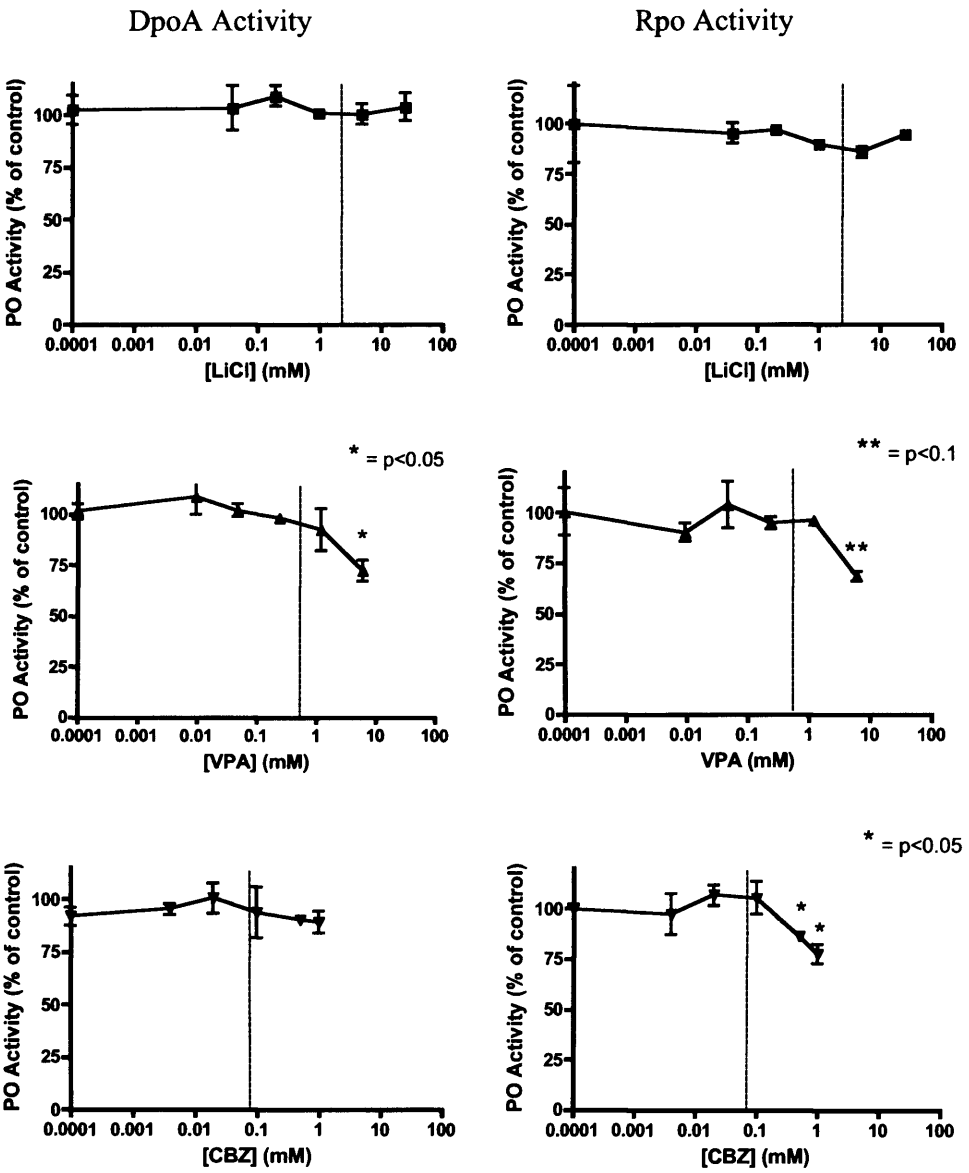


Figure 4-1 Effect of Mood Stabilising Drugs on PO Activity.

Activity of purified recombinant DpoA and Rpo, was assayed in the presence of the three mood stabilising drugs; LiCl, VPA and CBZ across a range of drug concentrations. Assays were carried out in 100mM HEPES buffer (pH 7.8) using the fluorigenic substrate Z-Gly-Pro-AMC at 25°C. The therapeutic dose concentration for each drug is marked on the graph by a dashed line. Results are the average of two repeats done in duplicate ± standard deviation.

4.2.2 VPA Inhibition Of Purified PO

The difference in VPA inhibition observed by Cheng et al. (2005) to that reported in this study could be accounted for by the use of a different assay pH. Cheng et al. observed the 80% inhibition of PO activity by VPA (6mM) in assay buffer adjusted to pH 7 and pH 7.2 whereas in this study a buffer adjusted to pH 7.8 was used. It has been shown previously in this study (section 3.2.3) that a change in pH from 7 to 8 may have a significant effect on enzyme activity. The PO activity assay using recombinant DpoA and Rpo was repeated at pH 7.0, 7.5 and 8.0 in the presence of 6mM VPA or a MeOH carrier control (Figure 4-2).

At pH 7.5 and 8.0 VPA inhibited both DpoA and Rpo by 25-30%. At pH 7.0 DpoA was inhibited to a lesser extent (20%) while Rpo showed no significant inhibition. The effect of VPA inhibition appears to increase in line with suggested ionisation of the histidine imidazole ring. It may be the case that this de-protonation is necessary for the inhibitory effect of VPA.

VPA is known to inhibit a number of other enzyme targets, including histone deacetalase (Gottlicher et al. 2001; Phiel et al. 2001), inositol-1-phosphate synthase (Shaltiel et al. 2004) and microsomal epoxide hydrolase (Spiegelstein et al. 2000). Investigation of the method of inhibition of these targets used VPA analogues to determine the significance of the carbon chain shape, including the side chain and the role of the carboxylic acid group, revealing the differing importance of the backbone structure and acid moiety depending on the target.

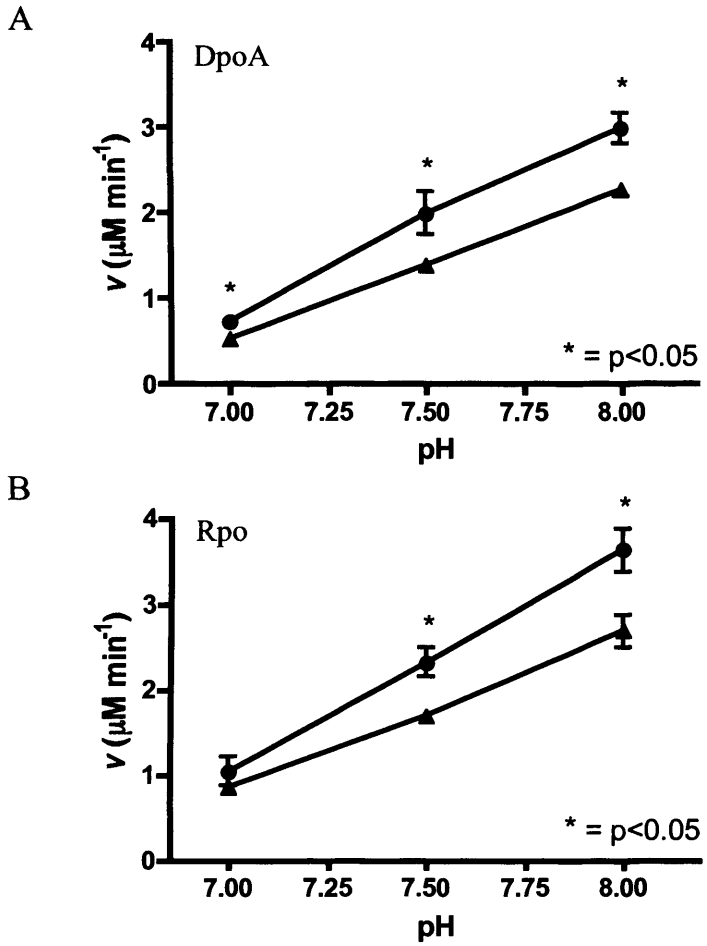
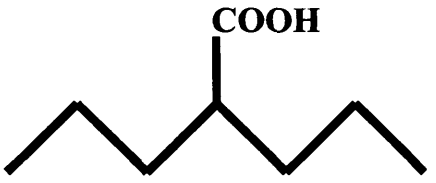
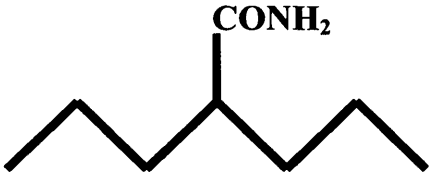


Figure 4-2 VPA Inhibition Of PO Activity Between pH 7 And pH 8.

Activity of purified recombinant DpoA, A, and Rpo, B, was assayed in 100mM HEPES buffer between pH 7 and 8 using the fluorigenic substrate Z-Gly-Pro-AMC at 25°C. Assays were carried out in the presence of 6mM VPA, ▲, or carrier control (MeOH), ●. Results are the average of at least 3 repeats ± standard deviation. Significant differences ($p<0.05$) between activity in the presence of VPA, compared with carrier control is indicated by * .



Valproic Acid



Valpromide

Octanoic Acid

Figure 4-3 Structure of VPA and Analogues

Stick diagram of VPA ($C_8H_{16}O_2$) and analogues including amide derivative, Valpromide ($C_8H_{17}NO$) and unbranched isomer, Octanoic acid ($C_8H_{16}O_2$).

VPA analogues were used to determine if VPA inhibition of PO required specific interactions with the branched carbon backbone or the acid group. Valpromide (VPM) ($C_8H_{17}NO$) is a VPA analogue in which the carboxylic acid moiety has been replaced by an amide group, octanoic acid ($C_8H_{16}O_2$) is an unbranched carboxylic acid with the same molecular formula as VPA (Figure 4-3).

Activity of recombinant DpoA and Rpo was measured in the presence of 6mM VPM or 6mM octanoic acid across the pH range from 7.0-8.0 (Figure 4-4). Inhibition of both DpoA and Rpo by VPM closely resembled that observed in the presence of VPA. VPM, as VPA, did not inhibit either enzyme at pH 7.0 but showed significant inhibition at pH 7.5 (32%, DpoA and 26%, Rpo) and pH 8.0 (26%, DpoA and 22% Rpo).

The same pattern of inhibition was not observed following addition of 6mM octanoic acid. This acid showed significant inhibition of both enzymes at pH 7.0 and pH 7.5. Inhibition of the mammalian enzyme, Rpo, (62% and 46% at pH 7 and 7.5) by this unbranched carboxylic acid was greater than that seen for the *Dictyostelium* enzyme (48% and 22% at pH 7 and 7.5). These observations imply that while the branched structure is more important at higher pH conditions the acid group may also play a role in inhibition at lower pH, especially in the case of the mammalian enzyme.

Human blood pH is strictly maintained at 7.4, the intracellular pH of human cells is close to this value but may vary dramatically within different cellular compartments (approximately 5.0 in lysosomes). The cytosolic pH of *Dictyostelium* is also estimated to be 7.4 ±0.2 (Martin et al. 1987). At pH 7.5, close to the physiological pH of 7.4 both the branched amide, VPM, and the unbranched carboxylic acid, octanoic acid, show significant inhibition of PO activity.

As changes of just pH 0.5 clearly affect PO activity it is of note that pH of the buffers was not altered by addition of 6mM VPA, VPM or octanoic acid, therefore the inhibitory effects observed are not simply a result of altered pH (data not shown).

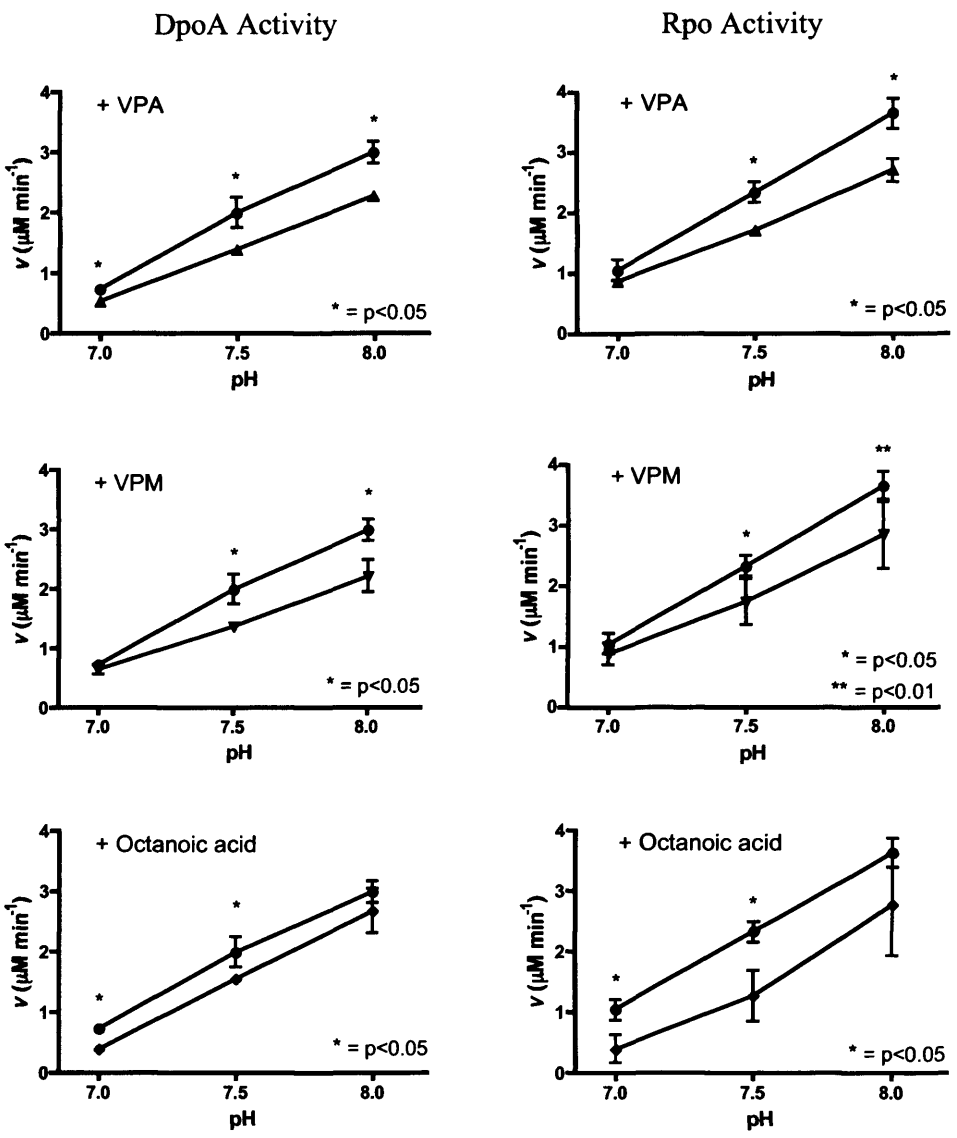


Figure 4-4 Effect Of pH On Inhibition Of PO Activity By VPA And Analogues.

Activity of purified recombinant DpoA and Rpo was assayed in 100mM HEPES buffer between pH 7 and 8 using the fluorigenic substrate Z-Gly-Pro-AMC at 25°C. Assays were carried out in the presence of 6mM VPA, ▲, VPM, ▼, Octanoic acid, ◆ or carrier control (MeOH), ●. Results are the average of at least 3 repeats ± standard deviation. Significant differences between activity in the presence of VPA, VPM or Octanoic acid compared with carrier control is indicated by * or **.

4.2.3 VPA Inhibition Of Cellular PO

VPA is able to inhibit purified recombinant PO activity. For PO to be a potential intracellular target of VPA it is important to determine if this inhibition occurs *in vivo*. The effect of VPA on intracellular PO activity was measured using the human lymphoblastoid cell line GC12139. Lymphoblast cells have been used previously in studies of *in vivo* inositol levels and found to mirror inositol levels in the frontal cortex of the brain (Belmaker et al. 2002). This suggests they show a similar pattern of inositol signalling as that observed in the brain, making them a useful model for studying the effects of mood stabilising drugs at the cellular level.

Lymphoblast cells growing in culture medium were treated overnight with increasing concentrations of VPA and analogues, VPM and octanoic acid. Cells were counted in the presence of Trypan blue to determine the effect of these compounds on cell viability. Following treatment with 10mM VPA, VPM or octanoic acid extensive cell death was observed. Treatment with 3mM resulted in a reduced amount of cell death caused by VPA and octanoic acid while VPM no longer had a noticeable effect on cell viability (Figure 4-5). The effect of the three compounds on cellular PO levels was subsequently measured up to a maximum concentration of 3mM. The possibility of these compounds to act in an additive way in combination with conventional PO inhibitors was determined by dual treatment of lymphoblast cells with VPA, VPM or octanoic acid and S17092.

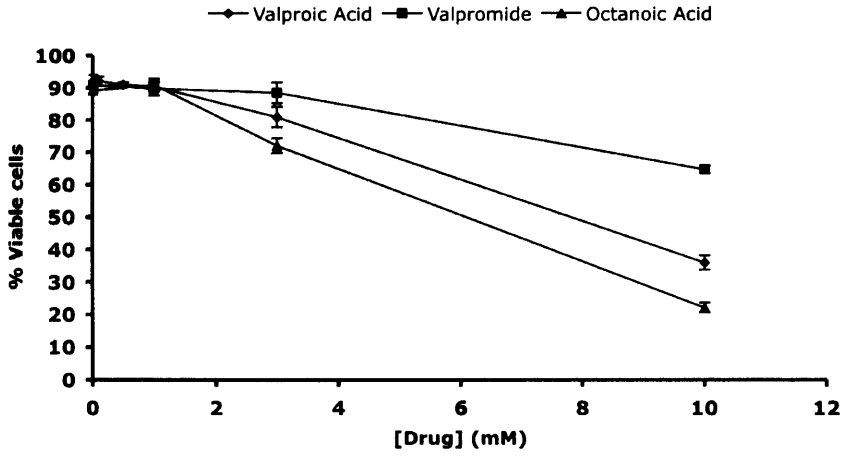
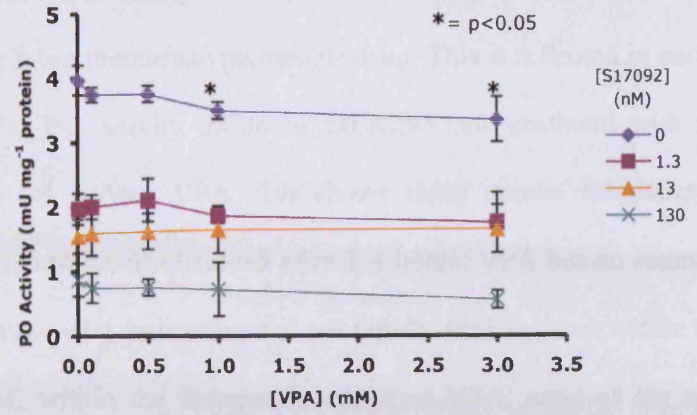


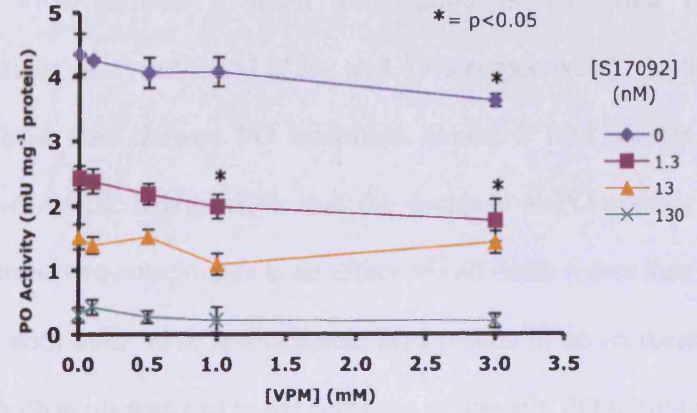
Figure 4-5 Lymphoblast Viability in Presence of VPA and Analogues

Lymphoblastoid cells in culture were treated overnight (16 hours) with VPA, VPM or Octanoic acid. Viable cells were determined by counting cells using a haemocytometer following addition of Trypan Blue. Results are average of 2-3 repeats, error bars represent the range of the repeats.

A.



B.



C.

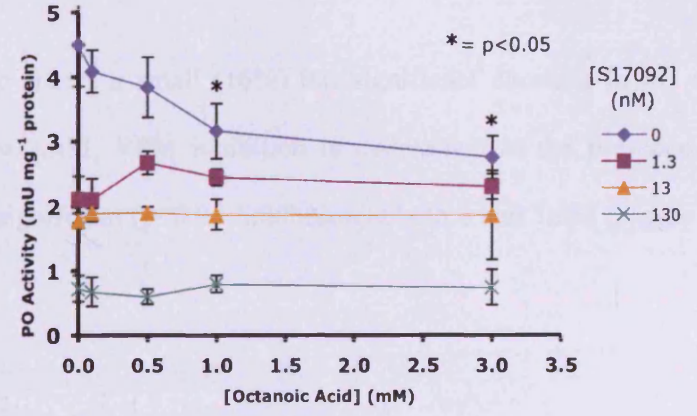


Figure 4-6 Intracellular Effect of VPA and Analogues

Lymphoblastoid cells in culture were treated overnight with VPA, graph A, VPM, graph B or Octanoic acid, graph C plus specific inhibitor S17092 or MeOH control. Following treatment cell extracts were taken and measured for PO activity. Results are average of three repeats done in duplicate ±SEM.

S17092 has a dose independent pharmacological index of oral bioavailability of 0.7 indicating it is a membrane permeable drug. This is reflected in complete inhibition of intracellular PO activity following HEK293 cell treatment with just 30nM S17092 (Barelli et al. 1999). VPA also shows rapid uptake following oral dosing with maximal serum levels observed after 1-4 hours. VPA has an estimated half-life of up to 16 hours *in vivo*, indicating it is not rapidly broken down within the cell.

At 0.5mM, within the therapeutic range of VPA, none of the compounds showed significant inhibition of PO activity (using students t-test and a cut off value of $p<0.05$). VPA showed a small yet significant inhibition of PO activity at concentrations of 1 and 3mM (12% and 15% respectively). At these concentrations octanoic acid also showed PO inhibition almost 2 fold greater than that of VPA (Figure 4-6 A&C). It is possible that the decrease in PO activity in the presence of either of these two compounds is an effect of cell death rather than specific inhibition, treatment with both VPA and octanoic acid results in an increase in cell death. The effect of both acids was lost in the presence of specific PO inhibitor S17092 at 1.3nM (50% inhibition of PO). S17092 treatment had no effect on cell viability (data not shown).

VPM also shows a small (16%) but significant decrease in PO activity yet no cell toxicity at 3mM, VPM inhibition is maintained in the presence of 1.3nM S17092 showing significant ($p<0.05$) inhibition at both 1 and 3mM (Figure 4-6 B).

4.2.4 VPA Enhances Not Inhibits LiCl Effect On Cellular Processes

Lithium treatment of *Dictyostelium* prevents aggregation due to modulation of inositol signalling, this effect is avoided by the lisA mutant lacking DpoA (Williams et al. 1999). VPA also prevents aggregation of wild type cells, again by modulation of inositol signalling, but does not prevent aggregation in the lisA mutant (Williams et al. 2002). Treatment with a PO inhibitor would be expected to attenuate Li^+ inhibition of aggregation. VPA does not act as a PO inhibitor to dampen the effect of lithium, but, increases lithium sensitivity preventing aggregation at lower LiCl concentrations. *Dictyostelium* were grown on agar plates containing 3mM VPA, VPM or Octanoic acid and increasing concentrations from 2-12mM of LiCl or NaCl control. In the presence of VPA a LiCl concentration of just 4mM was sufficient to prevent aggregation, *Dictyostelium* aggregation in the absence of any other compound was inhibited only at concentrations above 8mM, (Figure 4-7), NaCl did not affect *Dictyostelium* aggregation or development at any concentration (data not shown). VPA alone did not prevent aggregation but inhibited normal development of the fruiting body, this may be attributed to inhibition of alternative VPA targets.

Any PO inhibitory activity of VPM or octanoic acid is insufficient to have an effect on aggregation in the presence of LiCl, aggregation was affected to the same degree as that of *Dictyostelium* grown in the presence of LiCl alone. Neither VPM nor octanoic acid enhanced the effect of LiCl or showed any developmental defects. This implies that neither compound is able to target inositol-1-phosphate synthase, responsible for the inositol depletion effect of VPA (Shaltiel et al. 2004) or other VPA targets (possibly histone deacetylase or microsomal epoxide hydrolase) responsible for developmental defects.

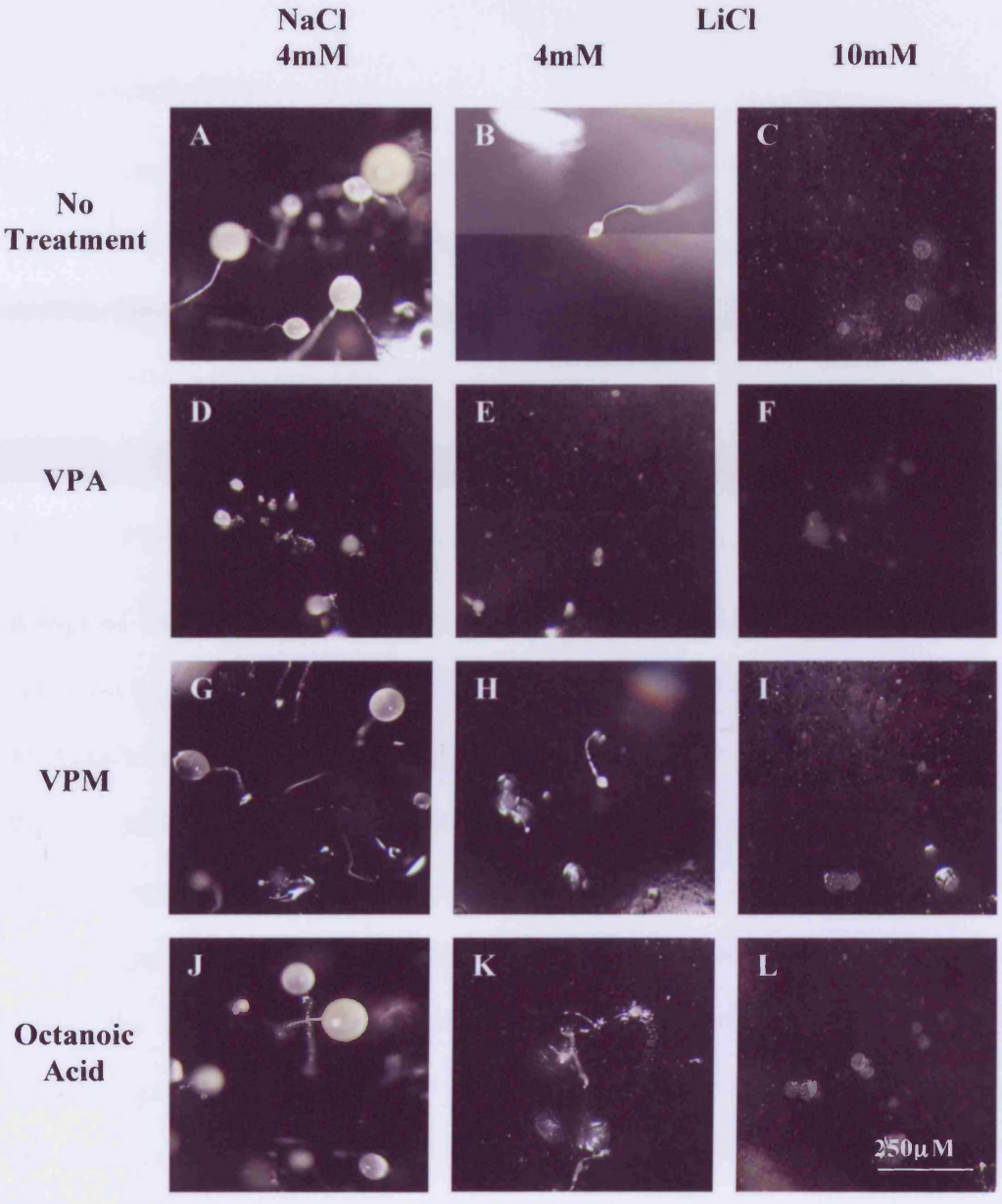


Figure 4-7 *Dictyostelium* Development in the Presence of VPA or Analogues

Development of *Dictyostelium* on agar supplemented with VPA (D,E,F), VPM (G,H,I), Octanoic acid (J,K,L) or MeOH (A,B,C) as well as 4mM (B,E,H,K), or 10mM (C,F,I,L) LiCl or NaCl salt control (A,D,G,J) was observed and photographed using a Zeiss stereo zoom dissecting microscope. Pictures are representative of 3-4 repeats.

4.3 Discussion

Initial results indicate that PO activity is not directly modulated by therapeutic concentration of mood stabilising drugs Li^+, VPA and CBZ. However, it has become evident that when considering PO activity and inhibition a small change in environmental conditions may have a significant effect on the behaviour of this enzyme.

4.3.1 VPA Inhibition Of PO Is pH Dependent

Assays of both the mammalian and *Dictyostelium* PO enzyme revealed little or no inhibition by VPA at pH 7.0 and inhibition of between 25 and 30% at pH 7.5 and 8.0. VPA inhibition may be dependent on the ionisation state of the enzyme, it is of note that the imidazole ring of the active site histidine is proposed to be deprotonated in this pH range. A similar inhibition was observed using the amide derivative of VPA, VPM, suggesting the effect of VPA on PO is not dependant on the carboxylic acid group. The unbranched VPA analogue octanoic acid was also able to inhibit PO activity at pH7.5 and 7.0 so the acid effect on the inhibition cannot be discounted.

PO inhibition by carboxylic acids has been reported previously by Park et al. (2006) who observed an inhibitory effect of a number of fatty acids. In their study it was concluded that the inhibition was dependant on the presence of a number of double bonds and the COOH moiety, no inhibition was observed with saturated fatty acids. Octanoic acid and VPA are shorter chain fatty acids than those previously tested therefore, I propose they may be able to overcome any steric effects preventing their longer, unsaturated family members from interacting with the binding site of the enzyme.

Studies of specific PO inhibitors often include a carbon chain ending in an aromatic ring that is able to enter the hydrophobic S3 pocket. Portevin et al. show the importance of side chain chirality and an optimum side chain length of $(CH_2)_3$, they also report that saturated side chain analogs were 2 fold less effective inhibitors than their unsaturated counterparts (Portevin et al. 1996). This is reminiscent of the situation in fatty acid inhibition and may support a steric effect in access to the S3 pocket.

A number of PO inhibitors, including Z-Pro-Prolinal and JTP-4819 (Table 1-1), contain a hydroxyl group on the proline binding the P1 position at the catalytic site. However, to my knowledge no inhibitors have been shown containing a carboxylic acid moiety in this domain, though the oxyanion binding site would be an obvious choice for binding of the carboxyl oxygen. If VPA, octanoic acid or VPM are interacting with PO at the active site, future studies may involve linking these compounds to a PO targeting peptide at both the S3 and S1 binding position.

The carboxylic acid domain of VPA is also not essential for the VPA inhibitory effect on human microsomal epoxide hydrolase (mEH) (Spiegelstein et al. 2000). mEH is an important metabolic enzyme responsible for hydrolysis of epoxides to their corresponding trans-dihydrodiols and is also a target for VPA inhibition. A study of the effect of both amide and acid VPA derivatives on mEH activity revealed inhibition requirements were limited to 8+ carbon atoms, including two saturated aliphatic alkyl side chains attached to the carbonyl. The presence of a carboxylic acid group was not necessary, in fact the compounds containing the amide moiety proved the more potent inhibitors (Spiegelstein et al. 2000). A similar study looking at the inhibition of another VPA target, the histone deacetylases (HDACs), revealed that while introduction of a double bond (to produce 2- or 4-ene VPA) was tolerated,

HDAC inhibition was severely depleted by amide substitution or structural alterations (Eyal et al. 2005).

From our observations using the VPA analogues VPM and octanoic acid it is not possible to differentiate between the importance of the branched structure or the carboxylic acid group for inhibition of PO activity.

4.3.2 *In Vivo* Effect Of VPA

The effect of VPA on endogenous human PO activity in lymphoblasts was complicated by the effect of VPA on cell viability. Both VPA and its unbranched analogue, octanoic acid, decrease cell viability by 12 and 19% respectively when added at 3mM. Eyal et al (2005) have also reported a decrease in cell viability (of 67%) in the presence of 2mM VPA using SW620 and 1106mel cells, but less than 20% in the presence of VPM.

In our assay, at 3mM both VPA and octanoic acid decreased lymphoblast PO activity by 16% and 39% respectively. At this concentration the decrease in cell viability must be considered and may account for a significant proportion of the lost PO activity. However, this raises the question of why this inhibition is lost in the presence of specific inhibitor S17092 when both compounds show the same effect on cell viability in the presence and absence of S17092 (data not shown).

Intracellular cytosolic pH of mammalian cells is tightly maintained at 7.4. Addition of acid or basic solutions to cells may alter intracellular pH (pH_i) only very transiently (5-10) minutes (Salvi et al. 2002) therefore it is unlikely that VPA or its analogues will affect pH_i. It is of note that cytosolic pH is decreased in apoptotic cells (Nilsson et al. 2003), as cell viability decreases decreased cytosolic pH may allow increased PO inhibition by octanoic acid.

The amide derivative of VPA, VPM, also showed a significant ($p<0.05$) decrease in PO activity, 17% decrease at 3mM, this is of particular interest as this compound did not have a significant effect on cell viability. As VPM only inhibits purified PO activity above pH 7.0 it is expected that VPM preferentially inhibits the de-protonated form of PO. VPM's inhibitory effect was also maintained in the presence of 50% PO inhibition by S17092,

Several studies have illustrated the ability of specific PO inhibitors to reverse the inositol depletion effects of LiCl. One of these effects is the inhibition of *Dictyostelium* aggregation, which is avoided in the mutant lacking DpoA. Neither VPA nor its analogues were able to overcome lithium inhibition of aggregation at 3mM, and VPA itself amplified the inhibition. This may merely imply an insufficient inhibition of PO for an effect to be seen, however, at increased concentrations of all three compounds normal cell viability was severely impaired (data not shown). Neither VPM nor octanoic acid amplified the LiCl inhibition of aggregation. Other VPA targets have been closely examined for structural requirements of inhibition, this has not been undertaken specifically for inositol-1-phosphate synthase, thought to be the VPA target responsible for its inositol depletion properties (Shaltiel et al. 2004). However, structural requirements of inositol depletion have been reported to be the presence of a carboxylic acid group as well as specific enantiomorphism (Eickholt et al. 2005; Shimshoni et al. 2007). I would not expect either of the above VPA analogues to affect inositol-1-phosphate synthase activity.

4.3.3 Summary

- Purified PO activity is not affected by therapeutic concentrations of mood stabilisers

- The inhibitory effect of VPA and its analogues on prolyl oligopeptidase is pH dependant.
- *In vivo* inhibition of PO by VPA is very slight and may be masked by the negative effect of VPA on cell viability.

5 Cellular Role Of PO

5.1 Introduction

A considerable amount of information is available on the PO enzyme structure, sequence, kinetics and inhibitors, some of which have even reached clinical trials. However, the true function of PO within the cell remains unresolved.

5.1.1 Potential Intracellular Functions Of PO

Prolyl Oligopeptidase is able to cleave small peptides following a proline residue, many bioactive peptides fall under this description and have proved, *in vitro*, to be effective substrates for PO. This has resulted in PO activity being linked to a number of different cellular processes, though it remains unclear whether all of these peptides are cleaved by PO *in vivo*.

PO may play a role in neuropeptide degradation. PO inhibition may result in neuroprotection (Checler et al. 1986b; Lew et al. 1994; Shishido et al. 1999a) and improvements in memory loss in both patients with cognitive memory disorders and animal models of amnesia (Yoshimoto et al. 1987; Toide et al. 1995a; Morain et al. 2000; Morain et al. 2002). PO also cleaves a number of bioactive peptides involved in osmoregulation, and altered PO levels are observed in rats following excessive salt or water treatments (Ferrario and Iyer 1998; Irazusta et al. 2001).

A function for PO involving less specific peptide cleavage has been suggested in general proteolysis. Constant protein turnover is central to healthy cellular metabolism, the presence of a proline residue within a peptide can protect against degradation by a number of general proteases. Thus, proteases specifically able to cleave proline-containing peptides are essential. Decreased PO levels have been observed at times of cell differentiation and cell stress both of which are associated

with altered levels of proteolysis (Pratt et al. 1989; Tsukahara et al. 1990b; Tsukahara et al. 1991a).

PO activity is also implicated in a number of cellular processes not obviously connected to cleavage of small peptides. The most prominent of these is its role in inositol signalling by inhibition of breakdown of higher order inositol phosphates to the second messenger IP_3 (Williams et al. 1999; Schulz et al. 2002). Another is POs potential role in DNA synthesis and therefore cell proliferation as observed in Swiss 3T3 and embryonic insect cells (Ohtuski et al. 1997; Ishino et al. 1998). Also, more recently, a role for PO in protein secretion has been identified. Inhibition of PO results in detection of increased overall protein secretion as well as an increase in secretion of β-amyloid peptides associated with Alzheimer's Disease (Rossner et al. 2005; Schulz et al. 2005).

Elevated mammalian PO levels have been associated with a number of inflammatory and autoimmune conditions (Cunningham and O'Connor 1997; Kakegawa et al. 2004; Seo et al. 2006). Bacterial PO is thought to be secreted from the bacterial cell and play a key role in infection by cleavage of the hosts extracellular matrix (Santana et al. 1997; Grellier et al. 2001; Bastos et al. 2005).

5.1.2 Localisation Of PO

Prolyl Oligopeptidase is a largely cytosolic enzyme, as has been observed in a number of mammalian tissues and in *Dictyostelium* cells (Goossens et al. 1995; Williams et al. 1999). A small proportion of cellular PO activity, between 10 and 30%, is associated with the particulate fraction rather than the soluble fraction (Dresdner et al. 1982; Agirregoitia et al. 2005). Reports of a membrane associated form of PO, which is largely removed following stringent washing suggests PO is interacting with other membrane bound proteins rather than integrated into the membrane (Lew et al. 1994).

The presence of PO within the nucleus has been observed in Swiss 3T3 cells and cultured insect cells, in both cases nuclear PO was present only in restricted regions of the nucleus while remaining distributed throughout the cytoplasm. Under certain conditions PO in cultured insect cells appeared to localise solely to the nucleus (Ohtuski et al. 1997; Ishino et al. 1998). In contrast, Schulz et al. have reported PO localisation to the perinuclear region and microtubules close to the nucleus, but excluded from the nucleus itself, in human glial and neuronal cell lines (Schulz et al. 2005).

Secretion of PO has been observed but only in bacteria (Chevallier et al. 1992; Kabashima et al. 1998), parasites (Bastos et al. 2005) and fungi (Yoshimoto et al. 1988; Sattar et al. 1990). PO is not secreted from mammalian or *Dictyostelium* cells (Williams et al. 1999; Schulz et al. 2005). This is at odds with reports detailing serum PO levels in relation to a number of disorders (Maes et al. 1995). Studies of PO levels in different tissues have reported PO activity in serum to be orders of magnitude less than that observed in other tissues (Agirregoitia et al. 2005). This activity may be accounted for by lysis of cells in the bloodstream, it is also reported that at least 60% of apparent PO activity observed in serum may be the result of ZIP activity (an enzyme showing similar specificity to PO) (Breen et al. 2004).

5.1.3 Inositol Signalling And PO Function

Inositol signalling is involved in a diverse array of cellular processes, therefore, any changes which affect these signalling pathways have the potential to bring about a number of effects.

PO inhibition of dephosphorylation of IP_6/IP_5 to IP_3 affects the levels of these higher order inositol phosphates as well as intracellular levels of IP_3. Therefore, as well as

affecting IP_3 mediated Ca^{2+} release and any downstream signalling events, a change in PO activity may affect downstream pathways of IP_6, IP_5 and IP_4.

The ratio of IP_6 to IP_4/IP_5 affects gene expression by negative (IP_6) and positive (IP_4/IP_5) regulation of chromatin remodelling factors (Shen et al. 2003; Steger et al. 2003). IP_6 is also essential for efficient mRNA export from the nucleus in yeast cells (York et al. 1999). Additionally, both IP_4 and IP_6 are able to inhibit endocytosis by interaction with proteins involved in clathrin coated pit formation (Palczewski et al. 1991; Gaidarov et al. 1996; Milano et al. 2006).

The enzyme, Multiple Inositol Polyphosphate Phosphatase (MIPP), dephosphorylates IP_6, IP_5 and IP_4 in both mammalian and *Dictyostelium* cells (Nogimori et al. 1991; Van Dijken et al. 1995). The mammalian enzyme is located largely in the rough and smooth endoplasmic reticulum (Ali et al. 1993; Craxton et al. 1995), while the *Dictyostelium* homologue is reportedly located at the inner face of the plasma membrane rather than the ER (Van Dijken et al. 1997).

5.1.4 AIM

This chapter will examine the cellular role of PO by establishing the intracellular localisation of this enzyme within *Dictyostelium*. It will also clarify the method by which PO is able to inhibit MIPP activity and thus affect inositol phosphate levels.

5.2 Results

5.2.1 Transfection Of *Dictyostelium* With GFP-Tagged DpoA

Wild type, Ax2, *Dictyostelium discoideum* were transfected with the pDXA-GFP2:DpoA vector, encoding a GFP tagged DpoA controlled by its endogenous promoter. Positive clones were identified by G418 resistance and maintained in high levels of G418 (100mg/l) to ensure sufficient expression for detection of fluorescence (for vector diagram and sequence see appendix I.III).

Transcription of the tagged DpoA was confirmed by Northern blot analysis of RNA extracted from two separate clones, RNA from untransformed Ax2 cells and LisA, the PO null cell line, were included as a control. Use of a PO specific probe revealed the presence of RNA encoding the endogenous DpoA, which was absent from LisA cells, and the presence of a larger DpoA RNA band in the transformed cells representing that encoding the tagged enzyme (Figure 5-1). The Northern blot was stripped and reprobed against Ig7, this gene encodes a microsomal rRNA subunit and is regularly used as a loading control.

Protein expression was then confirmed by visualisation of the tagged protein using a Zeiss Axioskop fluorescence microscope. Comparison of bright field and fluorescence pictures reveals the cells transformed with the tagged enzyme are clearly fluorescent while untransformed cells were not (Figure 5-2 images A-F). Subsequent transformation of LisA cells with the same vector was confirmed in the same way (Figure 5-2 images G-L).

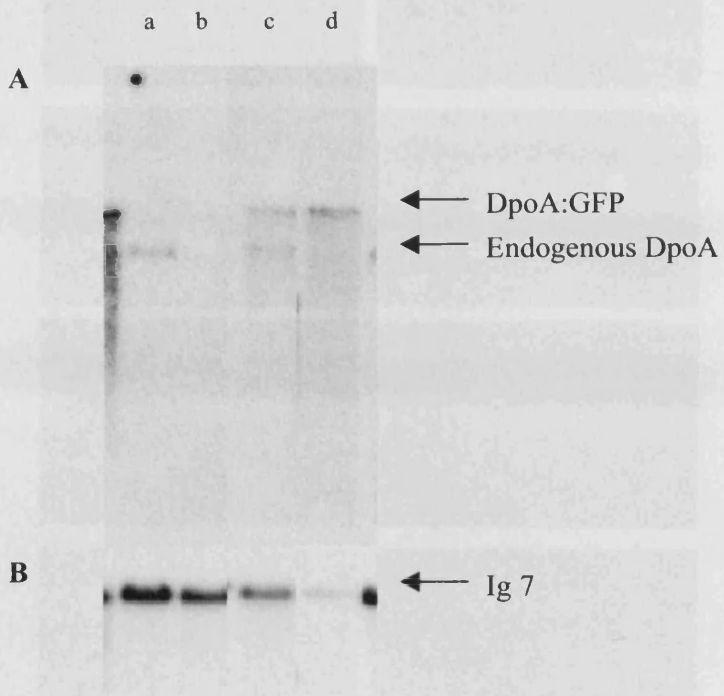


Figure 5-1 Expression of DpoA:GFP in *Dictyostelium*

A Northern blot was performed on *Dictyostelium* cell extracts (prepared from growing cells). Cell extracts were prepared from Ax2 (wild type) cells, a, LisA (PO null) cells, b and DpoA:GFP transfected Ax2 cells from two individual colonies, c and d. The blot was probed with a DpoA specific probe, **A** then stripped and re probed with an Ig7 specific probe as a loading control, **B** (for hybridisation conditions see Materials and Methods 2.3.12).

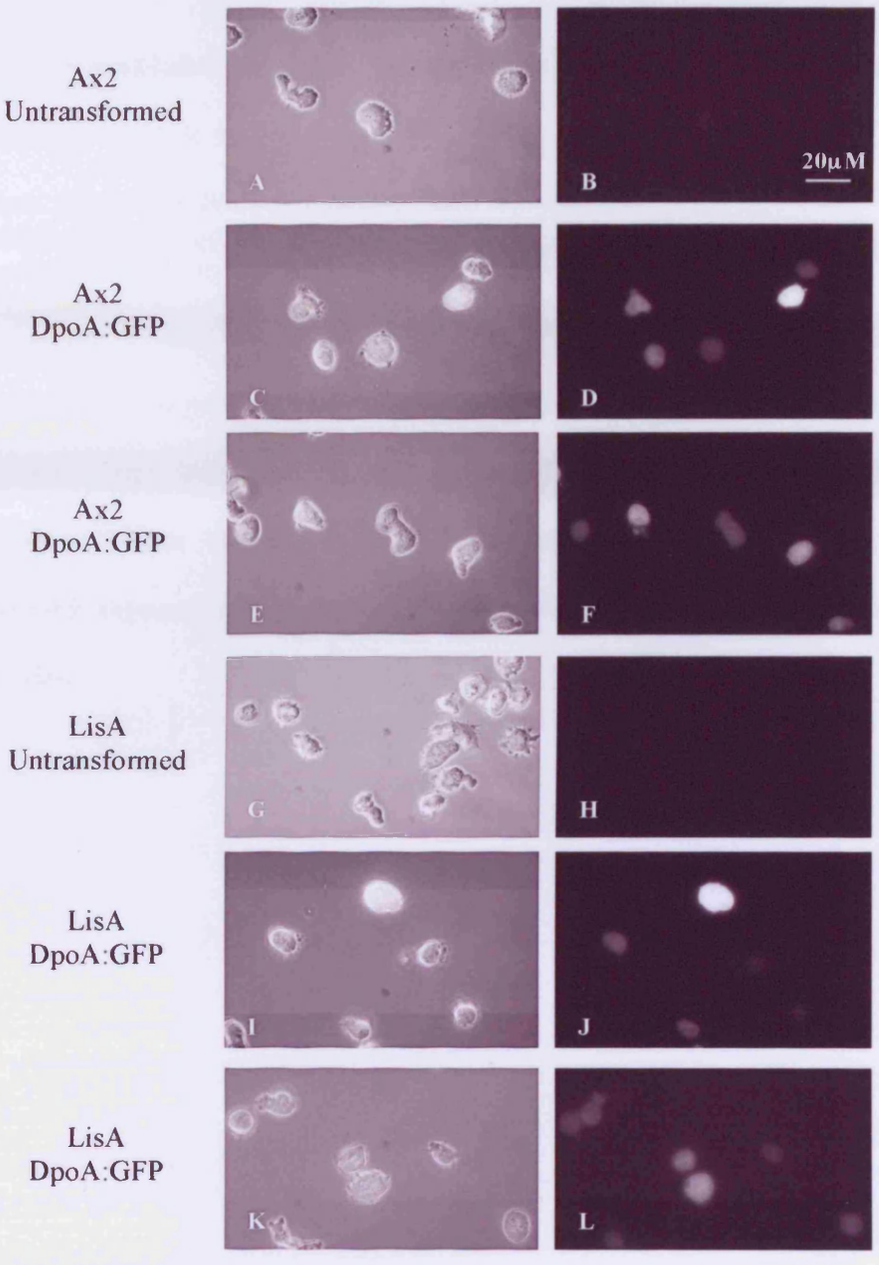


Figure 5-2 Visualisation of DpoA:GFP

Live *Dictyostelium* cells in KK2 media were captured using both bright field, pictures A,C,E,G,I,K and fluorescence, pictures B,D,F,H,J,L microscopy to identify cells expressing GFP tagged enzyme. Untransformed Ax2, A,B and LisA, G,H cells did not fluoresce while both Ax2, pictures C,D,E,F and LisA, pictures I,J,K,L cells expressing DpoA:GFP clearly fluoresced. Pictures are representative of total population.

It has been established previously that the PO enzyme containing the V5 tag retains enzyme activity (see section 3.2.2), the GFP tag also allows enzyme activity. Cell extracts were taken from transformed and untransformed Ax2 and LisA cells and assayed for PO activity using the substrate Z-Gly-Pro-pNA.

These assays revealed that DpoA:GFP expression resulted in an approximately 50% increase in PO activity in Ax2 cells (Figure 5-3). As expected PO activity was completely absent from the LisA cells. Importantly this also indicates the absence of any other enzyme showing a similar substrate specificity from *Dictyostelium*. DpoA:GFP expression in LisA cells returned PO activity levels to that seen in wild type cells.

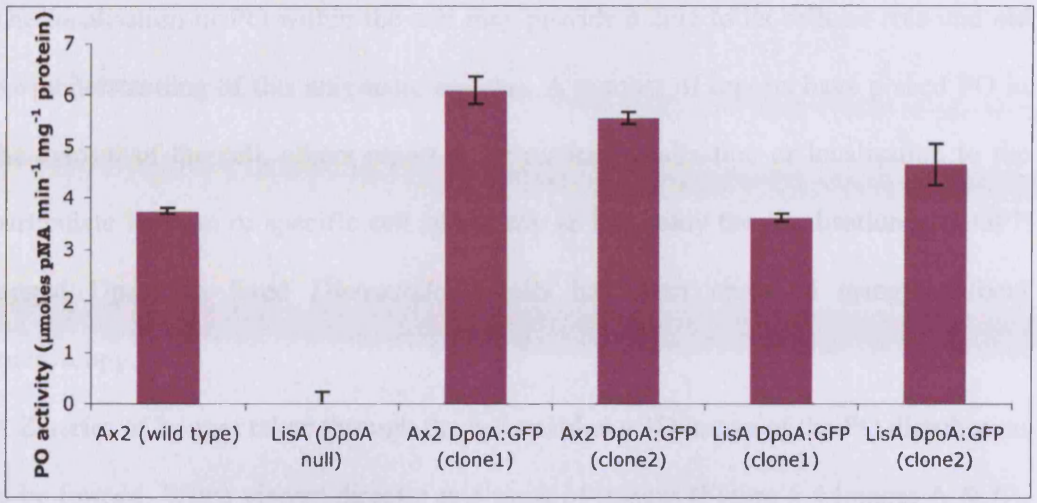


Figure 5-3 Activity of GFP Tagged DpoA in *Dictyostelium* Cell Extracts

Cell extracts of untransformed Ax2 and LisA cells and cells transformed with the GFP tagged DpoA expressing vector pDXA-GFP2:DpoA were incubated with the PO substrate Z-Gly-Pro-pNA PO activity was measured by the appearance of the product pNA (see materials and methods 2.6.5). Results are average of two repeats done in duplicate ± standard deviation.

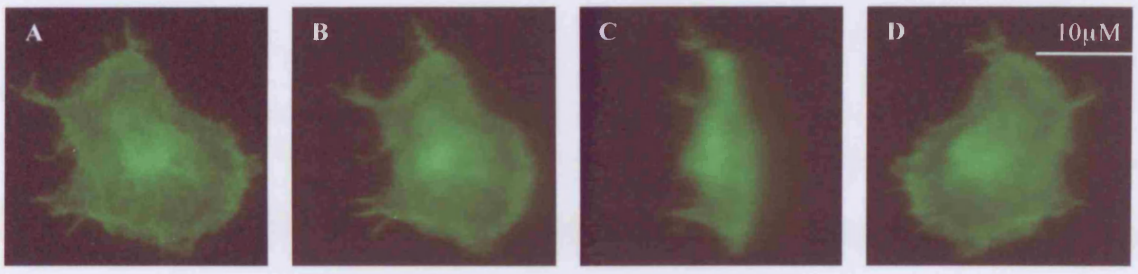
5.2.2 DpoA:GFP Is Distributed Throughout The Entire Cell

The localisation of PO within the cell may provide a clue to its cellular role and aid our understanding of this enigmatic enzyme. A number of reports have placed PO in the cytosol of the cell, others report some nuclear localisation or localisation to the particulate fraction or specific cell structures. In this study the localisation of a GFP tagged DpoA in fixed *Dictyostelium* cells has been observed using confocal microscopy.

A Z-series of images taken through the cell enabled a 3D image of the PO distribution to be formed. When viewed directly as a stack of images (Figure 5-4 images A & E), distribution appears increased around the centre of the cell and at the plasma membrane. However, rotation of the 3D image reveals that the increased volume of GFP at the centre of the cell may be attributed to the 'fried egg' shape of the cell. Enriched fluorescence around the plasma membrane may also be accounted for by the shape of the cell, a side view reveals membrane protrusions pointing upwards at the edge of the cell (Figure 5-4 images C & G). These images reveal PO to be distributed evenly throughout both the cytoplasm and nucleus.

Ax2 (wild type) *Dictyostelium* cells either; untransformed, expressing DpoA:GFP or expressing un-attached GFP were stained using DAPI to confirm presence of PO within the nucleus. Observation of single, unstacked confocal pictures of *Dictyostelium* expressing DpoA:GFP revealed a rather granular, slightly wispy distribution throughout the entire cell. Co-staining with DAPI showed a clear presence of DpoA:GFP, showing the same granular distribution, throughout the nucleus (Figure 5-5).

A Ax2 DpoA:GFP



B LisA DpoA:GFP

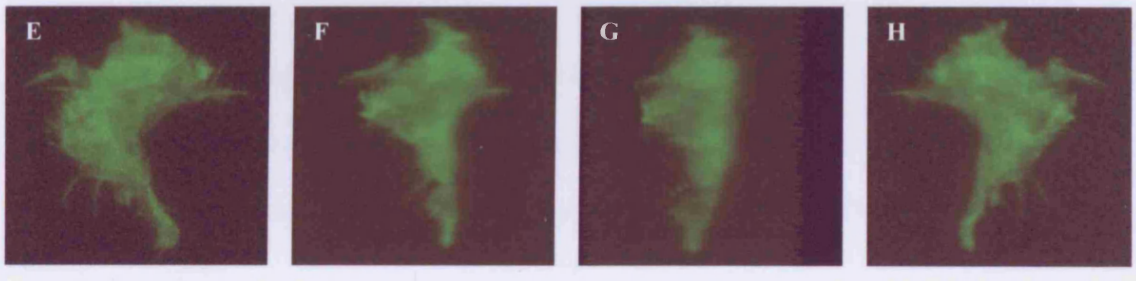


Figure 5-4 3D Projection Showing Cellular Distribution of DpoA:GFP

A Z-series of confocal images taken through Ax2 and LisA cells expressing DpoA:GFP were stacked together to produce a 3D image of the distribution of the tagged enzyme throughout the cell (see materials and methods 2.5.6). Pictures A-D show rotation of a single Ax2 cell around 180° while pictures E-H show the 180° rotation of a LisA cell. Both pictures are representative of all fluorescent cells observed.

Comparison of cells expressing DpoA:GFP with those expressing unattached GFP showed clear differences in cellular distribution (Figure 5-5). GFP alone showed a much more even distribution throughout the cytoplasm, it did not show the grainy distribution observed with DpoA:GFP. Another clear difference is the exclusion of GFP from vesicles present throughout the cell, this is not seen with the tagged protein suggesting the DpoA:GFP is able to freely enter these vesicles. This also implies distribution of DpoA:GFP is not governed by the GFP tag.

Subsequently the presence of DpoA:GFP was observed alongside staining of the endoplasmic reticulum. Several reports of PO activity in the particulate, non-soluble fraction of the cell suggest PO may be present within or associated with the ER or other cellular structures. Staining of the endoplasmic reticulum was achieved using a monoclonal antibody, mAb 221-64-1, against the ER marker Protein Disulphide Isomerase (PDI).

This antibody was a kind gift from Prof. M.Maniak, it targets the C-terminus of the PDI protein and is similar to mAb 221-135-2 described previously by Monnat et al. and used subsequently as an ER marker (Monnat et al. 1997; Williams et al. 1999).

Cell staining using the anti-PDI 1°Ab followed by a Texas Red conjugated secondary revealed a membranous structure within the cell. This was similar to that previously reported by Monnat using both anti-PDI Abs and a GFP:HDEL protein chimera containing GFP and the ER retention signal sequence HDEL (Monnat et al. 1997).

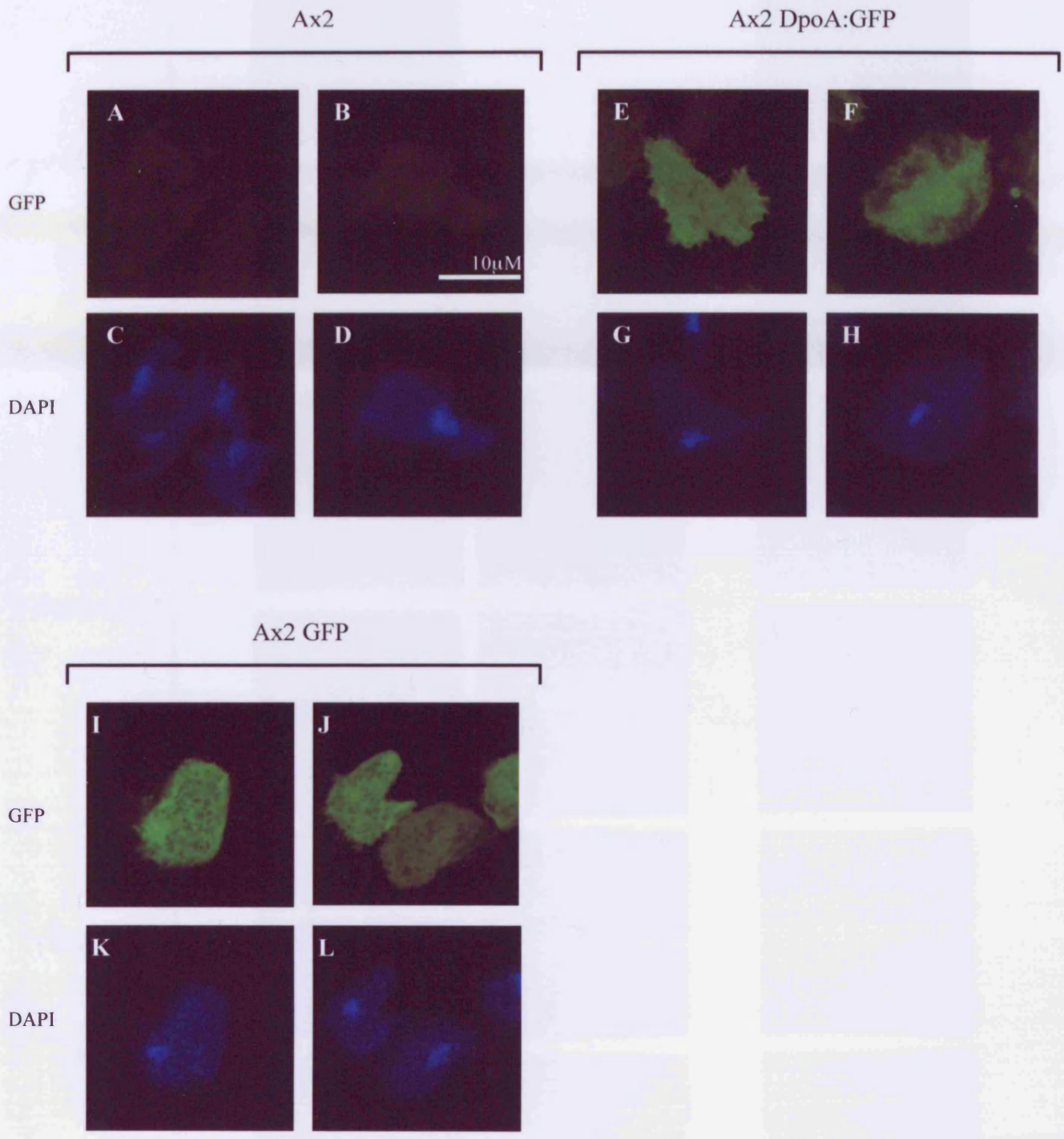


Figure 5-5 Co-Localisation of DAPI and DpoA:GFP

Confocal images of Ax2 cells; untransformed A-D, expressing DpoA:GFP E-H or expressing GFP alone I-L, co-stained with DAPI to identify the nucleus (see materials and methods 2.5). GFP and DAPI localisation are pictured for each cell.

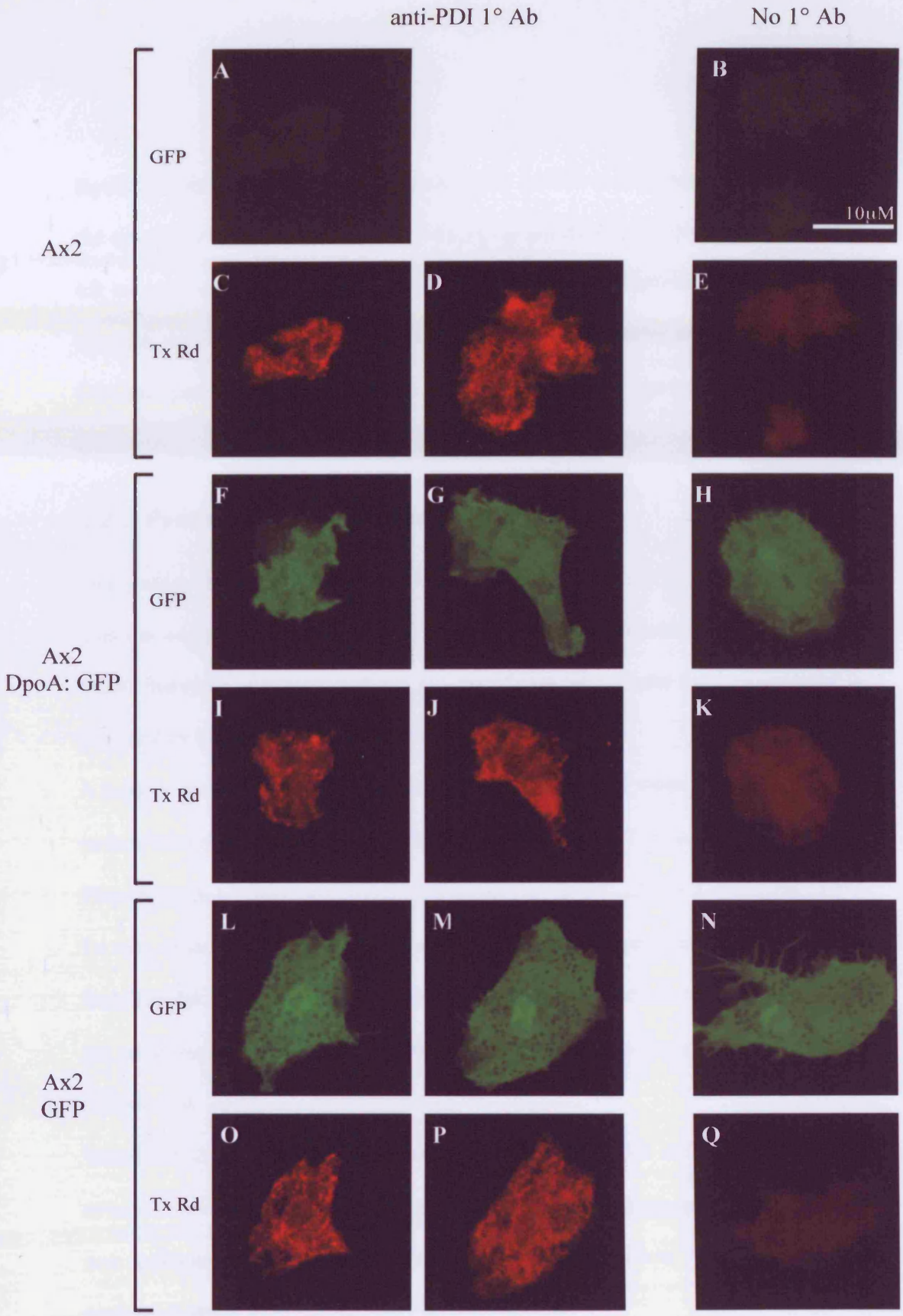


Figure 5-6 Comparison of DpoA:GFP Distribution with ER Marker

DpoA:GFP transformed, F-K, GFP transformed, L-Q and untransformed, A-E, Ax2 cells were stained with an anti-PDI 1°antibody followed by a Texas Red conjugated 2°antibody to identify the endoplasmic reticulum (see materials and methods 2.5). No 1°antibody was added in pictures, B,E,H,K,N &Q to identify any background signal.

DpoA:GFP did not specifically co-localise with the ER marker though the two stains did overlap in some regions (Figure 5-6). PO is not distributed throughout the entire ER but may show some localised association. In the cells expressing GFP alone the ER stain showed a clear lack of co-localisation with the vesicles excluding GFP. The size and position of these vesicles indicates they may represent endosomes or lysosomes.

5.2.3 Protein-Protein Interactions Of PO

The protein sequence of both mammalian and *Dictyostelium* prolyl oligopeptidase does not contain a classical nuclear, vesicular, plasma membrane or ER localisation signal, therefore, it is presumed that any significant intracellular localisation must be mediated by interaction with other proteins.

A number of other β-propeller domain proteins interact with other proteins via the top surface of the β-propeller domain (Jenne and Stanley 1987; Neer and Smith 1996). This region may, therefore, play an important part in the cellular localisation of PO. To date other than the peptides cleaved by PO the only protein-protein interaction described for PO is binding of α-tubulin. PO has been reported to bind α-tubulin in the same region as the binding site for microtubule-associated proteins (MAPs) (Schulz et al. 2005).

Tubulin forms an important structural component of the cell and α-tubulin staining reveals a network of fibres spread throughout the cytosol of the cell. This structure is best appreciated by viewing the maximum projection picture, built by stacking a confocal Z-series taken through the cell (Figure 5-7).

It is quite clear that DpoA:GFP and α-tubulin are not co-localised however this cannot discount an interaction between them, especially as the staining and GFP protein do overlap (Figure 5-7). The binding of α-tubulin to DpoA was further tested by investigating any interaction between these two proteins in extracellular extracts.

Dictyostelium cell extracts were taken from Ax2 cells expressing DpoA:GFP or unattached GFP. These extracts were incubated with magnetic beads conjugated to antiGFP antibodies and applied to a μMACs separation column. Any GFP tagged proteins and associated proteins are bound by magnetic force. A weak wash (20mM Tris-HCl, pH 7.5) is used to remove any unbound proteins while disrupting specific protein-protein interactions as little as possible. Following this a more stringent elution (50mM Tris-HCl pH6.8, 50mM DTT, 1% SDS, 1mM EDTA, 0.005% bromophenol blue, 10% glycerol) was used to remove the GFP proteins from the beads. An aliquot of the original cell extract along with the wash, eluted fraction and bead slurry was analysed by western blot for the presence of DpoA:GFP, GFP and α-tubulin.

Western blot analysis showed both DpoA:GFP and GFP clearly bound to the beads on the column and were only partly removed following the elution step as a proportion remained bound to the beads (Figure 5-8 B). α-Tubulin was not present in the column flow-through or preliminary wash however nor was it eluted along with DpoA. α-tubulin was only detected in the fraction containing the bead slurry, this indicates that α-tubulin may form a non-specific interaction with the antiGFP beads. It is not thought that its retention on the column is indicative of any interaction with PO as it remains bound following DpoA:GFP elution. In addition α-tubulin was also retained on the column in the absence of DpoA. The band identified by the α-tubulin antibody is smaller than expected (around 30 kDa rather than 51 kDa) suggesting the tubulin

has degraded. This may be caused by the stringent wash conditions or during removal of beads from the column.

This result does not give a definitive result but suggests that in our hands α-tubulin does not show specific interaction with DpoA.

A silver stain of the eluted fractions was also carried out, DpoA:GFP, GFP and α-tubulin were all visible in their respective fractions (Figure 5-8 A). Interestingly a number of bands were also visible representing proteins which apparently co-eluted with DpoA:GFP yet are absent from the GFP elution fraction. These bands (indicated by stars, Figure 5-8 A) may represent additional proteins that interact with DpoA.

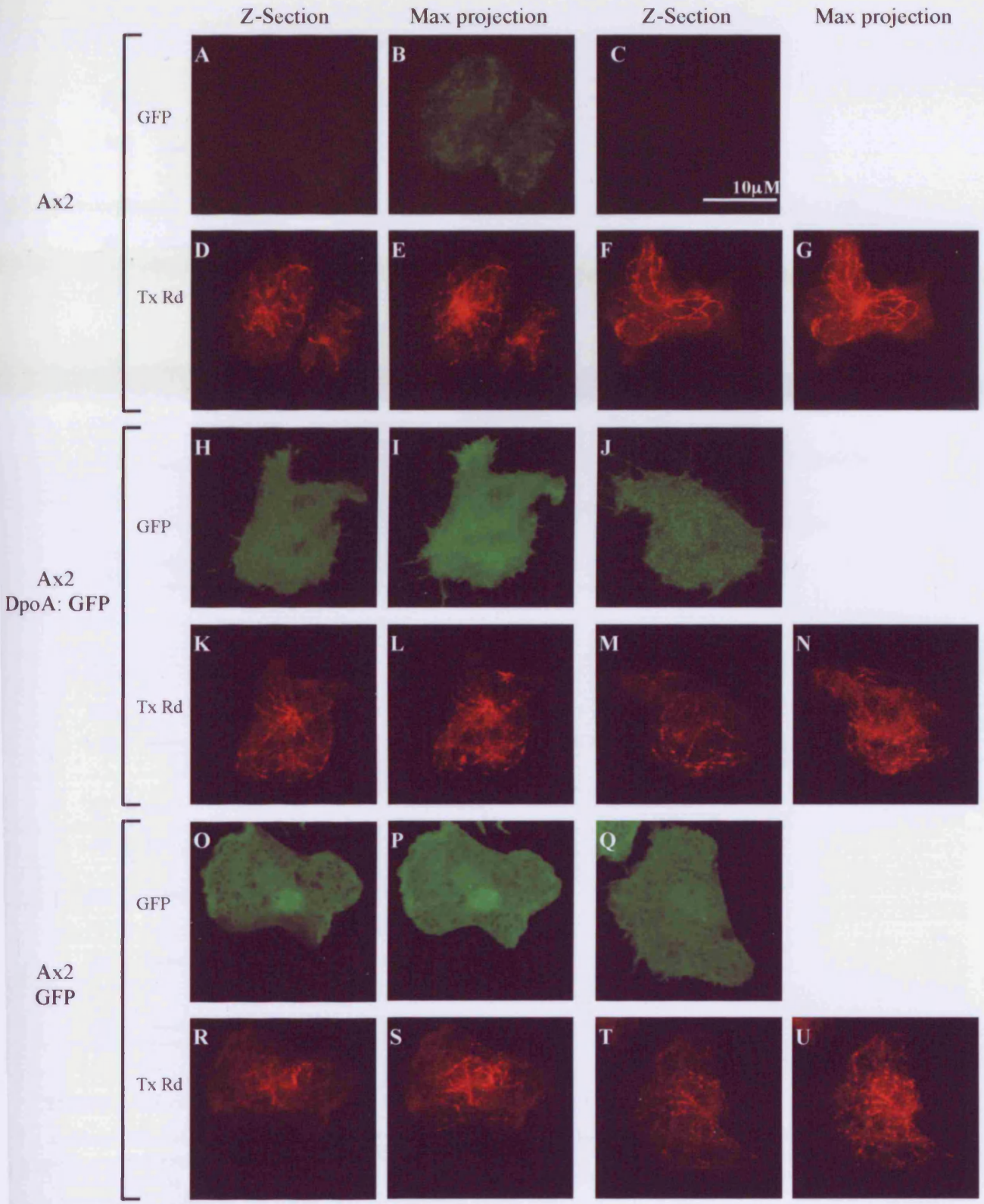


Figure 5-7 DpoA Does Not Co-Localise with α-Tubulin

Growing cells were fixed and stained with a 1°antibody against α-tubulin followed by a Texas Red conjugated 2°antibody. A series of Z-sections were taken through each cell and stacked to form a maximum projection picture of the tubulin through the depth of the cell (see materials and methods 2.5). Images from two cells of Ax2 untransformed, A-G, expressing DpoA:GFP, H-N or expressing GFP, O-U are representative of all cells observed. A maximum projection of the GFP fluorescence was only completed for one cell of each type.

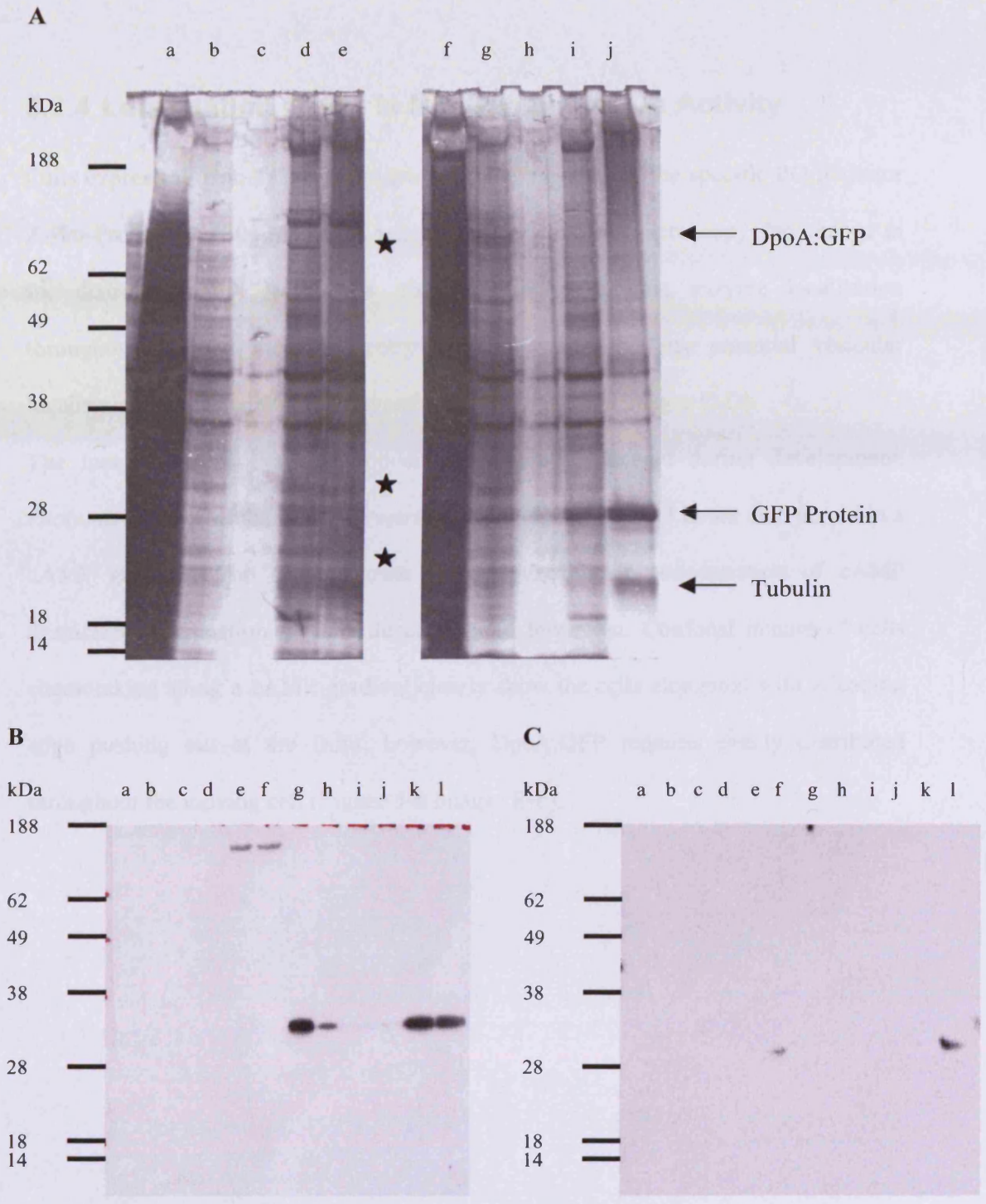


Figure 5-8 Protein-Protein Interaction of DpoA and α-Tubulin

A. Silver Stain of Proteins retained by DpoA:GFP on a GFP Binding Column. Cell extracts of DpoA:GFP, a-e, and GFP, f-j, expressing cells were incubated with GFP binding beads before being loaded onto a magnetic column. Unbound proteins were washed out in the flowthrough, a & f, and washes, b-c & g-h before elution of GFP proteins, d & i followed by release of the beads from the column, e & j. Bands representing DpoA:GFP, GFP and α-tubulin are indicated by arrows, stars indicate bands co-eluted with DpoA:GFP which were not observed with GFP alone. **B&C. Western Blot of GFP Binding Column Eluates.** Cell Extracts as for silver stain, A. DpoA:GFP, a-f, GFP, g-l, flowthrough, a & g, washes 1-3, b-d & h-j, elution, e & k, beads, f & l. Blot B was probed using anti-GFP-HRP antibody, blot C was probed using anti-α-tubulin 1°antibody followed by anti-mouse-HRP 2°antibody.

5.2.4 Localisation Of PO Is Not Dependent On Activity

Cells expressing DpoA:GFP were grown in the presence of the specific PO inhibitor Z-Pro-Prolinal at 130mM before being fixed for confocal microscopy. No change in the distribution of DpoA was observed, indicating that enzyme localisation throughout the cell including entry to the nucleus and any potential vesicular localisation is independent of enzyme activity (Figure 5-9 images C-D).

The localisation of PO also appears to remain unchanged during development. *Dictyostelium* cells in suspension were pulsed with cAMP for 5 hours then placed in a cAMP gradient. The cells migrate towards the higher concentration of cAMP mimicking aggregation of cells during mound formation. Confocal images of cells chemotaxing along a cAMP gradient clearly show the cells elongated with a leading edge pushing out at the front, however, DpoA:GFP remains evenly distributed throughout the moving cell (Figure 5-9 images E-F).

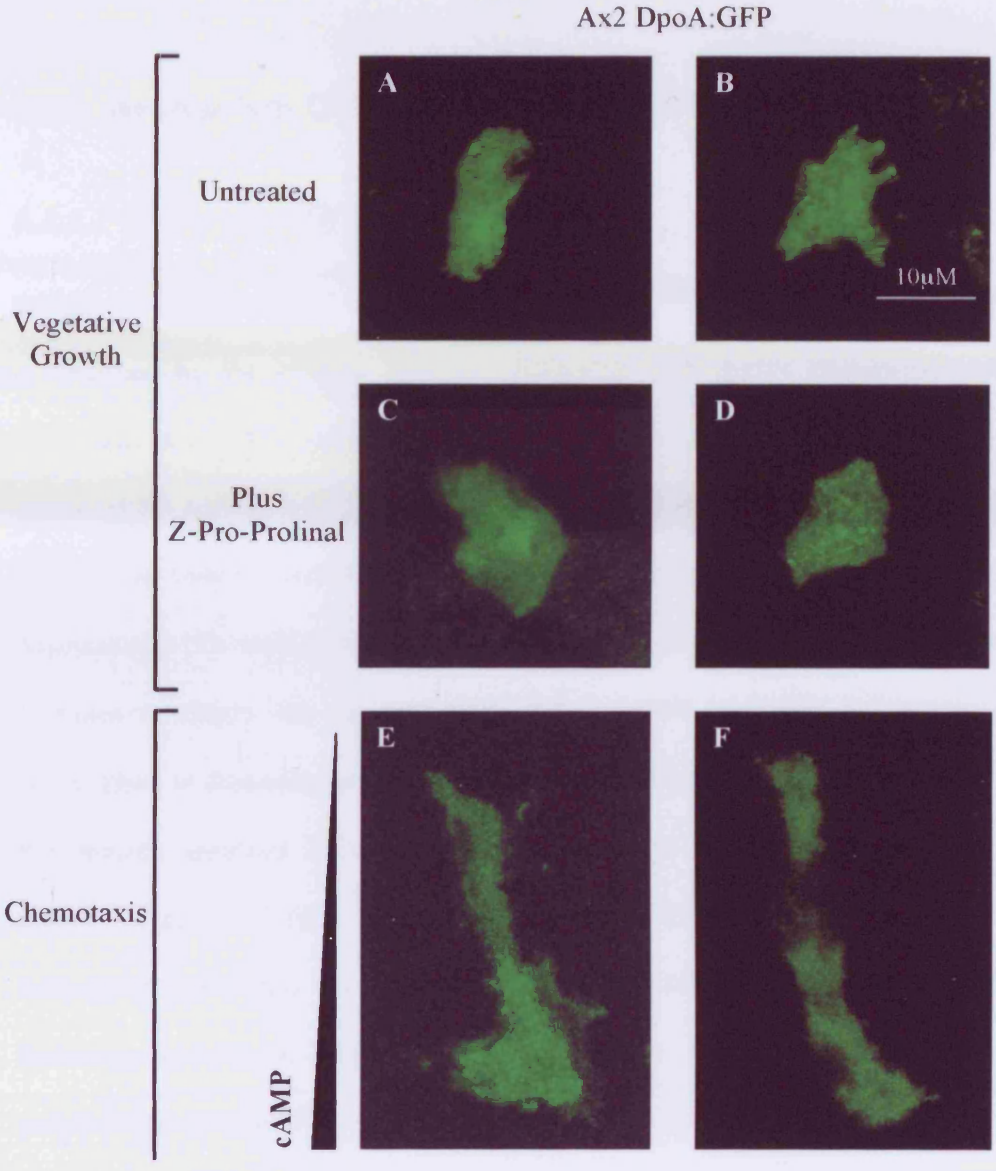


Figure 5-9 PO Localisation is Independent of Activity or Development

Confocal images of cells grown in the presence, C-D and absence A-B of the PO specific inhibitor Z-Pro-Prolinal, or grown in suspension, pulsed with cAMP for 5 hours and placed in a cAMP gradient, E-F. Cells were prepared as described in sections 2.5.3 and 2.5.4.

5.2.5 Mechanism Of PO Inhibition Of MIPP

5.2.5.1 Timecourse Of MIPP Inhibition

Inhibition of PO results in derepression of IP_5 dephosphorylation to IP_3, this reaction is catalysed by the enzyme Multiple Inositol Polyphosphate Phosphatase (MIPP) (Nogimori et al. 1991; Craxton et al. 1995). The mechanism by which PO is able to inhibit MIPP activity is not known. Direct cleavage of MIPP may be unlikely because of its size, MIPP activity could therefore be affected by a regulatory peptide. Alternatively PO could affect gene expression or protein synthesis of this enzyme.

In order to clarify the pathway from PO to MIPP inhibition a timecourse was investigated to determine any delay between inhibition of PO and activation of MIPP. PO specific inhibitor Z-Pro-Prolinal was added to *Dictyostelium* cells grown in culture, aliquots of cells were taken directly after addition of inhibitor and at subsequent time points. PO activity and IP_3 levels in cell samples were measured.

Following addition of Z-Pro-Prolinal, PO inhibition was immediate (6second time point) and maintained for over an hour. PO activity was decreased to less than 20% of that seen in control cells and did not increase in samples taken up to an hour after inhibitor treatment (Figure 5-10).

IP_3 levels were significantly higher in treated cells than those in control cells 30minutes after addition of PO inhibitor, indicating PO inhibitors are able to de-repress MIPP activity in under half an hour (Figure 5-11). This short time-course makes it unlikely that PO is acting via alteration of gene expression.

A steady increase in IP_3 was observed throughout the assay. As the assay progressed, levels of IP_3 in both treated and untreated cell cultures increased. This may be due to secretion of IP_3 into the cell culture media rather than an increase in intracellular IP_3.

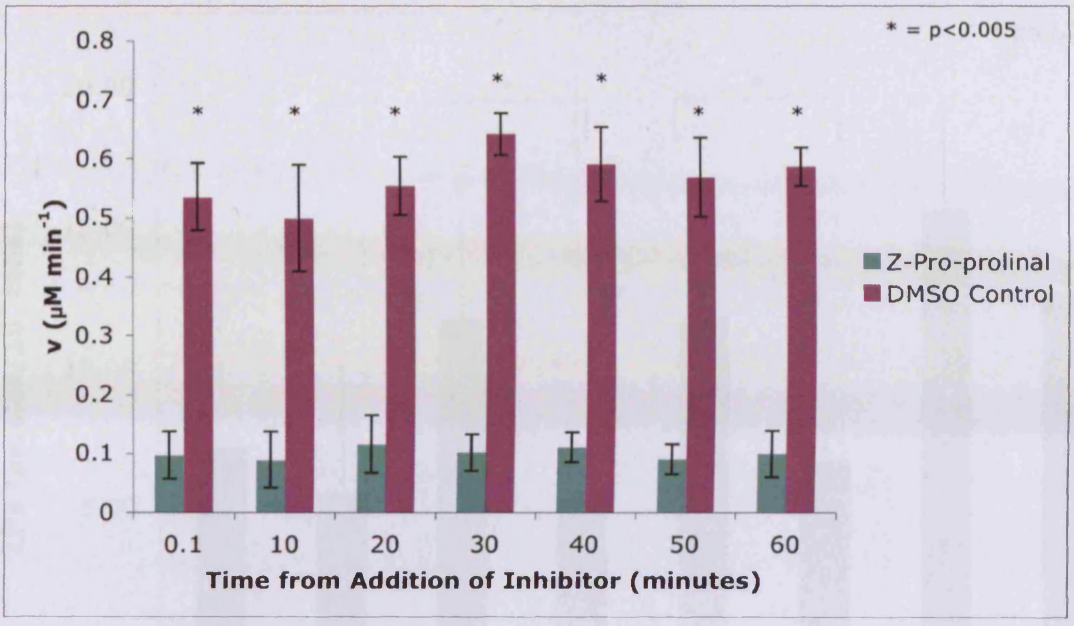


Figure 5-10 Time-course of PO Inhibition by Z-Pro-Prolinal

PO activity in cell extracts taken at 10minute intervals from growing cells following treatment with Z-Pro-Prolinal or DMSO, carrier control. Results are mean of 3 biological repeats done in duplicate ±SEM

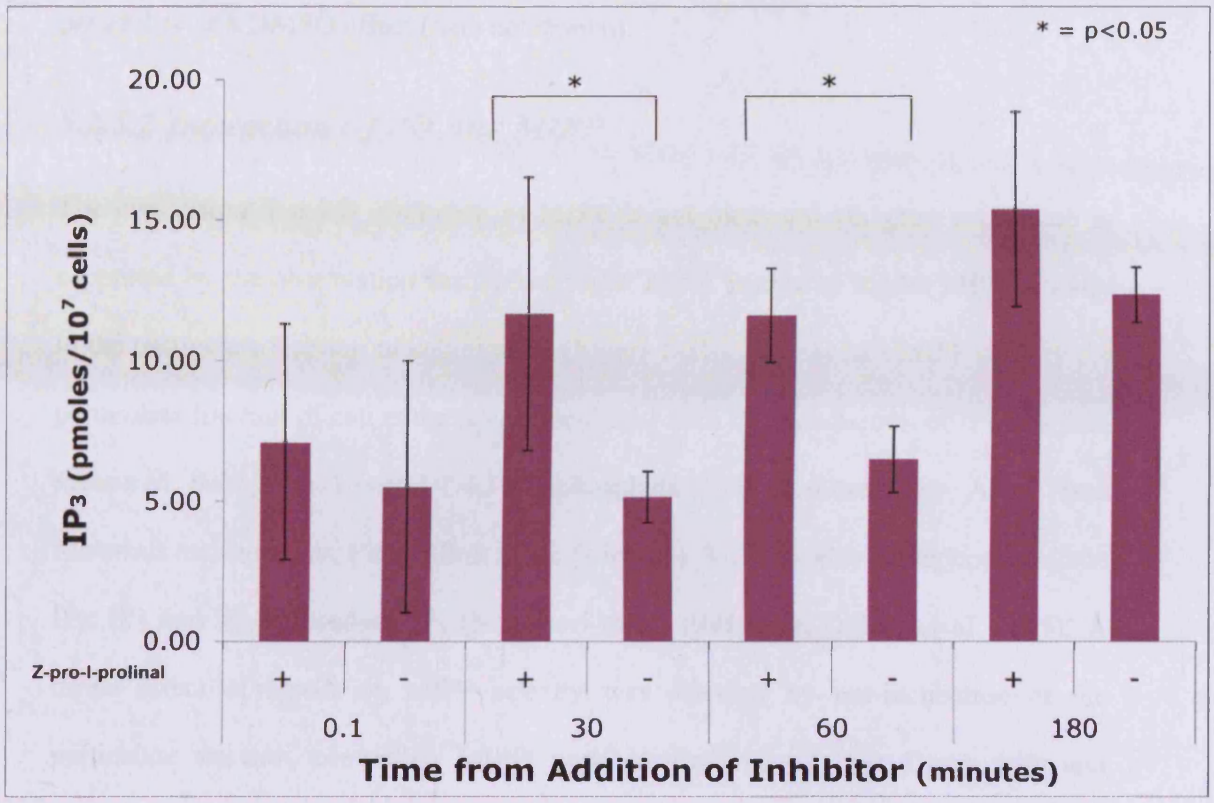


Figure 5-11 Time-course of Cellular IP_3 Changes Following Z-Pro-Prolinal Treatment

IP_3 level in cell extracts taken at 30minute intervals from growing cells following treatment with Z-Pro-Prolinal or DMSO, carrier control. Results are mean of at least 3 biological repeats done in duplicate ±SEM

This increase in IP_3 was also observed in the absence of DMSO eliminating the possibility of a DMSO effect (data not shown).

5.2.5.2 Interaction Of PO And MIPP

The conclusion that PO inhibition of MIPP is not mediated via gene expression is supported by the observation that recombinant DpoA is able to inhibit MIPP activity in the particulate fraction of cell extracts (Figure 5-12). To measure MIPP activity the particulate fraction of cell extracts was incubated with IP_6, production of IP_3 was then measured using the Inositol-1,4,5-Trisphosphate [^{3}H] Radioreceptor Assay (see Materials and methods, PerkinElmer Life Sciences). MIPP is able to dephosphorylate IP_6, IP_5 and IP_4 to produce IP_3 (Nogimori et al. 1991; Van Dijken et al. 1995). A direct effect of DpoA on MIPP activity was detected by pre-incubation of the particulate fraction, containing MIPP, with purified recombinant DpoA with and without Z-Pro-Prolinal.

Pre-incubation of MIPP with recombinant DpoA resulted in a significant decrease in MIPP activity, approximately 50%, compared with pre-incubation with buffer only. No decrease in activity was observed when MIPP was pre-incubated with both recombinant DpoA and its specific inhibitor (Figure 5-12).

This implies PO inhibition of MIPP activity is mediated by a direct interaction between PO and MIPP or via an intermediate protein or peptide present within the particulate fraction. This raises the question, could PO be able to cleave some proteins as well as small peptides and could PO potentially be acting directly on MIPP itself?

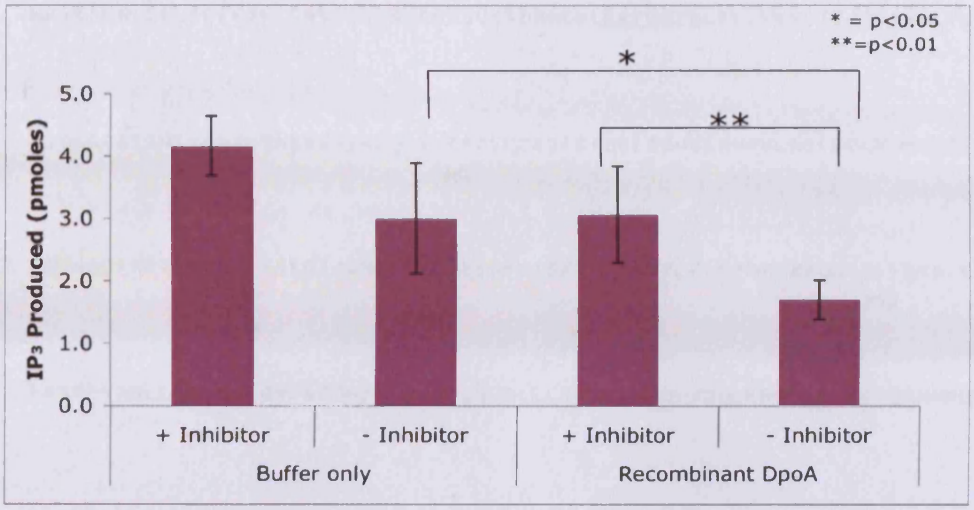


Figure 5-12 Direct Inhibition of MIPP by Recombinant DpoA

The particulate cell fraction containing MIPP A was incubated with and without purified recombinant DpoA in the presence of Z-Pro-Prolinal or DMSO. MIPP A activity was then assayed by addition of IP_6 followed by measurement of breakdown to IP_3. Results are average of at least 3 repeats done in duplicate ±SEM

***Dictyostelium* MIPP A (EAL64744)**

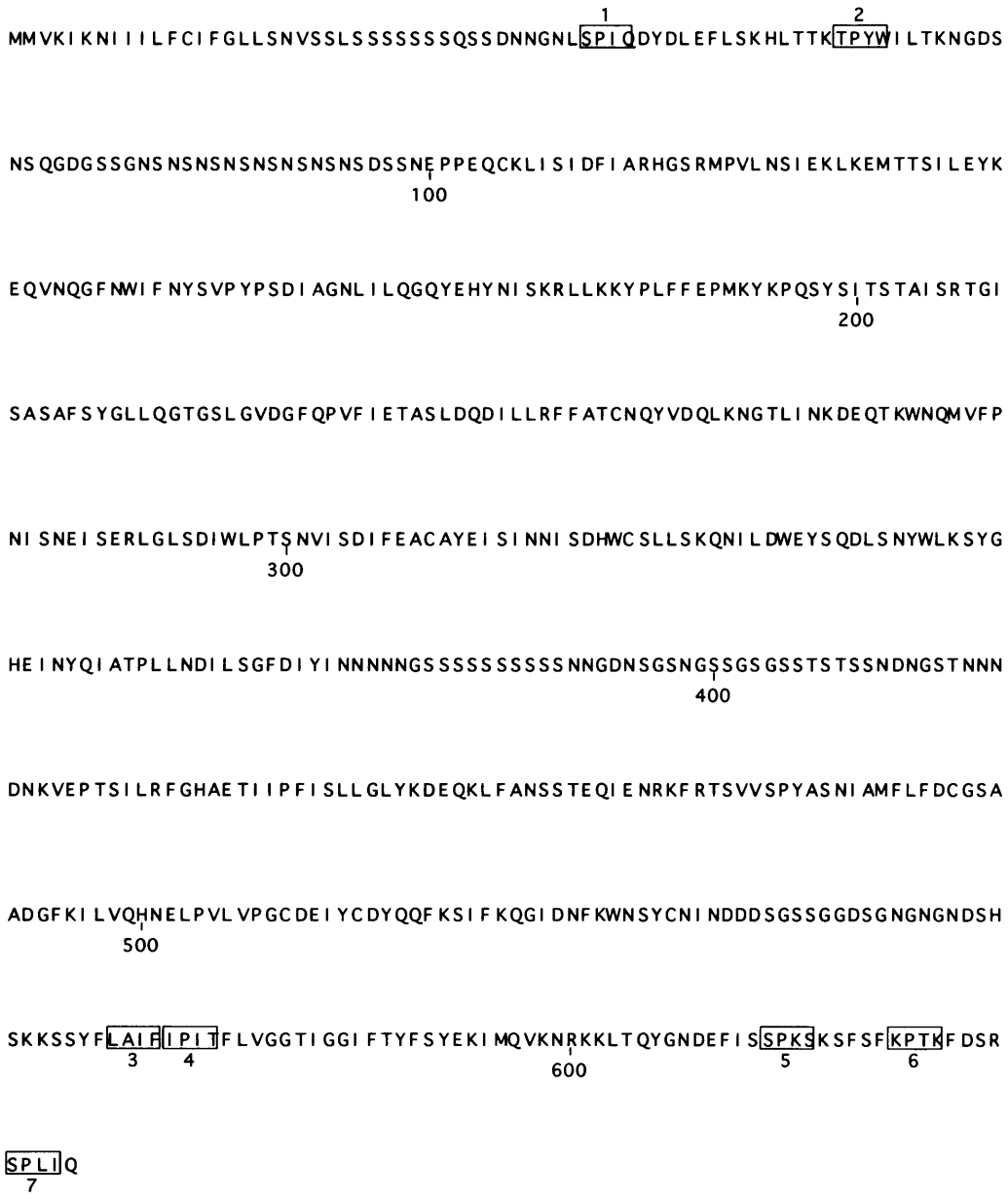


Figure 5-13 Protein Sequence of *Dictyostelium* MIPP A (EAL64744)

Potential Prolyl Oligopeptidase cleavage sites are identified by numbered boxes around four residues with the bond to be cleaved at the centre. Box 7 identifies the site most likely to be a true cleavage site.

The preferred cleavage site of PO is the carboxyl bond of a proline residue adjacent to a hydrophobic residue. PO is also able to cleave after an alanine residue, though with lower efficiency, under certain conditions (Sharma and Ortwerth 1994). Inspection of the protein sequence for *Dictyostelium* MIPP A (Accession No. EAL64744) identified seven potential PO cleavage sites within 100 residues of either end of the protein (Figure 5-13).

Six of these were proline residues, the seventh an alanine residue. Of these seven, five conformed to the Pro(Ala)-X motif where X is a hydrophobic residue (Figure 5-13 box 1,2,3,4,7). Cleavage of peptides by PO is reported to be restricted to sites within 4-5 residues of the peptide terminus therefore the only strong possibility as a cleavage site for PO is that outlined in box 7 Figure 5-13. Cleavage at this point would remove the three residues LIQ from the C-terminus of MIPP A.

5.2.6 Inactive DpoA Does Not Act As A Dominant Negative

The same active site mutations as introduced to the V5-tagged DpoA expressed in bacteria, were introduced to the GFP tagged DpoA in the *Dictyostelium* expression vector pDXA (Appendix I.III). Again, this was done using Quickchange mutagenesis and confirmed by sequencing of the vector. Both Ax2 and LisA cells were transformed with each of the mutants H730A, D693G and S609A, clones were selected using G418 and positive colonies confirmed by observation of fluorescence.

Activity of the GFP tagged mutants was measured following isolation on GFP binding magnetic columns. PO assays were carried out in the columns containing the bound GFP protein, the assay buffer was eluted into a 96 well plate, following completion, for spectrophotometric analysis as normal. The unbound cell extract (data not shown) and the flowthrough, were also assayed for PO activity (Figure 5-14).

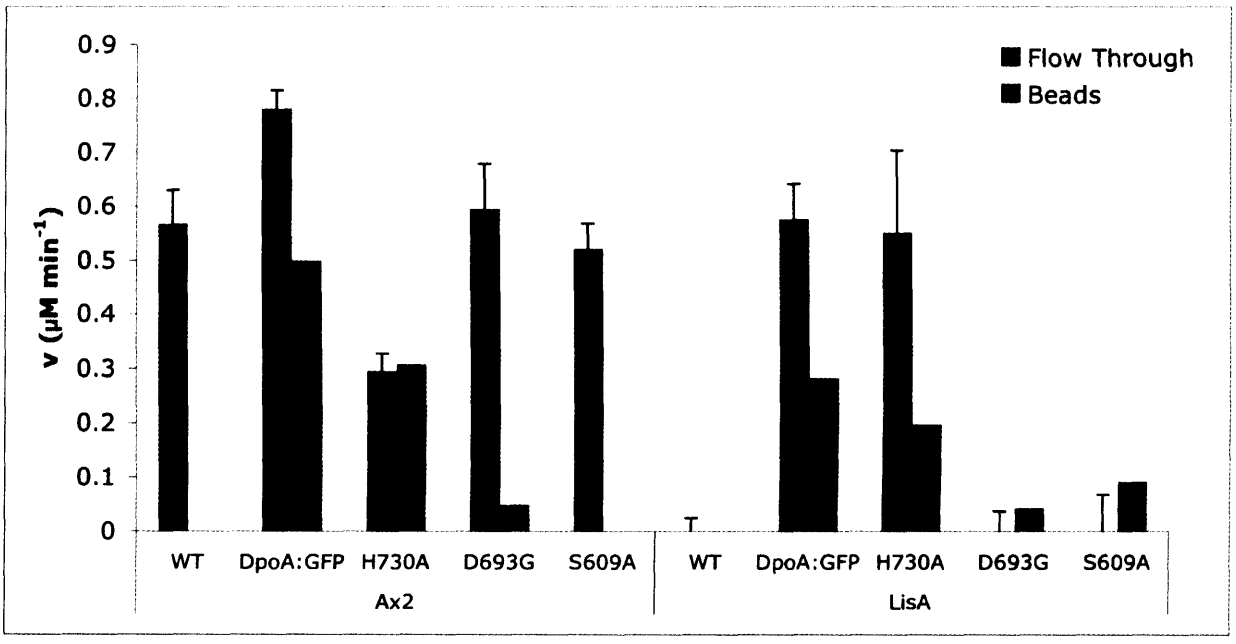


Figure 5-14 Activity of Mutant DpoA:GFP

Cell extracts were taken from wild type (WT) Ax2 and LisA cells as well as Ax2 and LisA cells expressing unaltered DpoA:GFP or DpoA:GFP containing the mutations H730A, D693G or S609A. These extracts were incubated with anti-GFP antibodies conjugated to magnetic beads and applied to a magnetic column. Flow-through, and GFP proteins bound to the columns were both assayed for PO activity.

As expected PO activity was observed in the unbound extract of all Ax2 cells as well as LisA cells expressing the wild type DpoA:GFP. Columns were loaded to saturation so some recombinant enzyme activity was present in the flowthrough. Also as expected, PO activity was observed bound to the columns in the cells expressing wild type DpoA:GFP. Very little or no activity was observed bound to the columns containing extracts from cells expressing S609A or D693G mutants or the respective flowthroughs when expressed in LisA cells.

Rather unexpectedly the H730A mutant retained its PO activity, this was not the case for the V5 tagged DpoA H730A mutant previously expressed in bacteria. The major difference between these two proteins is the C-terminal tag. The H730 residue is very close to the beginning of the tag sequence therefore its positioning may potentially be distorted by the presence of the tag. The GFP peptide is considerably larger than that of the V5 epitope tag so may have a greater effect on the context of the His residue at the active site

These mutants were observed to determine if they would behave as a dominant negative and show a similar insensitivity to LiCl as the LisA DpoA null mutants. Cells were plated out on agar containing increasing concentrations of LiCl or NaCl as a salt control.

Rather than acting as a dominant negative the mutant DpoA:GFP protein appears to increase sensitivity; aggregation of *Dictyostelium* expressing the mutant enzymes was inhibited at 5mM LiCl while both the untransformed cells and cells expressing the non-mutant tagged enzyme retained the ability to aggregate (Figure 5-15). *Dictyostelium* expressing the wild type DpoA:GFP showed a slight increased sensitivity to LiCl by showing inhibition of aggregation at a lower LiCl concentration than the untransformed cells (data not shown).

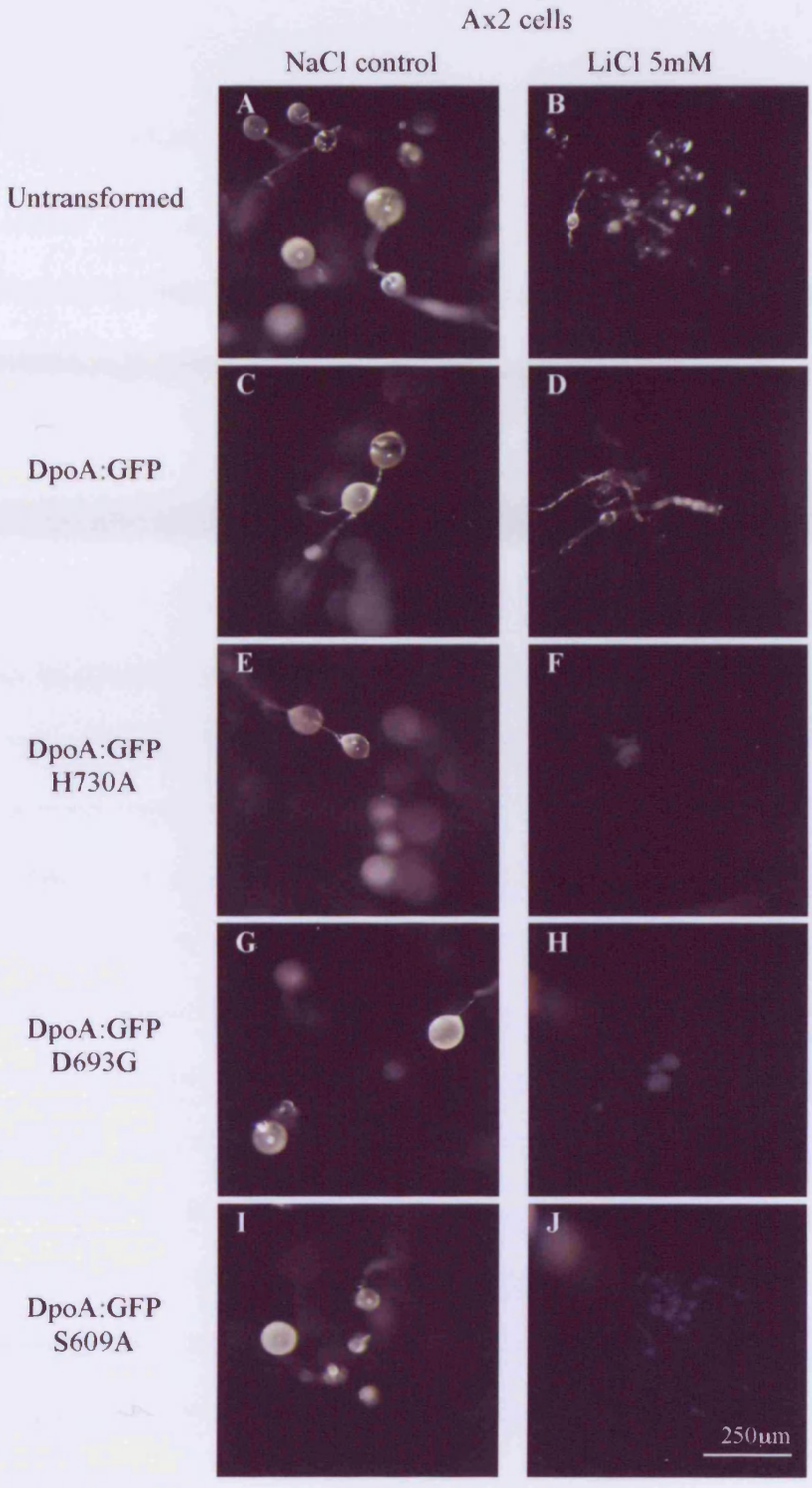


Figure 5-15 Sensitivity of Mutant DpoA Transformed *Dictyostelium* to LiCl

Ax2 cells expressing H730A, D693G or S609A mutant DpoA:GFP were allowed to develop in the presence of LiCl or NaCl salt control (see materials and methods 2.4.4).

5.3 Discussion

The cellular function of PO has been linked to a diverse array of processes. By studying intracellular localisation of this enzyme as well as specific enzyme interactions, it is possible to suggest potential mechanisms responsible for some of these effects.

5.3.1 Localisation Of DpoA; Indication Of PO Function

Confocal images of *Dictyostelium* cells expressing GFP tagged DpoA have revealed a diffuse localisation throughout the cell. Noticeably, DpoA:GFP was not excluded from intracellular vesicles that clearly excluded GFP alone. Distribution and size of these vesicles suggests they may represent endosomes or lysosomes. This conflicts with western blot analysis of different cellular fractions by Williams et al. (1999) which showed DpoA to be absent from the vesicular fraction. The potential presence of PO activity within these vesicles is interesting when considered in connection with the proposed existence of two pH forms of the enzyme (Polgar 1991a).

The high pH form of PO is active under physiological conditions, the vesicular localisation may propose a function of the low pH form as the pH within lysosomes is considerably lower than that of the cytoplasm. If PO is in fact functional within lysosomes (pH5) or late endosomes (pH5-6), it supports a previously proposed role in general proteolysis (Pratt et al. 1989; Tsukahara et al. 1991a; Tsukahara et al. 1991b). PO may be important for cells to be able to degrade and recycle peptides containing proline residues.

Staining of DpoA:GFP cells with DAPI revealed the presence of PO within the nucleus. Previous reports of nuclear PO have proposed a role in DNA synthesis (Ohtuski et al. 1997; Ishino et al. 1998). Another possibility is that of a nuclear

inositol signalling function. Although MIPP is reportedly localised to the endoplasmic reticulum or plasma membrane, a potential nuclear function is suggested by observation of the effect of ratio of IP_6 to IP_4/IP_5 in chromatin remodelling and gene expression (Shen et al. 2003; Steger et al. 2003). In *Dictyostelium*, changes in PO or MIPP activity are able to affect levels of gene expression of a number of enzymes in the inositol pathway (personal communication M.Keim and J.King).

The presence of DpoA within the nucleus is not dependent on enzyme activity, distribution was not affected by the presence of Z-Pro-Prolinal. There was also no change during chemotaxis, nuclear distribution was not decreased or accentuated. Ohtsuki et al. (1997) reported a decrease in nuclear PO accompanied by an increase in cytosolic PO following changes in cell culture conditions of insect embryonic cells. No such effects have been observed in *Dictyostelium* cells during aggregation or PO inhibition however, many other conditions may affect DpoA. Observation of DpoA localisation under different cellular conditions may yet reveal changes in its position within the cell.

PO distribution at the plasma membrane, the suggested localisation of MIPP in *Dictyostelium*, was not observed. There was also no apparent co-localisation of DpoA:GFP with the ER marker PDI as shown previously by Williams et al. (1999).

PO has been implicated in a number of functions that are localised to specific intracellular structures, for example gene expression and protein secretion. However, in this study even distribution of PO throughout the cytoplasm and nucleus is observed. This suggests that either PO acts sufficiently upstream in the signalling pathway that it does not localise to effecter proteins or, PO is able to play specific roles, by cleaving different substrates, in the different cellular compartments without increased localisation to these regions.

In order to completely discount the effect of the GFP tag on localisation of DpoA within the cell it would be necessary to carry out immunofluorescence studies of endogenous DpoA in untransformed cells. Unfortunately two DpoA antibodies raised against peptide fragments of the enzyme have proved ineffective.

5.3.2 Effects Of DpoA Mediated By Inositol Signalling

Previous reports have linked a loss of PO activity to increased vesicular transport and an increase in total protein secretion as well as a specific increase in secretion of β-amyloid (protein involved in plaque formation observed in Alzheimer's Disease) (Rossner et al. 2005; Schulz et al. 2005).

A function for PO in intracellular transport and peptide secretion would provide an alternative explanation to a number of contradictory hypotheses about the role of this enzyme. Numerous groups have reported an increase in a variety of neuropeptides including Substance P, α-MSH (Bellemere et al. 2003) and TRH (Shinoda et al. 1995) following PO inhibition. The current theory is that inhibition of PO results in a decrease in degradation of these peptides, this is slightly contentious as there is mounting evidence of the cytosolic, non-secreted localisation implying PO would not come in to contact with these peptides *in vivo*. Another explanation may be increased secretion of neuropeptides resulting from removal of the repressive effect of PO on vesicle trafficking and secretion. While these neuropeptides are clear substrates of PO *in vitro*, a significant number of enzymes are expressed *in vivo* which may result in the same cleavage.

These results have not identified PO localisation to the microtubules as previously observed (Schulz et al. 2005), however, this does not discount the role of PO in this process. It is possible that the effect of PO on vesicle trafficking and secretion is also mediated by the effect of PO on inositol phosphate levels. Inhibition of PO results in

increased dephosphorylation of IP_6, IP_5 and IP_4. Both IP_6 and IP_4 are able to bind a number of proteins involved in vesicle trafficking resulting in inhibition of endocytosis and vesicle recycling (Palczewski et al. 1991; Voglmaier et al. 1992; Gaidarov et al. 1996; Milano et al. 2006).

Inositol pyrophosphate IP_7 also shows a negative effect on clathrin coat formation, by binding proteins involved in assembly including AP-3 (Ye et al. 1995), and vesicle trafficking (Fleischer et al. 1994; Ali et al. 1995). While no change in pyrophosphate levels has been reported following inhibition of PO, it is known that in mammalian cells up to 50% of the IP_6 pool goes through cycles of phosphorylation and dephosphorylation to IP_7 and IP_8 every hour (Menniti et al. 1993). Therefore, an increase in IP_6 breakdown has the potential to cause a knock-on effect to the levels of these compounds.

5.3.3 Specific Intracellular Interactions

Other proteins containing a β-propeller domain are able to form protein-protein interactions via the top surface of the propeller (e.g. interactions of both hemopexin and vitronectin with cellular receptors and binding of Gβ to the α and γ subunits (Jenne and Stanley 1987; Neer and Smith 1996)). This domain provides a potential site for PO to interact with other intracellular proteins. PO is present throughout the cytoplasm, nucleus and potentially a number of intracellular vesicles and able to influence a number of cellular functions. Specific protein interactions may be important in targeting PO to a particular localisation or particular signalling pathway. Silver stain analysis of proteins bound by DpoA:GFP trapped in a GFP binding column revealed a number of proteins retained in the presence of DpoA:GFP but not GFP alone. These results showing potential DpoA-binding proteins highlight an

exciting progression, perhaps using yeast-2-hybrid methods, for work on this enzyme however, this is beyond the scope of this particular investigation.

The addition of PO inhibitors resulting in increased IP_3 levels has been well documented (Williams et al. 1999; Schulz et al. 2002). Increased IP_3 is the result of increased dephosphorylation of higher order inositol phosphates, a reaction catalysed by the enzyme MIPP. Time course and activity studies have enabled further insight into how PO may act to inhibit MIPP activity. MIPP is not an obvious target for cleavage by PO, whose activity is reportedly restricted to peptides of less than 30 amino acids. This restriction has been proposed to be due to the presence of secondary structures in larger peptides preventing access to the active site, therefore, PO is potentially also able to cleave unstructured regions at the N or C-terminus of a larger protein.

MIPP activity was increased within 30 minutes of PO inhibition making it unlikely that the effect of PO is mediated by changes in gene expression. In fact a much more direct interaction between these two enzymes is highly likely, addition of purified DpoA is able to inhibit MIPP activity in a cell extract particulate fraction. Three possible scenarios of MIPP inhibition by PO are proposed; cleavage of MIPP itself, peptide cleavage to create an inhibitory peptide or cleavage of a stimulatory peptide. Despite the size restriction preventing MIPP cleavage by PO a potential PO cleavage site is present at the C-terminus of the enzyme.

5.3.4 Effect Of Catalytically Inactive DpoA

In contrast to the V5 tagged DpoA the H730A mutant of DpoA:GFP retained its ability to hydrolyse Z-Gly-Pro-pNA. The only difference between these two recombinant enzymes is the C-terminal tag, suggesting the presence of the larger GFP tag may distort the last member of the catalytic triad or the area around it such that

another residue may compensate for the loss of His. The literature contains a number of examples where the mutated amino acid may be compensated for; Corey et al. (1992) found that the function of the mutated Asp residue in trypsin may be replaced by the introduction of a nearby acidic residue.

A number of mechanisms have been proposed to explain the activity of the catalytic triad in the prolyl oligopeptidase family enzymes. These include models of charge relay, hydrogen bond formation and the existence of two active diads rather than a single active triad (Polgar and Halasz 1982; Liao et al. 1992). The role of the histidine residue is that of a proton acceptor, stabilising the active serine residue and the tetrahedral intermediate (Polgar and Bender 1969). During catalysis the His imidazole ring gains a proton in the transition state and is stabilised by Asp.

The exact position of the His residue is flexible. Carter and Wells (1987) reported site-directed mutagenesis of the catalytic His residue in subtilisin can be partially compensated by substrate assisted catalysis, His at the P2 position of the substrate is able to compensate for the missing catalytic residue. The Gly residue in our synthetic substrate would not act in this way, however, it is possible another residue close to the active site would. In a small subset of serine proteases (Sedolisins SB, S53) the His residue function is in fact carried out by a glutamic acid residue whose carboxyl group is able to accept a proton from serine (Polgar 2005).

The region surrounding the catalytic triad of DpoA, 30 aa upstream of Ser to 30 aa downstream of His, contains 26 possible proton acceptors; 7 His, 13 Asp and 6 Glu residues, of which 4 His, 8 Asp and 2 Glu residues lie outside of predicted secondary structures while a further 2 His residues are at the terminus of α-helices (Fulop et al. 1998). If the active site is slightly distorted by the presence of the GFP tag one of these residues may be able to align close to the active site and compensate to a certain

extent for the loss of the catalytic His. A structural model of the active site of DpoA was created using the Swiss Model application using the X-ray crystal structure of the porcine enzyme, 1h2w, as a template. In this structure it appears that three of these possible proton acceptors, Glu742, Asp 632 and Asp 694 are in the vicinity of the active and have the potential to compensate for loss of the His residue (Figure 5-16). Further mutagenesis studies would be required to determine if this was the case.

Surprisingly, following development in the presence of LiCl the transformants containing the mutant DpoA:GFP did not behave as a dominant negative, instead aggregation was hypersensitive to the presence of lithium as would be expected in a transformant showing over-expression of the active enzyme. Hypersensitivity to LiCl of *Dictyostelium* expressing the mutant enzymes may indicate that although mutant PO is no longer able to cleave its target it can still bind it, thus sequestering it from its appropriate role. This is consistent with a model whereby PO is cleaving either MIPP directly or a stimulatory peptide rather than creating an inhibitory peptide.

The transformant expressing active DpoA:GFP was also hypersensitive to Lithium treatment but to a slightly lesser extent than those expressing the mutant versions. No difference in hypersensitivity was observed between the active H730A mutant and the inactive S609A and D693G mutants suggesting activity of the His mutant may not be maintained in the cell. The role of the catalytic triad become much more important in cleavage of a stronger peptide bond rather than an activated substrate (Corey and Craik 1992). Szeltner et al. (2002) also found that stabilisation of the transition state during catalysis was less important for activity if the substrate contained a good leaving group such that only a weak bond was being cleaved. This may account for the ability of the His mutant to cleave Z-Gly-Pro-pNA but show the same phenotype as the other mutants.

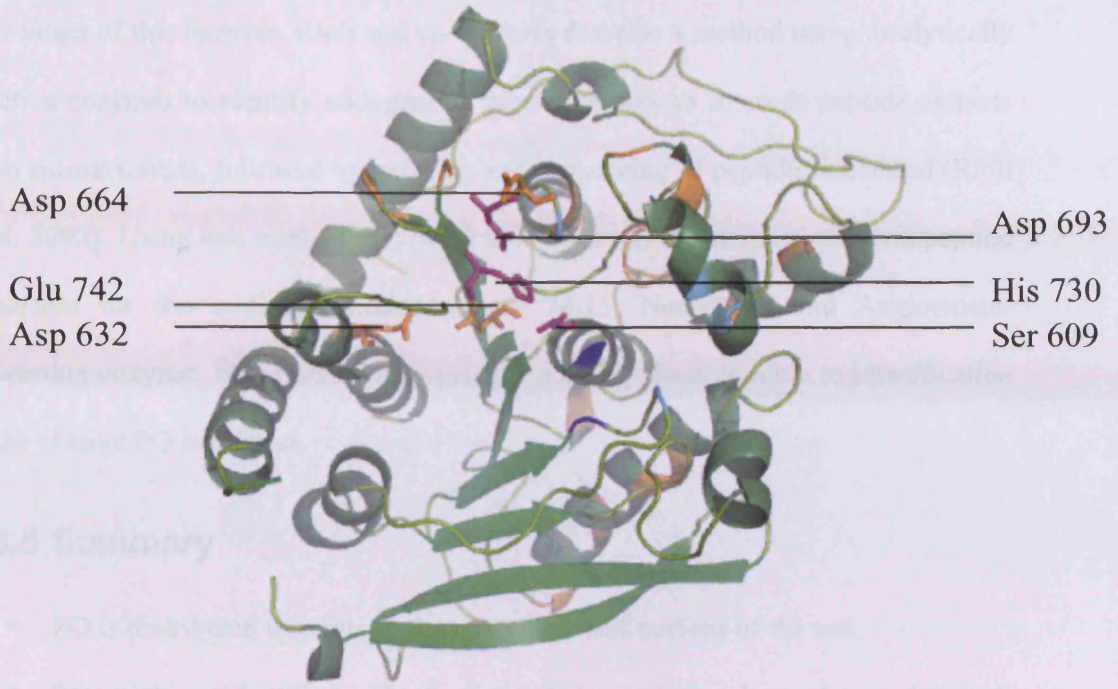


Figure 5-16. Potential Proton Acceptors close to the active site

Ribbon representation of the structure of the *Dictyostelium* PO, DpoA, as modelled by SwissModel using prolyl oligopeptidase from porcine brain, accession no. 1h2w, as a template. Images were produced using MacPyMol software. Only the catalytic domain is represented in this image. Active site residues are shown by purple stick structures (Ser609, Asp693 and His730). The 26 possible proton acceptors, which may compensate for the loss of the catalytic His, in the region surrounding the catalytic triad of DpoA, 30 aa upstream of Ser to 30 aa downstream of His, are highlighted in orange. Stick structures are shown (in orange) for three of these possible proton acceptors, Glu742, Asp 632 and Asp 694, which may be close to the active site. Also highlighted, are the proline binding residues (light blue) and the oxyanion binding residues (dark blue).

Catalytically inactive DpoA mutants may prove valuable for identification of the *in vivo* target of this enzyme. Rioli and co-workers describe a method using catalytically inactive enzymes to identify endogenous substrates present in crude peptide extracts from animal tissues, followed by isolation and sequencing of peptides identified (Rioli et al. 2003). Using this method they were able to describe a number of novel peptide substrates for the enzymes; Endopeptidase 24.15, Neurolysin and Angiotensin-converting enzyme. This method may provide a highly feasible route to identification of the elusive PO substrates.

5.3.5 Summary

- PO is distributed throughout the cytoplasm and nucleus of the cell.
- Potential, unidentified, DpoA interacting proteins have been detected, however, a previously reported interaction with α-tubulin has not been reproduced.
- DpoA is able to inhibit MIPP A in an isolated particulate fraction implying a direct mechanism of inhibition.
- Mutant studies of inactive enzyme are consistent with a model of PO cleavage of MIPP or a MIPP stimulatory peptide.

6 Discussion

6.1 Introduction

PO was first identified in 1971 as an oxytocin degrading enzyme (Walter et al. 1971). More than 30 years on and the true intracellular targets and functions of prolyl oligopeptidase are still not fully understood. In this study, sequence analysis, biochemical kinetics and cell biology have been used to study this enzyme in order to further understand its function as well as its potential as a therapeutic target.

6.2 Dictyostelium Provides A Good Model For Inositol Phosphate Signalling

Since its first discovery in the 1930s *Dictyostelium discoideum* has enjoyed an ever-increasing popularity as a model organism (Raper 1935), not least because of its single celled amoeboid growth, division and chemotaxis followed by cellular differentiation and development to form a multicellular organism. Studies using this organism have enabled insights into our understanding of some important biological processes including chemoattractant sensing, cell movement, and signal transduction (Harwood 2001).

Identification of the PO homologue, DpoA, in *Dictyostelium* and its role in inositol signalling adds weight to the argument for use of this organism to study the inositol phosphate pathway. This pathway is central to cell function and while *Dictyostelium* shows some differences to mammalian cells in its inositol phosphate chemistry, inositol phosphate signalling and the enzymes involved are highly conserved in this organism (van Haastert and van Dijken 1997).

Sequence analysis of the prolyl oligopeptidase enzyme across a number of species from the three different kingdoms has revealed a number of points. A striking observation was that a number of database sequences annotated as PO showed closer phylogenetic relatedness to other members of the Prolyl Oligopeptidase (S9) family than to PO suggesting they may be mis-annotated. The majority of these protein sequences lacked regions of homology to either the β-propeller or the catalytic domain. It may be the case that these sequences could be more accurately annotated as DPPIV, OB or AP, this observation highlights the essential nature of detailed sequence analysis on database sequences.

The prolyl oligopeptidase sequence is conserved across Eukaryotes, Bacteria and Archaea. Within the Prolyl Oligopeptidase phylogeny the Eukaryotes form a separate cluster from the Bacterial and Archaea proteins. DpoA, present in the eukaryote cluster, surprisingly shows a greater evolutionary distance from the mammalian enzyme than the plant (*O.sativa* and *A.thaliana*) or parasitic (*T.cruzi* and *T.brucei*) enzymes. Despite this evolutionary distance the more detailed sequence analysis shows a very high sequence homology around the active site and β-propeller domain, site directed mutagenesis has confirmed both the identity and necessity of the residues of the catalytic triad.

The use of the *Dictyostelium* homologue as a model for the mammalian enzyme is further supported by biochemical analysis of activity of recombinant DpoA and Rpo against the PO specific substrate Z-Gly-Pro-AMC. Enzyme kinetics studies revealed a similar K_M and V_{max} for both enzymes as well as very similar inhibition profiles in the presence of the specific PO inhibitors Z-Pro-prolinal and S17092.

The mechanism by which PO is able to control dephosphorylation of higher order inositol phosphates is currently under investigation. The similarity of DpoA to the mammalian enzyme makes *Dictyostelium* an ideal model for further understanding how this complex pathway is controlled. *Dictyostelium* may prove a more suitable choice than a number of other model organisms, the enzyme annotated as PO in the nematode *C.elegans* was one of those lacking the β-propeller domain while yeast completely lack PO. The two commonly used model organisms *D.melanogastor* and *D.rerio* contain PO sequences showing a closer relatedness to the mammalian enzyme than that of *Dictyostelium*. However, preliminary studies indicate that the recombinant *D.melanogaster* PO expressed in bacteria does not hydrolyse the PO specific substrate Z-Gly-Pro-pNA (unpublished data). In addition, while *D.rerio* PO may represent a

closer model of the mammalian enzyme, this organism is much harder to maintain and manipulate in a laboratory environment.

The two plant enzymes also show good homology to the mammalian enzyme. Signalling in these organisms may be complicated by identification of multiple PO homologues, some showing a closer homology to the bacterial enzyme and some the mammalian enzyme.

6.3 Understanding Of Inositol Pathways In Mood Disorders

Changes in inositol signalling, inositol or inositol phosphate levels have been reported in a number of mood disorders and other neurological disorders including Bipolar Disorder (Berridge et al. 1989), Schizophrenia (Shimon et al. 1998) and Down's Syndrome (Berry et al. 1999; Beacher et al. 2005). These represent complex disorders with both genetic and environmental causes. The underlying cause of most mood disorders remains poorly understood and treatment requires a combination of drugs, some with potentially serious side effects, and careful dose control (Burt and Rasgon 2004; Freeman and Freeman 2006; Young and Newham 2006). Understanding of signal transduction pathways potentially underlying these disorders may aid identification of more relevant and specific therapeutic targets.

One common treatment for Bipolar Disorder, valproic acid (VPA), is also used to treat epilepsy and migraines. The effects of VPA in Bipolar treatment are thought to be mediated by its effect on inositol-1-phosphate synthase resulting in inositol depletion; LiCl, another BD treatment, also causes inositol depletion though by inhibition of IMPase (Berridge et al. 1982; Inhorn and Majerus 1987; Shaltiel et al. 2004). A recent report by Cheng et al. (2005) identified an inhibitory effect of VPA on PO and proposed a model in which VPA is able to control inositol levels by acting on different parts of the pathway to both deplete and increase inositol. This suggests a mechanism to control both manic and depressive episodes, which may be related to increased and decreased inositol levels in bipolar patients.

In this study using recombinant His-tagged DpoA and Rpo (the *Dictyostelium* and rat prolyl oligopeptidase homologues) VPA inhibition was observed but to a much lesser extent than previously reported (Cheng et al. 2005). Effects of VPA analogues valpromide (VPM) and octanoic acid on PO activity indicate that both the carboxylic

acid domain and the branched structure may play a role in PO inhibition. At pH 7.5 both the unbranched carboxylic acid, octanoic acid, and the VPA amide derivative, valpromide, are able to inhibit recombinant DpoA and Rpo.

Physiological pH of mammalian cells, blood and cerebral spinal fluid (CSF) is around pH 7.4 and very little variation in pH is observed. At this pH either the carboxylic acid domain, the branched structure or both may be important for PO inhibition. To examine the effect of VPA *in vivo*, lymphoblast cells were treated overnight with VPA or its analogues. A small yet significant decrease in activity was observed in the presence of VPM. A small but significant inhibition of PO was also observed following treatment with VPA or octanoic acid, both of these treatments also led to a decrease in cell viability, therefore it is possible that the decrease in PO activity is a result of cell stress rather than direct inhibition.

The model proposed by Cheng et al. (2005) of altered functions of VPA during periods of mania compared with periods of depression could potentially be explained by the pH dependency of VPA inhibition of PO. This is an intriguing idea as small decreases in brain intracellular pH (changes of less than pH 0.1) have been associated with bipolar disorder (Kato et al. 1998; Hamakawa et al. 2004). Even more intriguingly a mitochondrial DNA polymorphism has been associated with Bipolar Disorder, Alzheimers Disease and Parkinson Disease, cells containing this polymorphism showed decreased mitochondrial matrix pH (approximately pH 0.1) and increased intracellular Ca^{2+} signalling (Kazuno et al. 2006); VPA has recently been reported to decrease Ca^{2+} levels only in cells containing this polymorphism (Kazuno et al. 2007).

VPM was able to inhibit recombinant PO at a physiological pH, this ability was maintained in vivo, VPM was able to demonstrate small yet significant PO inhibition

in lymphoblast cells without affecting cell viability. However, this inhibition was not sufficient to provide any visible lithium resistance when applied to developing *Dictyostelium.*

These results combined with a previous inhibitor study of unsaturated fatty acids on PO (Park et al. 2006), enable the suggestion that the important structural requirements for PO inhibition are the presence of one or more double bonds, a branched or a relatively short carbon chain and, at low pH, a carboxylic acid group. Other reports on the essential structural requirements for inhibition of alternative targets of VPA showed differing importance of the branched structure and the carboxylic acid (Spiegelstein et al. 2000; Eyal et al. 2005). These differences may be key in separating desired therapeutic effects of VPA from those causing unwanted side effects.

The structure required for VPA inhibition of inositol-1-phosphate synthase may prove valuable in future treatment of mood disorders. Studies of VPA derivatives have found the main structural requirement for inositol depletion to be the presence of a carboxylic acid group. The side chain length and branching appeared less important, however, inositol depletion was enantiomer specific suggesting a stereo-specific interaction is involved (Eickholt et al. 2005; Shimshoni et al. 2007). These reported essential structural requirements for inositol depletion differ from the potential requirements for PO inhibition, therefore, it may be possible to separate these two opposing effects of VPA on inositol signalling.

6.4 Cellular Interactions Of PO

The importance of non-inositol phosphate signalling roles of PO should not be overlooked. As is evident from phylogenetic analysis, a large number of bacterial POs that show strong similarity to the mammalian enzyme have been identified. Bacteria do not contain inositol polyphosphates therefore, the role of PO in these organisms is exclusively un-related to MIPP signalling. Elucidation of these roles may be aided by identification of PO binding partners or intracellular substrates.

PO does not contain a nuclear localisation signal or other targeting sequences. It is therefore expected that PO is able to interact with other proteins within the cytoplasm to facilitate nuclear localisation, entry to vesicles or even transient localisation to the plasma membrane or ER to interact with MIPP. The presence of PO within intracellular vesicles is supported by observation of a GFP tagged DpoA. This protein is localised throughout the cytoplasm while GFP alone showed clear exclusion from a number of small intracellular vesicles. Additionally, up to 30% of PO in the cell may be present in the particulate fraction (Agirregoitia et al. 2005). If this is true it suggests the existence of two separate pools of PO, which may be carrying out different functions. This is an interesting proposition as PO has been linked to varied biological functions.

To date the only protein reported to bind to PO other than peptide substrates is α-tubulin (Schulz et al. 2005). In this study I have been unable to replicate this interaction, however, very preliminary data indicates PO may bind a number of other proteins. Further research is needed to identify binding partners of this enzyme, an ideal step would be to complete yeast-2-hybrid studies. It will also be important to determine if any interactions identified in *Dictyostelium* also occur with the mammalian enzyme.

A final question, which has not been addressed in this study, is the upstream control of PO. PO levels and localisation are altered in response to different cell stresses indicating this enzyme is under specific control (Pratt et al. 1989; Tsukahara et al. 1990b; Sharma and Ortwerth 1994; Ohtsuki et al. 1997). In *Dictyostelium* cells localisation was not affected by addition of inhibitors, nor did it change during chemotaxis. A previous report describes an endogenous inhibitor for PO (Salers 1994), identification of proteins interacting with PO should identify any intracellular inhibitors. To determine if PO expression rather than activity is altered during different cell stress conditions, gene expression and promoter studies may be needed.

6.5 Potential Direct MIPP inhibition By PO

Loss of PO is known to result in increased MIPP activity. I propose that PO inhibition of MIPP is mediated by either direct cleavage of MIPP by PO or PO cleavage of a MIPP stimulatory peptide present in the particulate fraction.

Following PO inhibition MIPP activity, as measured by accumulation of IP_3, is increased in less than 30 minutes. In addition, purified PO is able to decrease MIPP activity when added to the particulate fraction of cell extracts. This rapid downstream effect as well as the ability to interact in the absence of whole cells indicates a relatively direct interaction between PO and MIPP. Three models to fit this pathway include (a) PO cleavage of MIPP itself, (b) cleavage of a stimulatory peptide or (c) cleavage of an additional protein or peptide to release an inhibitory peptide (Figure 6-1).

Localisation of these enzymes does not disagree with these models, MIPP is localised within the endoplasmic reticulum or potentially at the plasma membrane (Ali et al. 1993; Craxton et al. 1995; Van Dijken et al. 1997). The action of MIPP within the cell is to dephosphorylate higher order inositol phosphates to produce IP_3. These IPs are involved in a number of signalling pathways occurring within the cytoplasm however the ratio of IP_6 to IP_5 and IP_4 also affects gene expression, by activation and inhibition of chromatin remodelling complexes (Shen et al. 2003; Steger et al. 2003), therefore MIPP may also play a nuclear role.

PO's presence throughout the cytosol may indicate that it is unlikely to come into contact with MIPP. However, reports showing PO in the particulate fraction (Dresdner et al. 1982; Agirregoitia et al. 2005) and observation of the DpoA:GFP localisation within the nucleus as well as avoiding vesicle exclusion, indicates that at least a proportion of cellular PO may be able to access MIPP.

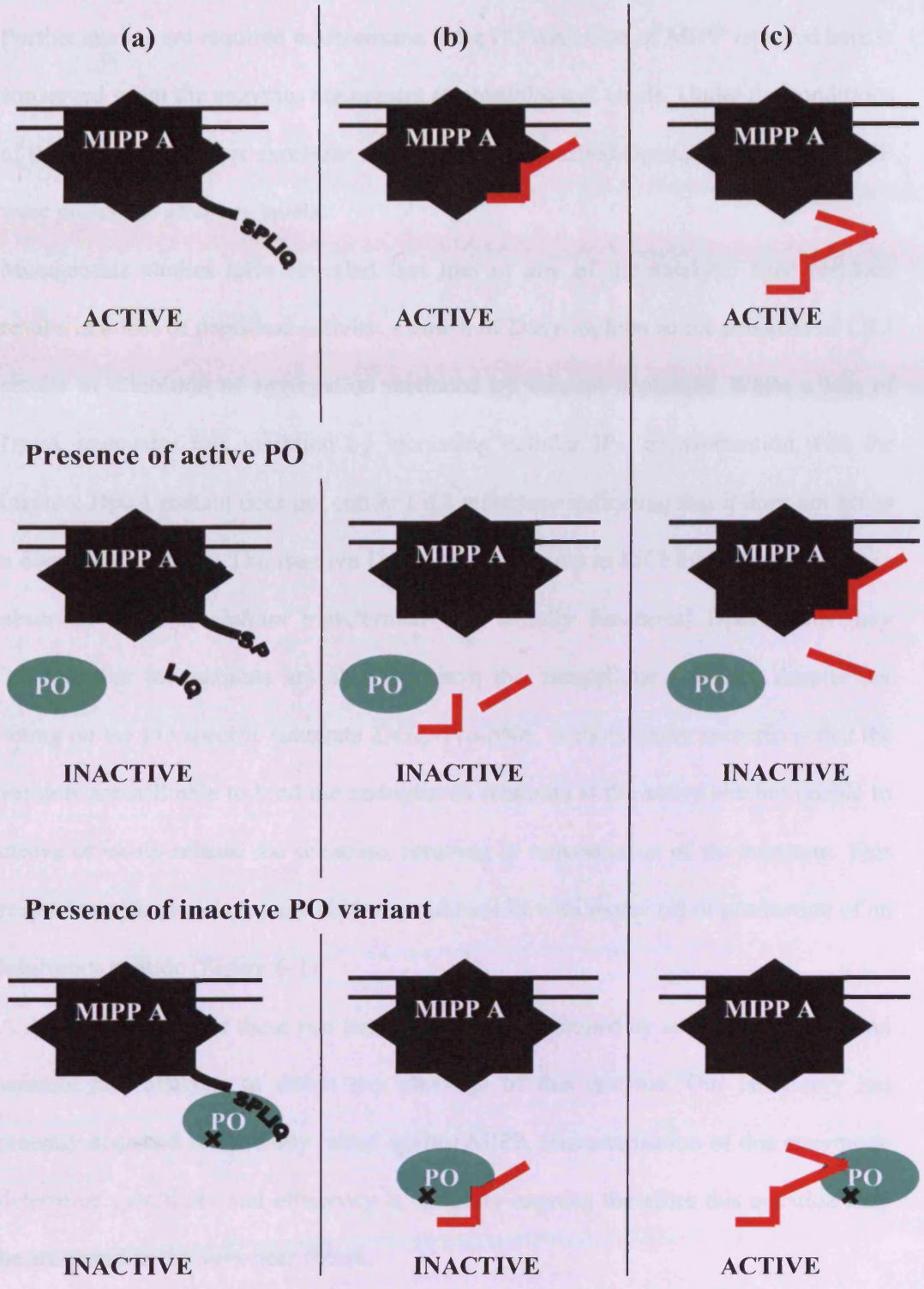


Figure 6-1 Potential Models of PO Inhibition of MIPP

I propose three possible models of MIPP inhibition by PO; (a) PO cleavage of MIPP itself, (b) cleavage of a stimulatory peptide or (c) cleavage of an additional peptide to release an inhibitory peptide. Only models (a) and (b) agree with observations of over-expression of the inactive PO variant in *Dictyostelium*.

Further studies are required to determine if the PO inhibition of MIPP reported here is conserved when the enzymes are present at physiological levels. Under the conditions of the assay, MIPP over expresser cell extracts and purified DpoA, both PO and MIPP were present at arbitrary levels.

Mutagenesis studies have revealed that loss of any of the catalytic triad residues results in a loss of peptidase activity. Growth of *Dictyostelium* in the presence of LiCl results in inhibition of aggregation mediated by inositol depletion. While a loss of DpoA attenuates this inhibtion by increasing cellular IP_3, transformation with the inactive DpoA mutant does not confer LiCl resistance indicating that it does not act as a dominant negative. The inactive DpoA mutant results in LiCl hypersensitivity as is observed in *Dictyostelium* transformed with a fully functional DpoA. This may indicate that the variants are able to cleave the intracellular substrate despite not acting on the PO specific substrate Z-Gly-Pro-pNA. A more likely scenario is that the variants are still able to bind the endogenous substrate at the active site but unable to cleave or easily release the substrate, resulting in sequestration of the substrate. This result fits with models (a) and (b) but would not fit with model (c) of production of an inhibitory peptide (Figure 6-1).

A distinction between these two models may be determined by isolation of MIPP and western blot analysis to detect any cleavage of this enzyme. Our laboratory has recently acquired an antibody raised against MIPP, characterisation of this enzyme to determine specificity and efficiency is currently ongoing therefore this question may be answered in the very near future.

6.6 Summary

Prolyl Oligopeptidase has been identified in archaea, bacteria and eukaryotes and is highly conserved between different species. In this study both phylogenetic and biochemical analysis have revealed the *Dictyostelium* PO homologue, DpoA, to be similar to the mammalian enzyme. While much work is still required to understand this enzyme fully *Dictyostelium* appears to be a highly suitable model for studying this pathway.

Inhibition of PO is able to reverse the action of a number of mood stabilising drugs by reversing their inositol depletion effect. This does not appear to involve direct interaction between the drugs and PO. However, in contrast to this an inhibitory effect of VPA on PO has been observed *in vitro* at physiological pH. This inhibition may be dependent on the presence of a carboxylic acid domain and a short or branched carbon backbone. The negative effect of VPA on cell viability leaves its inhibitory effect *in vivo* unclear.

Identification of PO binding proteins including its intracellular substrate and any regulatory peptides or proteins may prove integral to understanding the cellular role of PO. Initial results from isolation of the GFP tagged enzyme indicate the presence of potential PO binding partners. Further research using the peptidase dead mutants described in this study to identify *in vivo* targets of this enzyme has the potential to finally throw some light on this mysterious protein.

Finally, following observation of a time-course of enzyme activity inhibition *in vivo* as well as *in vitro* enzyme assays and mutant enzyme over expression, I have proposed three models to explain PO inhibition of MIPP activity.

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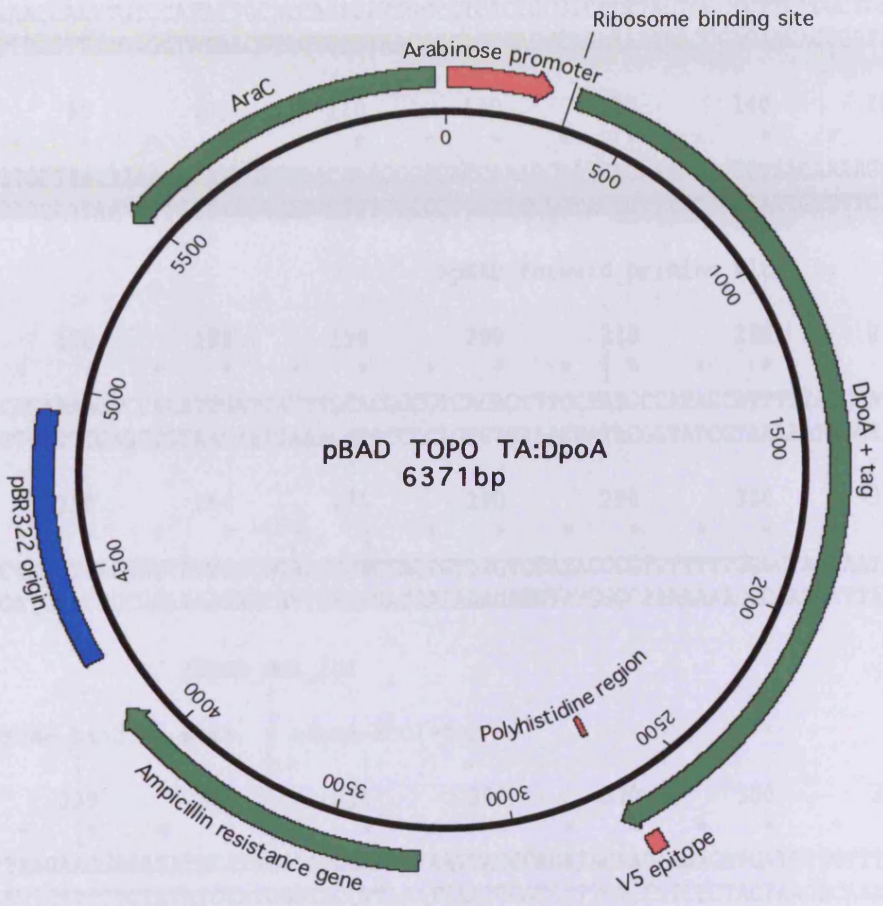
I Appendices

I.I Species List

Archaea	Euryarchaeota	Halobacteria	*Haloarcula marismortui*
		Thermococci	*Pyrococcus furiosus*
		Thermoplasmata	*Thermoplasma volcanium*
			Picrophilus torridus
Bacteria	Actinobacteria	Actinobacteria (class)	*Corynebacterium glutamicum*
			Mycobacterium tuberculosis
			Corynebacterium diphtheriae
	Bacteroidetes/Chlorobi group	Bacteroidetes	*Flavobacterium meningosepticum*
			Elizabethkingia meningoseptica
	Cyanobacteria	Gloeobacteria	*Gloeobacter violaceus*
		Nostocales	*Nostoc sp*
	Deinococcus-Thermus	Deinococci	*Deinococcus radiodurans*
	Firmicutes	Bacilli	*Bacillus cereus*
			Streptococcus pneumoniae
			Streptococcus mutans
			Streptococcus pyogenes
	Planctomycetes	Planctomycetacia	*Rhodopirellula baltica*
	Proteobacteria	Alphaproteobacteria	*Erythrobacter litoralis*
			Rickettsia typhi
			Gluconobacter oxydans
			Caulobacter crescentus
			Novosphingobium capsulatum
		Betaproteobacteria	*Chromobacterium violaceum*
			Burkholderia pseudomallei
		Bdellovibrionaceae	*Bdellovibrio bacteriovorus*
		Cystobacterineae	*Myxococcus xanthus*
		Gammaproteobacteria	*Xanthomonas axonopodis*
			Xanthomonas oryzae
			Shewanella oneidensis
			Idiomarina loihiensis
			Aeromonas punctata
			Aeromonas hydrophila
			Vibrio parahaemolyticus
			Vibrio fischeri
			Photorhabdus luminescens
			Salmonella enterica
			Escherichia coli
			Coxiella burnetii
	Spirochaetes	Spirochaetes (class)	*Treponema denticola*
Eukaryota	Euglenozoa	Kinetoplastida	*Trypanosoma cruzi*
			Trypanosoma brucei
	Fungi/Metazoa group	Fungi	*Aspergillus fumigatus*
			Trichophyton rubrum
		Metazoa	*Drosophila melanogaster*
			Danio rerio
			Mus musculus
			Rattus Norvegicus
			Bos Taurus
			Sus scrofa
			Homo sapiens
			Pan troglodytes
			Caenorhabditis elegans
	Mycetozoa	Dictyosteliida	*Dictyostelium discoideum*
	Viridiplantae	Streptophyta	*Arabidopsis thaliana*
			Oryza sativa

I.II pBAD TOPO: DpoA

I.II.I Vector Diagram

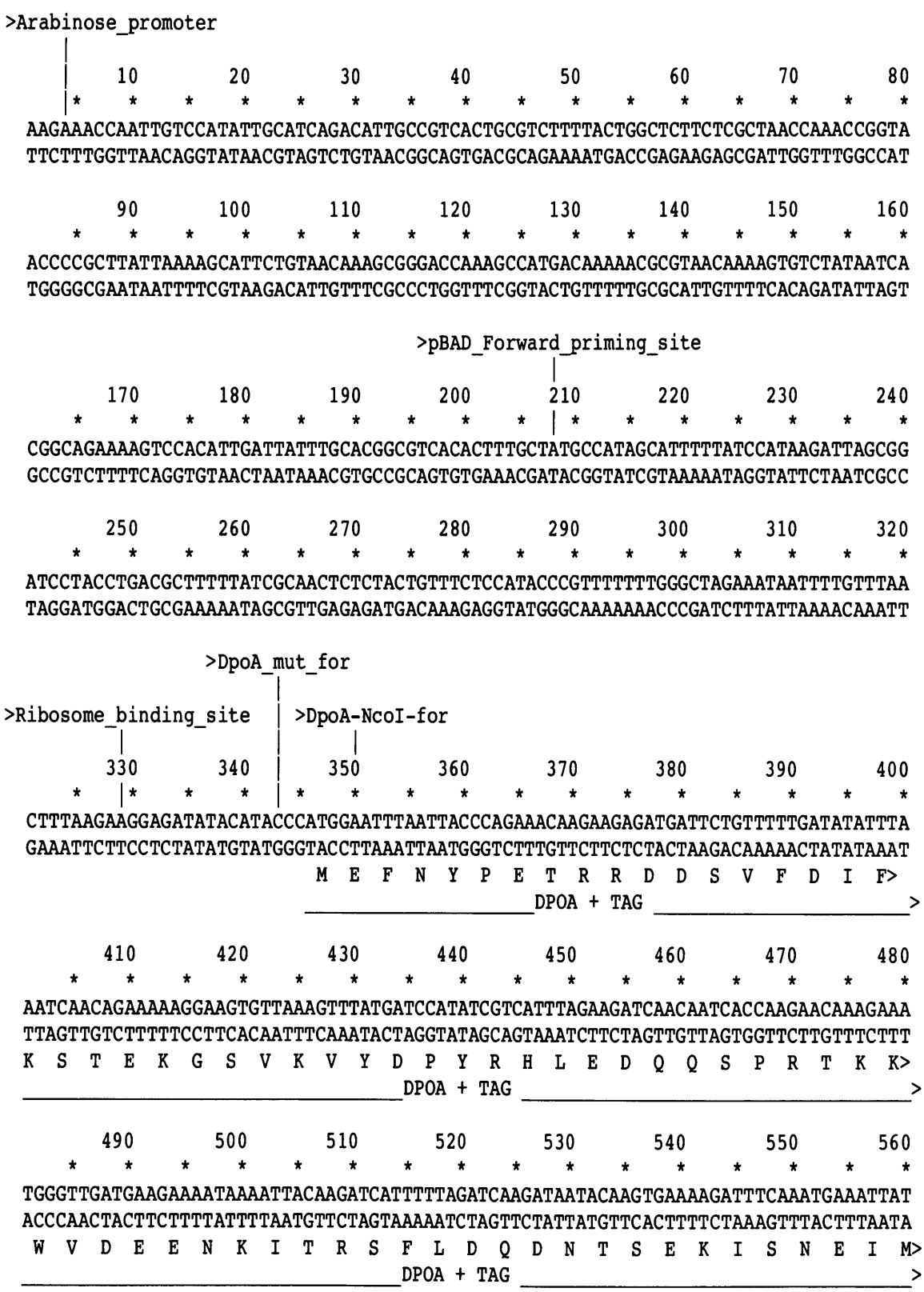


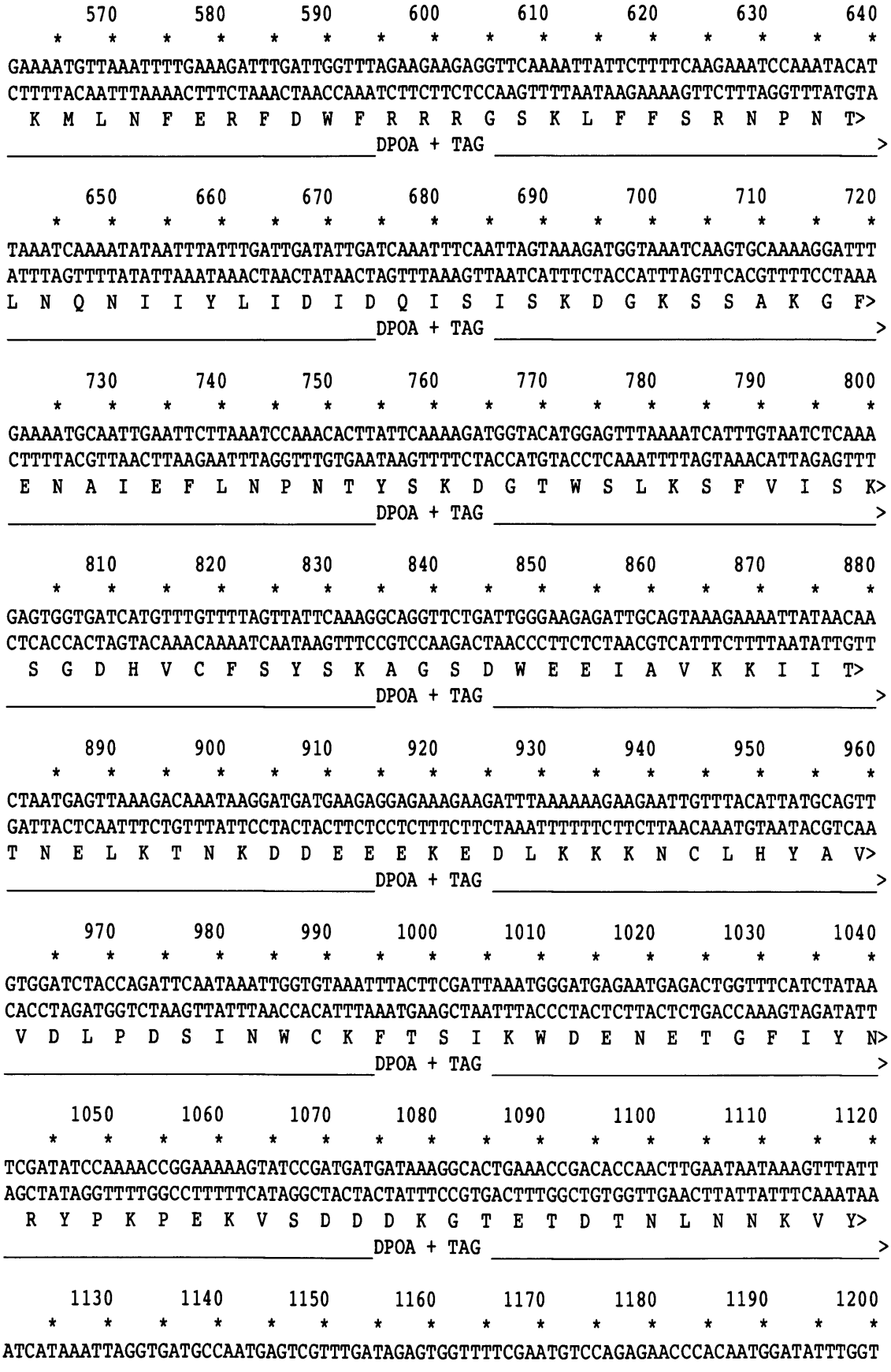
Features

4-276	Arabinose promoter
329-332	Ribosome binding site
346-2718	DpoA:V5His
4262-4935	pBR322 origin
3258-4117	Ampicillin resistance gene
6345-5466	AraC

I.II.II Sequence

Sequence Range: 1 to 6371

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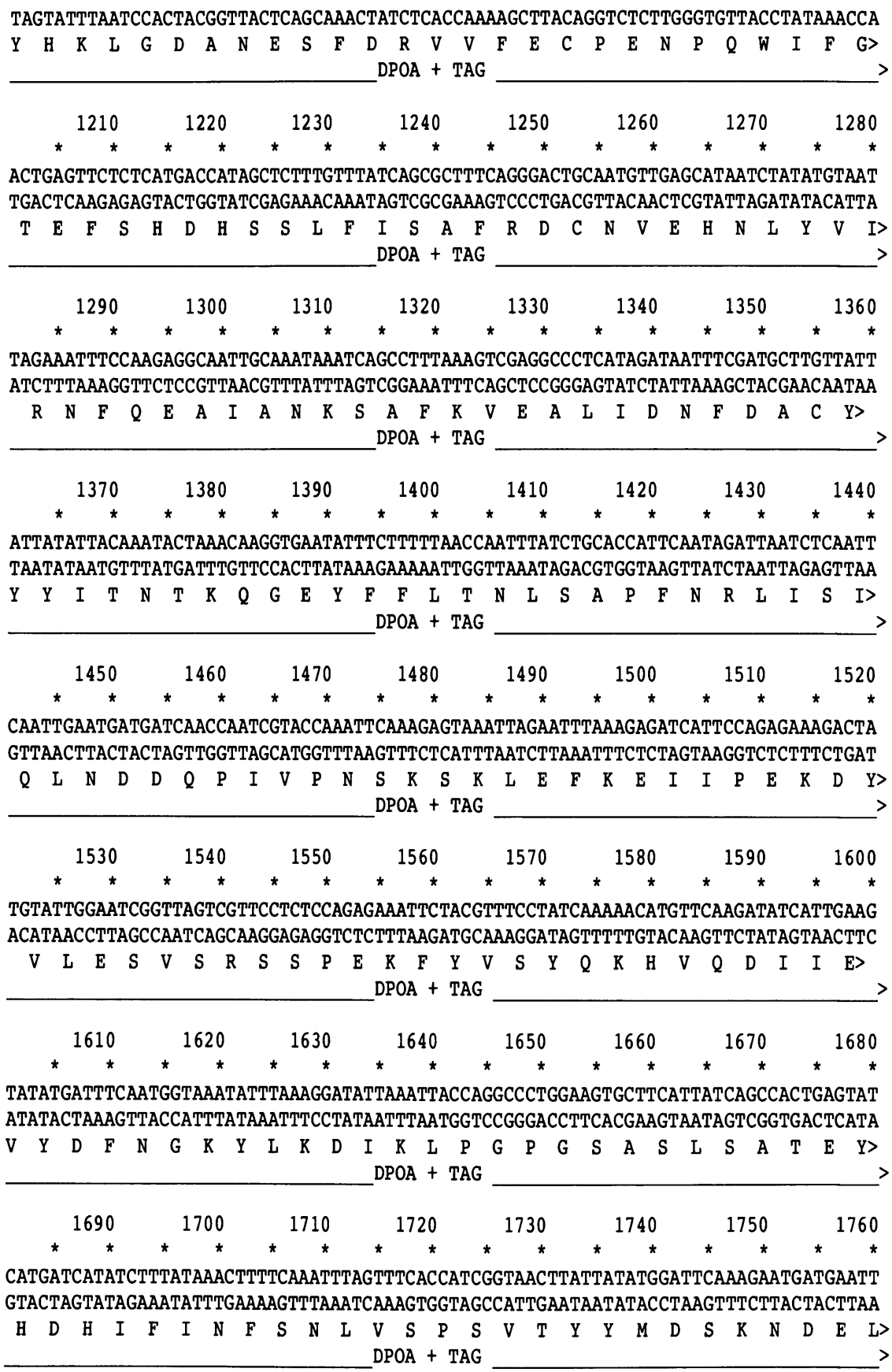
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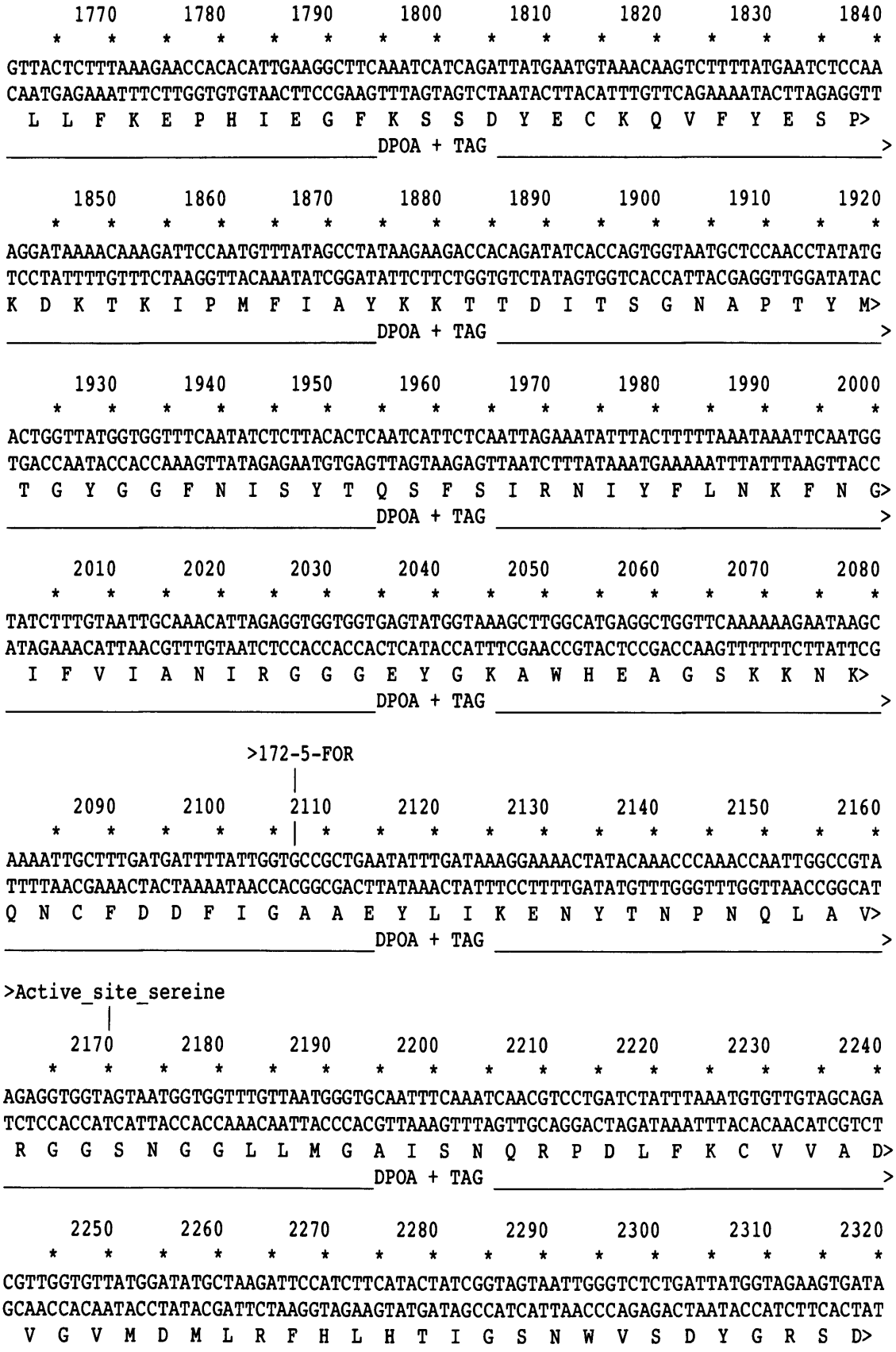
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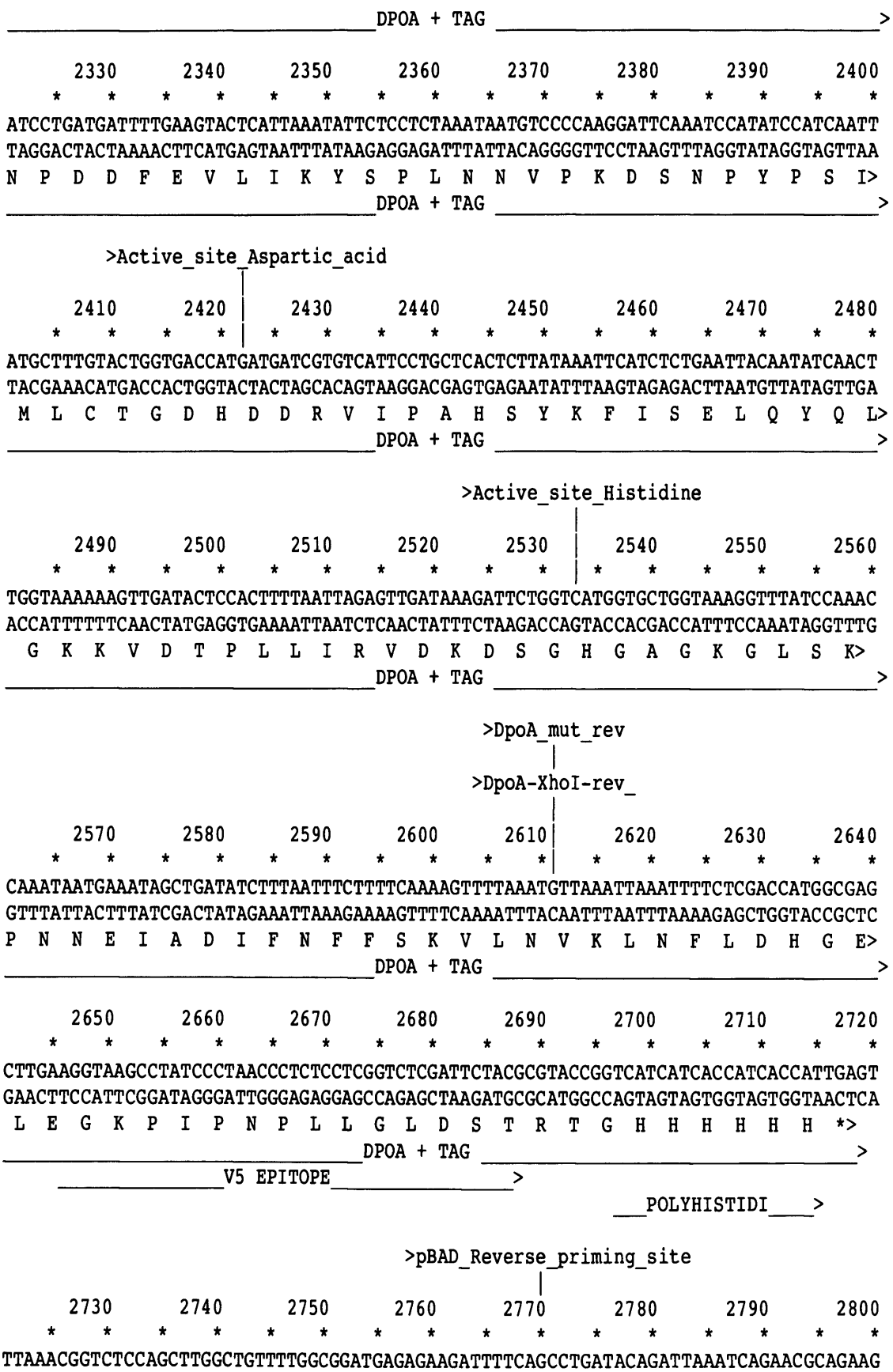
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GCCAGACTATTTTGTCTTAAACGGACCGCCGTCATCGCGCCACCAGGGTGGACTGGGGTACGGCTTGAGTCTTCACTTTG

      2890      2900      2910      2920      2930      2940      2950      2960
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCA
CGGCATCGCGGCTACCATCACACCCCAGAGGGGTACGCTCTCATCCCTTGACGGTCCGTAGTTTATTTTGCTTTCCGAGT

      2970      2980      2990      3000      3010      3020      3030      3040
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGG
CAGCTTTCTGACCCGGAAAGCAAAATAGACAACAAACAGCCACTTGCGAGAGGACTCATCCTGTTTAGGCGGCCCTCGCC

      3050      3060      3070      3080      3090      3100      3110      3120
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAG
TAAACTTGCAACGCTTCGTTGCCGGGCCTCCCACCGCCCGTCCTGCGGGCGGTATTTGACGGTCCGTAGTTTAATTCGTC

      3130      3140      3150      3160      3170      3180      3190      3200
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTCAAATATGTAT
TTCCGGTAGGACTGCCTACCGGAAAAACGCAAAGATGTTTGAGAAAAACAAATAAAAAGATTTATGTAAGTTTATACATA

      3210      3220      3230      3240      3250      3260      3270      3280
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGT
GGCGAGTACTCTGTTATTGGGACTATTTACGAAGTTATTATAACTTTTTCCTTCTCATACTCATAAGTTGTAAAGGCACA
                                                      M  S  I  Q  H  F  R  V>
                                                    ___AMPICILLIN RESIS____>

      3290      3300      3310      3320      3330      3340      3350      3360
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTG
GCGGGAATAAGGGAAAAAACGCCGTAAAACGGAAGGACAAAAACGAGTGGGTCTTTGCGACCACTTTCATTTTCTACGAC
  A  L  I  P  F  F  A  A  F  C  L  P  V  F  A  H  P  E  T  L  V  K  V  K  D  A>
_____________________AMPICILLIN RESISTANCE GENE_____________________>

      3370      3380      3390      3400      3410      3420      3430      3440
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAA
TTCTAGTCAACCCACGTGCTCACCCAATGTAGCTTGACCTAGAGTTGTCGCCATTCTAGGAACTCTCAAAAGCGGGGCTT
E  D  Q  L  G  A  R  V  G  Y  I  E  L  D  L  N  S  G  K  I  L  E  S  F  R  P  E>
_____________________AMPICILLIN RESISTANCE GENE_____________________>

      3450      3460      3470      3480      3490      3500      3510      3520
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
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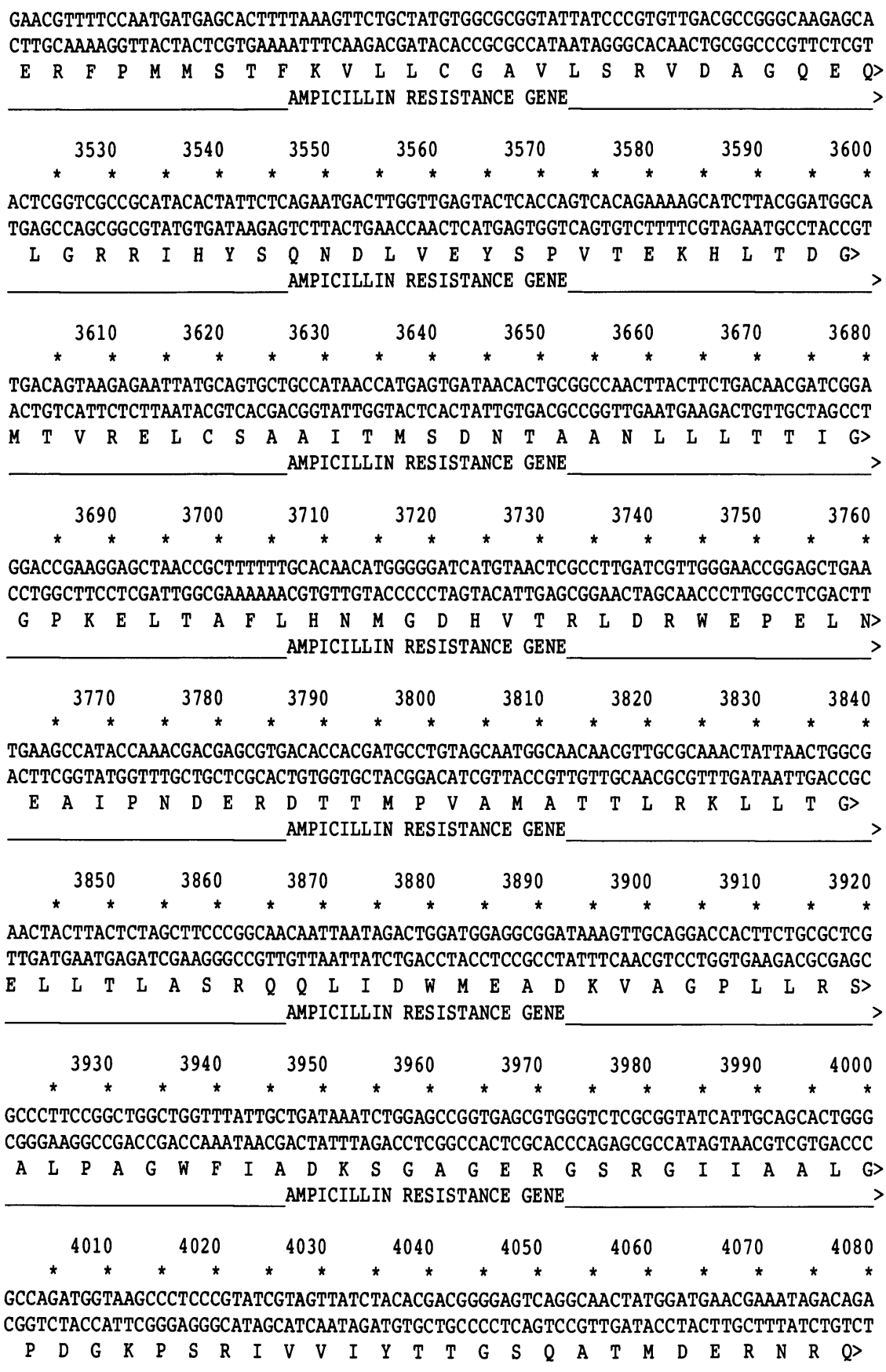
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AMPICILLIN RESISTANCE GENE
3530 3540 3550 3560 3570 3580 3590 3600
ACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCA
TGAGCCAGCGGCGTATGTGATAAGAGTCTTACTGAACCAACTCATGAGTGGTCAGTGTCTTTTCGTAGAATGCCTACCGT
L G R R I H Y S Q N D L V E Y S P V T E K H L T D G>
AMPICILLIN RESISTANCE GENE
3610 3620 3630 3640 3650 3660 3670 3680
TGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGA
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M T V R E L C S A A I T M S D N T A A N L L L T T I G>
AMPICILLIN RESISTANCE GENE
3690 3700 3710 3720 3730 3740 3750 3760
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CCTGGCTTCCTCGATTGGCGAAAAAACGTGTTGTACCCCCTAGTACATTGAGCGGAACTAGCAACCCTTGGCCTCGACTT
G P K E L T A F L H N M G D H V T R L D R W E P E L N>
AMPICILLIN RESISTANCE GENE
3770 3780 3790 3800 3810 3820 3830 3840
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ACTTCGGTATGGTTTGCTGCTCGCACTGTGGTGCTACGGACATCGTTACCGTTGTTGCAACGCGTTTGATAATTGACCGC
E A I P N D E R D T T M P V A M A T T L R K L L T G>
AMPICILLIN RESISTANCE GENE
3850 3860 3870 3880 3890 3900 3910 3920
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AMPICILLIN RESISTANCE GENE
3930 3940 3950 3960 3970 3980 3990 4000
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A L P A G W F I A D K S G A G E R G S R G I I A A L G>
AMPICILLIN RESISTANCE GENE
4010 4020 4030 4040 4050 4060 4070 4080
GCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGA
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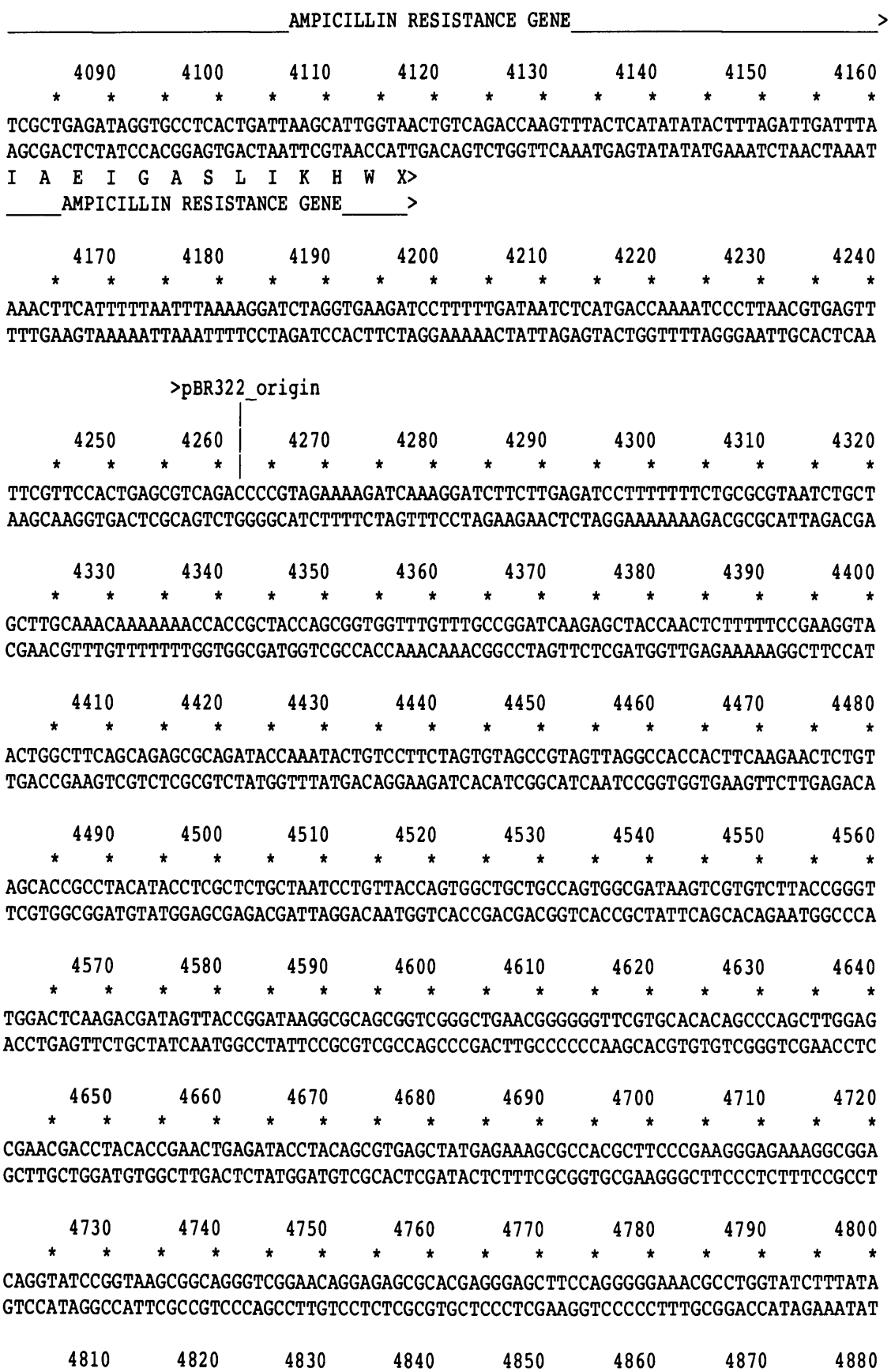
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I A E I G A S L I K H W X>
AMPICILLIN RESISTANCE GENE
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4330 4340 4350 4360 4370 4380 4390 4400
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4410 4420 4430 4440 4450 4460 4470 4480
ACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGT
TGACCGAAGTCGTCTCGCGTCTATGGTTTATGACAGGAAGATCACATCGGCATCAATCCGGTGGTGAAGTTCTTGAGACA
4490 4500 4510 4520 4530 4540 4550 4560
AGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGT
TCGTGGCGGATGTATGGAGCGAGACGATTAGGACAATGGTCACCGACGACGGTCACCGCTATTCAGCACAGAATGGCCCA
4570 4580 4590 4600 4610 4620 4630 4640
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ACCTGAGTTCTGCTATCAATGGCCTATTCCGCGTCGCCAGCCCGACTTGCCCCCCAAGCACGTGTGTCGGGTCGAACCTC
4650 4660 4670 4680 4690 4700 4710 4720
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GCTTGCTGGATGTGGCTTGACTCTATGGATGTCGCACTCGATACTCTTTCGCGGTGCGAAGGGCTTCCCTCTTTCCGCCT
4730 4740 4750 4760 4770 4780 4790 4800
CAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATA
GTCCATAGGCCATTCGCCGTCCCAGCCTTGTCCTCTCGCGTGCTCCCTCGAAGGTCCCCCTTTGCGGACCATAGAAATAT
4810 4820 4830 4840 4850 4860 4870 4880

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    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
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CAGGACAGCCCAAAGCGGTGGAGACTGAACTCGCAGCTAAAAACACTACGAGCAGTCCCCCCGCCTCGGATACCTTTTTG

      4890      4900      4910      4920      4930      4940      4950      4960
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGA
CGGTCGTTGCGCCGGAAAAATGCCAAGGACCGGAAAACGACCGGAAAACGAGTGTACAAGAAAGGACGCAATAGGGGACT

      4970      4980      4990      5000      5010      5020      5030      5040
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAG
AAGACACCTATTGGCATAATGGCGGAAACTCACTCGACTATGGCGAGCGGCGTCGGCTTGCTGGCTCGCGTCGCTCAGTC

      5050      5060      5070      5080      5090      5100      5110      5120
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TGAGCGAGGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGC
ACTCGCTCCTTCGCCTTCTCGCGGACTACGCCATAAAAGAGGAATGCGTAGACACGCCATAAAGTGTGGCGTATACCACG

      5130      5140      5150      5160      5170      5180      5190      5200
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCT
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      5210      5220      5230      5240      5250      5260      5270      5280
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGT
CGCGGGGCTGTGGGCGGTTGTGGGCGACTGCGCGGGACTGCCCGAACAGACGAGGGCCGTAGGCGAATGTCTGTTCGACA

      5290      5300      5310      5320      5330      5340      5350      5360
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCAGATCAATTCGCGCG
CTGGCAGAGGCCCTCGACGTACACAGTCTCCAAAAGTGGCAGTAGTGGCTTTGCGCGCTCCGTCGTCTAGTTAAGCGCGC

      5370      5380      5390      5400      5410      5420      5430      5440
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CGAAGGCGAAGCGGCATGCATAATGTGCCTGTCAAATGGACGAAGCAGGGATTCTGCAAACCCTATGCTACTCCGTCAAG
GCTTCCGCTTCGCCGTACGTATTACACGGACAGTTTACCTGCTTCGTCCCTAAGACGTTTGGGATACGATGAGGCAGTTC

      5450      5460      5470      5480      5490      5500      5510      5520
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CCGTCAATTGTCTGATTCGTTACCAATTATGACAACTTGACGGCTACATCATTCACTTTTTCTTCACAACCGGCACGGAA
GGCAGTTAACAGACTAAGCAATGGTTAATACTGTTGAACTGCCGATGTAGTAAGTGAAAAAGAAGTGTTGGCCGTGCCTT
                        <*  S  L  K  V  A  V  D  N  V  K  E  E  C  G  A  R  F
                      <_____________________ARAC _____________________________

      5530      5540      5550      5560      5570      5580      5590      5600
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CTCGCTCGGGCTGGCCCCGGTGCATTTTTTAAATACCCGCGAGAAATAGAGTTGATCGTCAAAACCAACATTGCGACCGA
GAGCGAGCCCGACCGGGGCCACGTAAAAAATTTATGGGCGCTCTTTATCTCAACTAGCAGTTTTGGTTGTAACGCTGGCT
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<_____________________________________ARAC ____________________________________
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      5610       5620       5630       5640       5650       5660       5670       5680
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CGGTGGCGATAGGCATCCGGGTGGTGCTCAAAAGCAGCTTCGCCTGGCTGATACGTTGGTCCTCGCGCCAGCTTAAGACG
GCCACCGCTATCCGTAGGCCCACCACGAGTTTTCGTCGAAGCGGACCGACTATGCAACCAGGAGCGCGGTCGAATTCTGC
  <T  A  I  P  M  R  T  T  S  L  L  L  K  A  Q  S  I  R  Q  D  E  R  W  S  L  V
<_____________________________________ARAC _____________________________________

      5690       5700       5710       5720       5730       5740       5750       5760
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CTAATCCCTAACTGCTGGCGGAAAAGATGTGACAGACGCGACGGCGACAAGCAAACATGCTGTGCGACGCTGGCGATATC
GATTAGGGATTGACGACCGCCTTTTCTACACTGTCTGCGCTGCCGCTGTTCGTTTGTACGACACGCTGCGACCGCTATAG
<S  I  G  L  Q  Q  R  F  L  H  S  L  R  S  P  S  L  C  V  H  Q  A  V  S  A  I  D
<_____________________________________ARAC _____________________________________

      5770       5780       5790       5800       5810       5820       5830       5840
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAAATTGCTGTCTGCCAGGTGATCGCTGATGTACTGACAAGCCTCGCGTACCCGATTATCCATCGGTGGATGGAGCGACT
TTTTAACGACAGACGGTCCACTAGCGACTACATGACTGTTCGGAGCGCATGGGCTAATAGGTAGCCACCTACCTCGCTGA
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<_____________________________________ARAC _____________________________________

      5850       5860       5870       5880       5890       5900       5910       5920
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CGTTAATCGCTTCCATGCGCCGCAGTAACAATTGCTCAAGCAGATTTATCGCCAGCAGCTCCGAATAGCGCCCTTCCCCT
GCAATTAGCGAAGGTACGCGGCGTCATTGTTAACGAGTTCGTCTAAATAGCGGTCGTCGAGGCTTATCGCGGGAAGGGGA
  <N  I  A  E  M  R  R  L  L  L  Q  E  L  L  N  I  A  L  L  E  S  Y  R  G  E  G
<_____________________________________ARAC _____________________________________

      5930       5940       5950       5960       5970       5980       5990       6000
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TGCCCGGCGTTAATGATTTGCCCAAACAGGTCGCTGAAATGCGGCTGGTGCGCTTCATCCGGGCGAAAGAACCCCGTATT
ACGGGCCGCAATTACTAAACGGGTTTGTCCAGCGACTTTACGCCGACCACGCGAAGTAGGCCCGCTTTCTTGGGGCATAA
<Q  G  A  N  I  I  Q  G  F  L  D  S  F  H  P  Q  H  A  E  D  P  R  F  F  G  T  N
<_____________________________________ARAC _____________________________________

      6010       6020       6030       6040       6050       6060       6070       6080
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GGCAAATATTGACGGCCAGTTAAGCCATTCATGCCAGTAGGCGCGCGGACGAAAGTAAACCCACTGGTGATACCATTCGC
CCGTTTATAACTGCCGGTCAATTCGGTAAGTACGGTCATCCGCGCGCCTGCTTTCATTTGGGTGACCACTATGGTAAGCG
<A  F  I  S  P  W  N  L  W  E  H  W  Y  A  R  P  R  F  Y  V  W  Q  H  Y  W  E  R
<_____________________________________ARAC _____________________________________

      6090       6100       6110       6120       6130       6140       6150       6160
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GAGCCTCCGGATGACGACCGTAGTGATGAATCTCTCCTGGCGGGAACAGCAAAATATCACCCGGTCGGCAAACAAATTCT
CTCGGAGGCCTACTGCTGGCATCACTACTTAGAGAGGACCGCCCTTGTCGTTTTATAGTGGGCCAGCCGTTTGTTTAAGA
  <A  E  P  H  R  G  Y  H  H  I  E  G  P  P  F  L  L  I  D  G  P  R  C  V  F  E
<_____________________________________ARAC _____________________________________

      6170       6180       6190       6200       6210       6220       6230       6240
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
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 CGTCCCTGATTTTTCACCACCCCCTGACCGCGAATGGTGAGATTGAGAATATAACCTTTCATTCCCAGCGGTCGGTCGAT
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<R  G  Q  N  K  V  V  G  Q  G  R  I  T  L  N  L  I  Y  G  K  M  G  L  P  R  D  I
<_____________________________________ARAC _____________________________________

      6250       6260       6270       6280       6290       6300       6310       6320
     *    *     *    *     *    *     *    *     *    *     *    *     *    *     *    *
 AAAAAAATCGAGATAACCGTTGGCCTCAATCGGCGTTAAACCCGCCACCAGATGGGCATTAAACGAGTATCCCGGCAGCA
 TTTTTTTAGCTCTATTGGCAACCGGAGTTAGCCGCAATTTGGGCGGTGGTCTACCCGTAATTTGCTCATAGGGCCGTCGT
 <F  F  D  L  Y  G  N  A  E  I  P  T  L  G  A  V  L  H  A  N  F  S  Y  G  P  L  L
<_____________________________________ARAC _____________________________________

      6330       6340       6350       6360       6370
     *    *     *    *     *    *     *    *     *    *
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 CCCCTAGTAAAACGCGAAGTCGGTATGAAAAGTATGAGGGCGGTAAGTCTC
  <P  D  N  Q  A  E  A  M
<_________ARAC ___________
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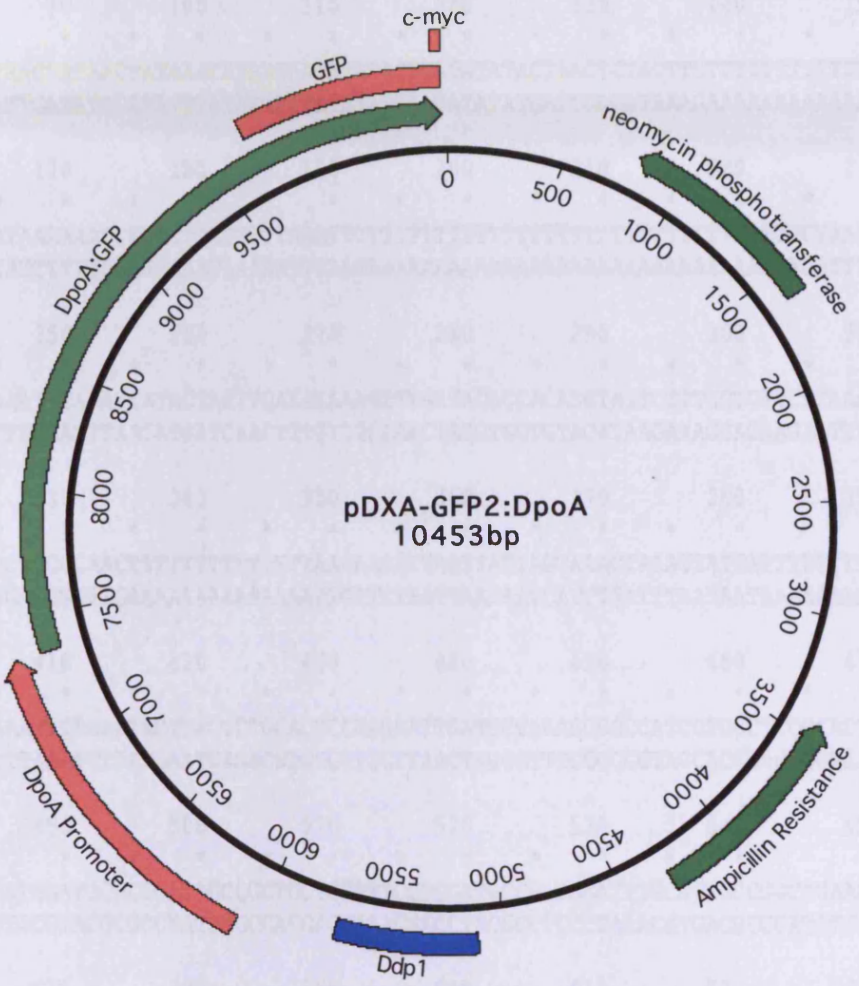
I.III pDXA GFP2: DpoA

I.III.I Vector Diagram



Features

1616-786	Neomycin Phosphotransferase
4299-3439	Ampicillin Resistance
5106-5694	Ddp1
6067-7353	DpoA Promoter
7353-10430	DpoA:GFP

I.III.II Sequence

Sequence Range: 1 to 10453

```
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TTAAGTTCAAAATGGTATTTTATTTTTTTTTTTTTATTTTTTTTTGTTTAGGTTAACAAAAATATAATTTATTAATTTTAT

        90        100       110       120       130       140       150       160
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
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TTACATTGATATTGATATTTATATTATTATATATGAATATATATGATTGAGATAAAGAAAAAAAAAAAAAAAAAAATCT

        170       180       190       200       210       220       230       240
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
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        250       260       270       280       290       300       310       320
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GTATAAATTTAAATTATACTAGTTGAAACAAAGTTTGATACACCACATGTATTCTTTCCTCTTCTTAAATAAATGTATCC
CATATTTAAATTTAATATGATCAACTTTGTTTCAAACTATGTGGTGTACATAAGAAAGGAGAAGAATTTATTTACATAGG

        330       340       350       360       370       380       390       400
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
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AACAAGAGGGGTTGAAAAAAAAAAAAAAATTGTTTTAATTAATAATCATTTGATTTAATAATAAAAAAAATAATAATAATA

        410       420       430       440       450       460       470       480
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
TTTAAAATTTAAAATACTTACTCTGCACCCCAAGAATTGATCCCAAAGCGGCCATCGTGCCTCCCCACTCCTGCAGTTCG
AAATTTTAAATTTTATGAATGAGACGTGGGGTTCTTAACTAGGGTTTCGCCGGTAGCACGGAGGGGTGAGGACGTCAAGC

        490       500       510       520       530       540       550       560
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GGGGCATGGATGCGCGGATAGCCGCTGCTGGTTTCCTGGATGCCGACGGATTTGCACTGCCGGTAGAACTCCGCGAGGTC
CCCCGTACCTACGCGCCTATCGGCGACGACCAAAGGACCTACGGCTGCCTAAACGTGACGGCCATCTTGAGGCGCTCCAG

        570       580       590       600       610       620       630       640
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GTCCAGCCTCAGGCAGCAGCTGAACCAACTCGCGAGGGGATCGAGCCCGGGCCGGGTGGGCGAAGAACTCCAGCATGAGA
CAGGTCGGAGTCCGTCGTCGACTTGGTTGAGCGCTCCCCTAGCTCGGGCCCGGCCCACCCGCTTCTTGAGGTCGTACTCT

        650       660       670       680       690       700       710       720
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
TCCCCGCGCTGGAGGATCATCCAGCCGGCGTCCCGGAAAACGATTCCGAAGCCCAACCTTTCATAGAAGGCGGCGGTGGA
AGGGGCGCGACCTCCTAGTAGGTCGGCCGCAGGGCCTTTTGCTAAGGCTTCGGGTTGGAAAGTATCTTCCGCCGCCACCT

        730       740       750       760       770       780       790       800
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
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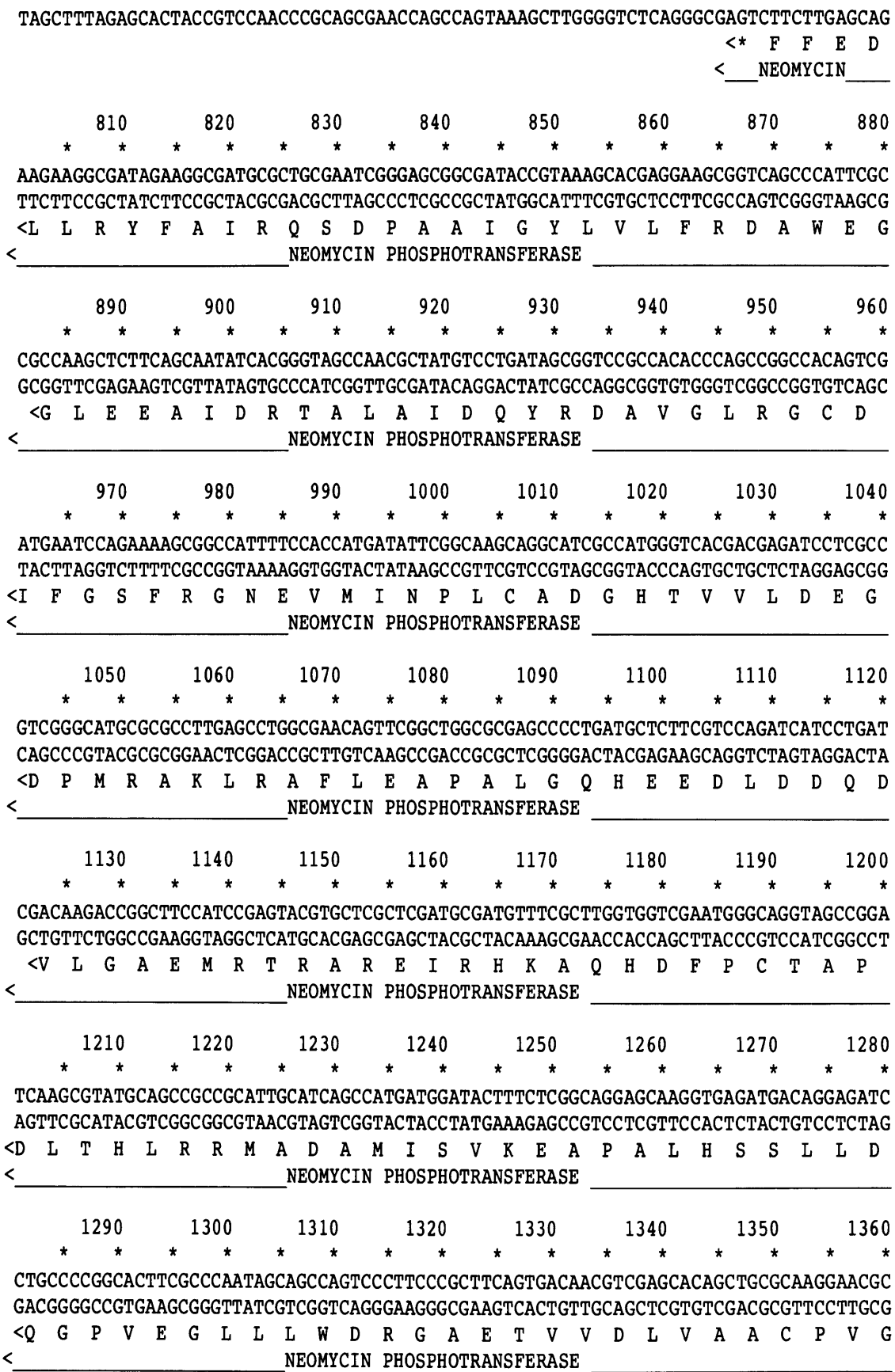
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<I F G S F R G N E V M I N P L C A D G H T V V L D E G
<_____NEOMYCIN PHOSPHOTRANSFERASE _____
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<D P M R A K L R A F L E A P A L G Q H E E D L D D Q D
<_____NEOMYCIN PHOSPHOTRANSFERASE _____
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<V L G A E M R T R A R E I R H K A Q H D F P C T A P
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<D L T H L R R M A D A M I S V K E A P A L H S S L L D
<_____NEOMYCIN PHOSPHOTRANSFERASE _____
1290 1300 1310 1320 1330 1340 1350 1360
CTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGC
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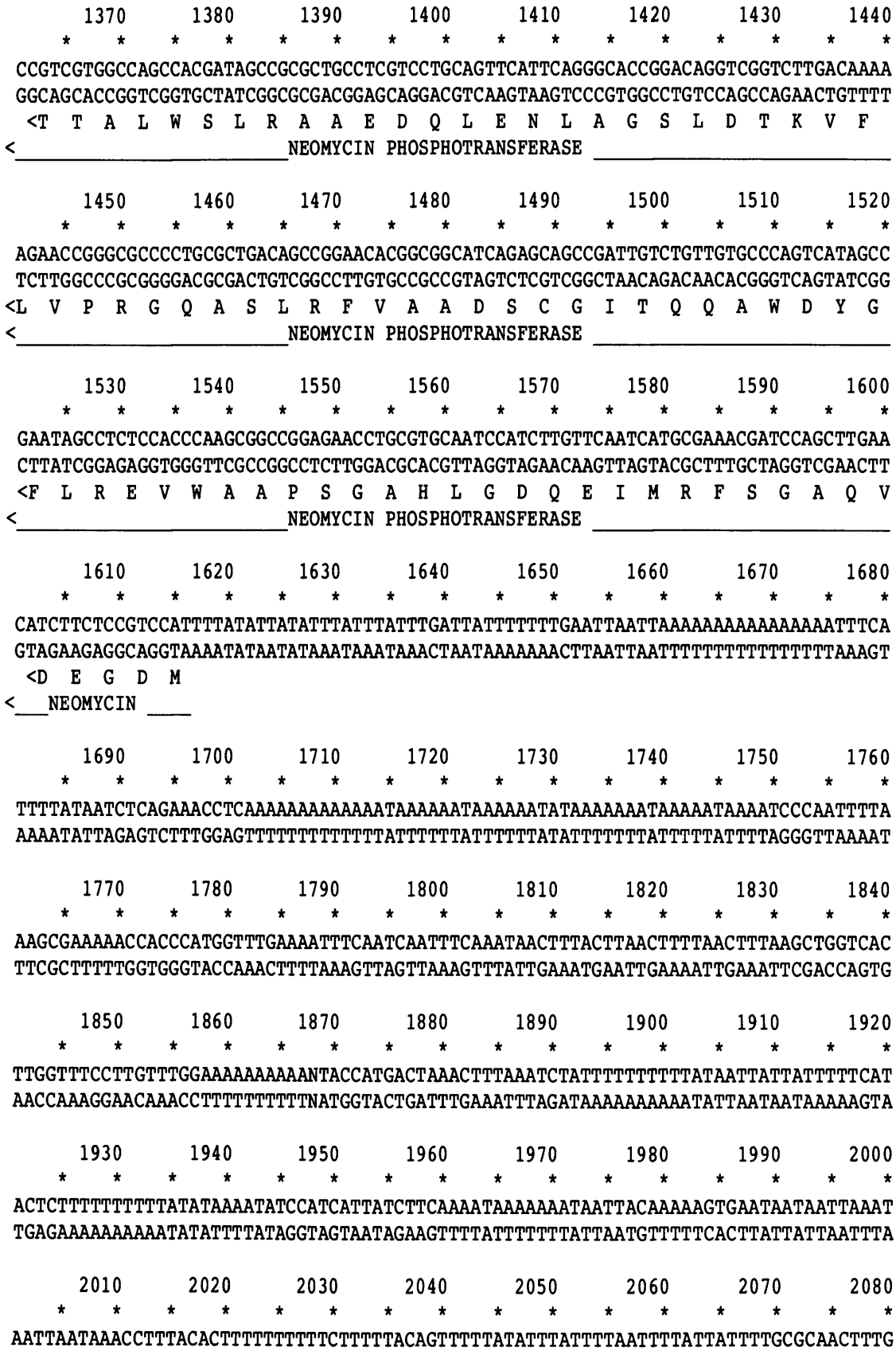
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<T T A L W S L R A A E D Q L E N L A G S L D T K V F
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TCTTGGCCCGCGGGGACGCGACTGTCGGCCTTGTGCCGCCGTAGTCTCGTCGGCTAACAGACAACACGGGTCAGTATCGG
<L V P R G Q A S L R F V A A D S C G I T Q Q A W D Y G
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<D E G D M
<NEOMYCIN
1690 1700 1710 1720 1730 1740 1750 1760
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1770 1780 1790 1800 1810 1820 1830 1840
AAGCGAAAAACCACCCATGGTTTGAAAATTTCAATCAATTTCAAATAACTTTACTTAACTTTTAACTTTAAGCTGGTCAC
TTCGCTTTTTGGTGGGTACCAAACTTTTAAAGTTAGTTAAAGTTTATTGAAATGAATTGAAAATTGAAATTCGACCAGTG
1850 1860 1870 1880 1890 1900 1910 1920
TTGGTTTCCTTGTTTGGAAAAAAAAAANTACCATGACTAAACTTTAAATCTATTTTTTTTTTATAATTATTATTTTTCAT
AACCAAAGGAACAAACCTTTTTTTTTTNATGGTACTGATTTGAAATTTAGATAAAAAAAAAATATTAATAATAAAAAGTA
1930 1940 1950 1960 1970 1980 1990 2000
ACTCTTTTTTTTTTATATAAAATATCCATCATTATCTTCAAAATAAAAAAATAATTACAAAAAGTGAATAATAATTAAAT
TGAGAAAAAAAAAAATATATTTTATAGGTAGTAATAGAAGTTTTATTTTTTTATTAATGTTTTTCACTTATTATTAATTTA
2010 2020 2030 2040 2050 2060 2070 2080
AATTAATAAACCTTTACACTTTTTTTTTTCTTTTTACAGTTTTTATATTTATTTTAATTTTATTATTTTGCGCAACTTTG

```
TTAATTATTTGGAAATGTGAAAAAAAAAAGAAAAATGTCAAAAATATAAATAAAATTAAAATAATAAAACGCGTTGAAAC

      2090      2100      2110      2120      2130      2140      2150      2160
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAAAAATAAAAAAGATAAAGTTTTTATAAATATCTATCAAAAAAGATATCGTTGTTGATTGATTATTTTGGATAATTTTT
TTTTTTATTTTTTCTATTTCAAAAATATTTATAGATAGTTTTTTCTATAGCAACAACTAACTAATAAAACCTATTAAAAA

      2170      2180      2190      2200      2210      2220      2230      2240
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CTTTAAATTGGGGCAAATCTGTAATTTTCAGATTAATAAGATTTTTCCTTTTTATTTTTCTTTTTACTTTTTGTAATTTT
GAAATTTAACCCCGTTTAGACATTAAAAGTCTAATTATTCTAAAAAGGAAAAATAAAAAGAAAAATGAAAAACATTAAAA

      2250      2260      2270      2280      2290      2300      2310      2320
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TAAAAATAAACCTCTGAATTAATTCGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATT
ATTTTTATTTGGAGACTTAATTAAGCCGCATTAGTACCAGTATCGACAAAGGACACACTTTAACAATAGGCGAGTGTTAA

      2330      2340      2350      2360      2370      2380      2390      2400
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT
GGTGTGTTGTATGCTCGGCCTTCGTATTTCACATTTCGGACCCCACGGATTACTCACTCGATTGAGTGTAATTAACGCAA

      2410      2420      2430      2440      2450      2460      2470      2480
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCG
CGCGAGTGACGGGCGAAAGGTCAGCCCTTTGGACAGCACGGTCGACGTAATTACTTAGCCGGTTGCGCGCCCCTCTCCGC

      2490      2500      2510      2520      2530      2540      2550      2560
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCA
CAAACGCATAACCCGCGAGAAGGCGAAGGAGCGAGTGACTGAGCGACGCGAGCCAGCAAGCCGACGCCGCTCGCCATAGT

      2570      2580      2590      2600      2610      2620      2630      2640
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCA
CGAGTGAGTTTCCGCCATTATGCCAATAGGTGTCTTAGTCCCCTATTGCGTCCTTTCTTGTACACTCGTTTTCCGGTCGT

      2650      2660      2670      2680      2690      2700      2710      2720
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATC
TTTCCGGTCCTTGGCATTTTTCCGGCGCAACGACCGCAAAAAGGTATCCGAGGCGGGGGGACTGCTCGTAGTGTTTTTAG

      2730      2740      2750      2760      2770      2780      2790      2800
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGC
CTGCGAGTTCAGTCTCCACCGCTTTGGGCTGTCCTGATATTTCTATGGTCCGCAAAGGGGGACCTTCGAGGGAGCACGCG

      2810      2820      2830      2840      2850      2860      2870      2880
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTC
AGAGGACAAGGCTGGGACGGCGAATGGCCTATGGACAGGCGGAAAGAGGGAAGCCCTTCGCACCGCGAAAGAGTTACGAG
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     2890      2900      2910      2920      2930      2940      2950      2960
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGGTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACC
TGCGACATCCATAGAGTCAAGCCACATCCAGCAAGCCAGGTTCGACCCGACACACGTGCTTGGGGGGCAAGTCGGGCTGG

     2970      2980      2990      3000      3010      3020      3030      3040
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGT
CGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGACCGTCGTCGGTGACCA

     3050      3060      3070      3080      3090      3100      3110      3120
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAG
TTGTCCTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTC

     3130      3140      3150      3160      3170      3180      3190      3200
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GACACTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAA
CTGTGATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGCCTTTTTCTCAACCATCGAGAACTAGGCCGTTTGTTT

     3210      3220      3230      3240      3250      3260      3270      3280
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTG
GGTGGCGACCATCGCCACCAAAAAAACAAACGTTCGTCGTCTAATGCGCGTCTTTTTTTCCTAGAGTTCTTCTAGGAAAC

     3290      3300      3310      3320      3330      3340      3350      3360
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGAT
TAGAAAAGATGCCCCAGACTGCGAGTCACCTTGCTTTTGAGTGCAATTCCCTAAAACCAGTACTCTAATAGTTTTTCCTA

     3370      3380      3390      3400      3410      3420      3430      3440
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CTTCACCTAGATCCTTTTAAATTAAAATGAAGTATTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTT
GAAGTGGATCTAGGAAAATTTAATTTTACTTCATAAATTTAGTTAGATTTCATATATACTCATTTGAACCAGACTGTCAA
                                                                              <*
                                                                              <__

     3450      3460      3470      3480      3490      3500      3510      3520
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTG
TGGTTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAAAGCAAGTAGGTATCAACGGACTGAGGGGCAGCAC
  <W  H  K  I  L  S  A  G  I  E  A  I  Q  R  N  R  E  D  M  T  A  Q  S  G  T  T
<_____________________AMPICILLIN RESISTANCE_____________________

     3530      3540      3550      3560      3570      3580      3590      3600
   *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCC
ATCTATTGATGCTATGCCCTCCCGAATGGTAGACCGGGGTCACGACGTTACTATGGCGCTCTGGGTGCGAGTGGCCGAGG
<Y  I  V  V  I  R  S  P  K  G  D  P  G  L  A  A  I  I  G  R  S  G  R  E  G  A  G
<_____________________AMPICILLIN RESISTANCE_____________________

     3610      3620      3630      3640      3650      3660      3670      3680
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    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGT
TCTAAATAGTCGTTATTTGGTCGGTCGGCCTTCCCGGCTCGCGTCTTCACCAGGACGTTGAAATAGGCGGAGGTAGGTCA
<S  K  D  A  I  F  W  G  A  P  L  A  S  R  L  L  P  G  A  V  K  D  A  E  M  W  D
<_____________________________AMPICILLIN RESISTANCE_____________________________

      3690      3700      3710      3720      3730      3740      3750      3760
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGC
GATAATTAACAACGGCCCTTCGATCTCATTCATCAAGCGGTCAATTATCAAACGCGTTGCAACAACGGTAACGATGTCCG
  <I  L  Q  Q  R  S  A  L  T  L  L  E  G  T  L  L  K  R  L  T  T  A  M  A  V  P
<_____________________________AMPICILLIN RESISTANCE_____________________________

      3770      3780      3790      3800      3810      3820      3830      3840
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCC
TAGCACCACAGTGCGAGCAGCAAACCATACCGAAGTAAGTCGAGGCCAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGG
<M  T  T  D  R  E  D  N  P  I  A  E  N  L  E  P  E  W  R  D  L  R  T  V  H  D  G
<_____________________________AMPICILLIN RESISTANCE_____________________________

      3850      3860      3870      3880      3890      3900      3910      3920
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCA
GTACAACACGTTTTTTCGCCAATCGAGGAAGCCAGGAGGCTAGCAACAGTCTTCATTCAACCGGCGTCACAATAGTGAGT
<M  N  H  L  F  A  T  L  E  K  P  G  G  I  T  T  L  L  L  N  A  A  T  N  D  S  M
<_____________________________AMPICILLIN RESISTANCE_____________________________

      3930      3940      3950      3960      3970      3980      3990      4000
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
TGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACC
ACCAATACCGTCGTGACGTATTAAGAGAATGACAGTACGGTAGGCATTCTACGAAAAGACACTGACCACTCATGAGTTGG
  <T  I  A  A  S  C  L  E  R  V  T  M  G  D  T  L  H  K  E  T  V  P  S  Y  E  V
<_____________________________AMPICILLIN RESISTANCE_____________________________

      4010      4020      4030      4040      4050      4060      4070      4080
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAG
TTCAGTAAGACTCTTATCACATACGCCGCTGGCTCAACGAGAACGGGCCGCAGTTATGCCCTATTATGGCGCGGTGTATC
<L  D  N  Q  S  Y  H  I  R  R  G  L  Q  E  Q  G  A  D  I  R  S  L  V  A  G  C  L
<_____________________________AMPICILLIN RESISTANCE_____________________________

      4090      4100      4110      4120      4130      4140      4150      4160
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCA
GTCTTGAAATTTTCACGAGTAGTAACCTTTTGCAAGAAGCCCCGCTTTTGAGAGTTCCTAGAATGGCGACAACTCTAGGT
<L  V  K  F  T  S  M  M  P  F  R  E  E  P  R  F  S  E  L  I  K  G  S  N  L  D  L
<_____________________________AMPICILLIN RESISTANCE_____________________________

      4170      4180      4190      4200      4210      4220      4230      4240
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACA
CAAGCTACATTGGGTGAGCACGTGGGTTGACTAGAAGTCGTAGAAAATGAAAGTGGTCGCAAAGACCCACTCGTTTTTGT
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  <E  I  Y  G  V  R  A  G  L  Q  D  E  A  D  K  V  K  V  L  T  E  P  H  A  F  V
<_____________________AMPICILLIN RESISTANCE_____________________________________

      4250       4260       4270       4280       4290       4300       4310       4320
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTA
CCTTCCGTTTTACGGCGTTTTTTCCCTTATTCCCGCTGTGCCTTTACAACTTATGAGTATGAGAAGGAAAAAGTTATAAT
<P  L  C  F  A  A  F  F  P  I  L  A  V  R  F  H  Q  I  S  M
<_________________AMPICILLIN RESISTANCE_____________________

      4330       4340       4350       4360       4370       4380       4390       4400
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTC
AACTTCGTAAATAGTCCCAATAACAGAGTACTCGCCTATGTATAAACTTACATAAATCTTTTTATTTGTTTATCCCCAAG

      4410       4420       4430       4440       4450       4460       4470       4480
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGT
GCGCGTGTAAAGGGGCTTTTCACGGTGGACTGCAGATTCTTTGGTAATAATAGTACTGTAATTGGATATTTTTATCCGCA

      4490       4500       4510       4520       4530       4540       4550       4560
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCAC
TAGTGCTCCGGGAAAGCAGAGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGTG

      4570       4580       4590       4600       4610       4620       4630       4640
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGC
TCGAACAGACATTCGCCTACGGCCCTCGTCTGTTCGGGCAGTCCCGCGCAGTCGCCCACAACCGCCCACAGCCCCGACCG

      4650       4660       4670       4680       4690       4700       4710       4720
    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGA
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      4730       4740       4750       4760       4770       4780       4790       4800
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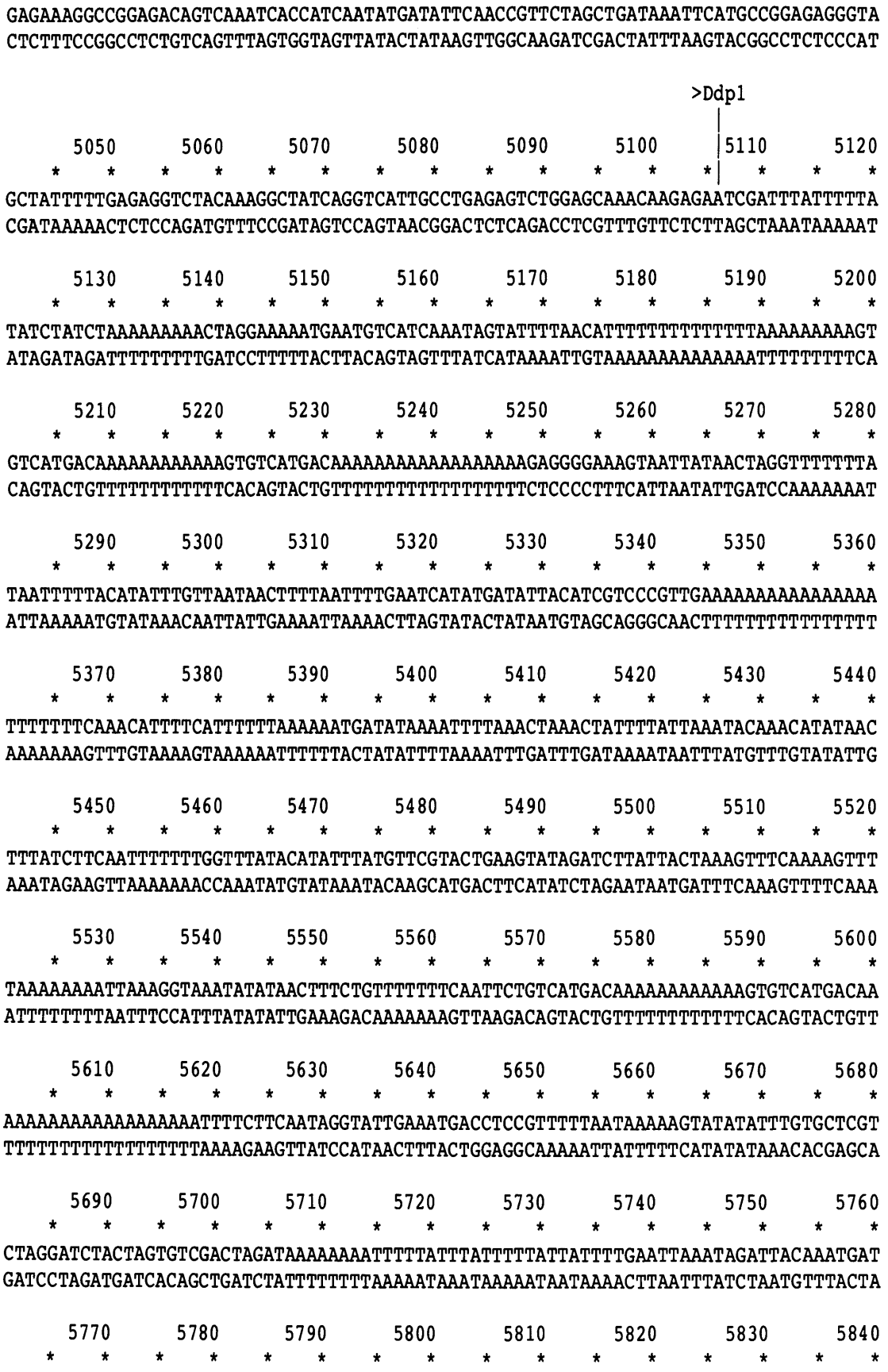
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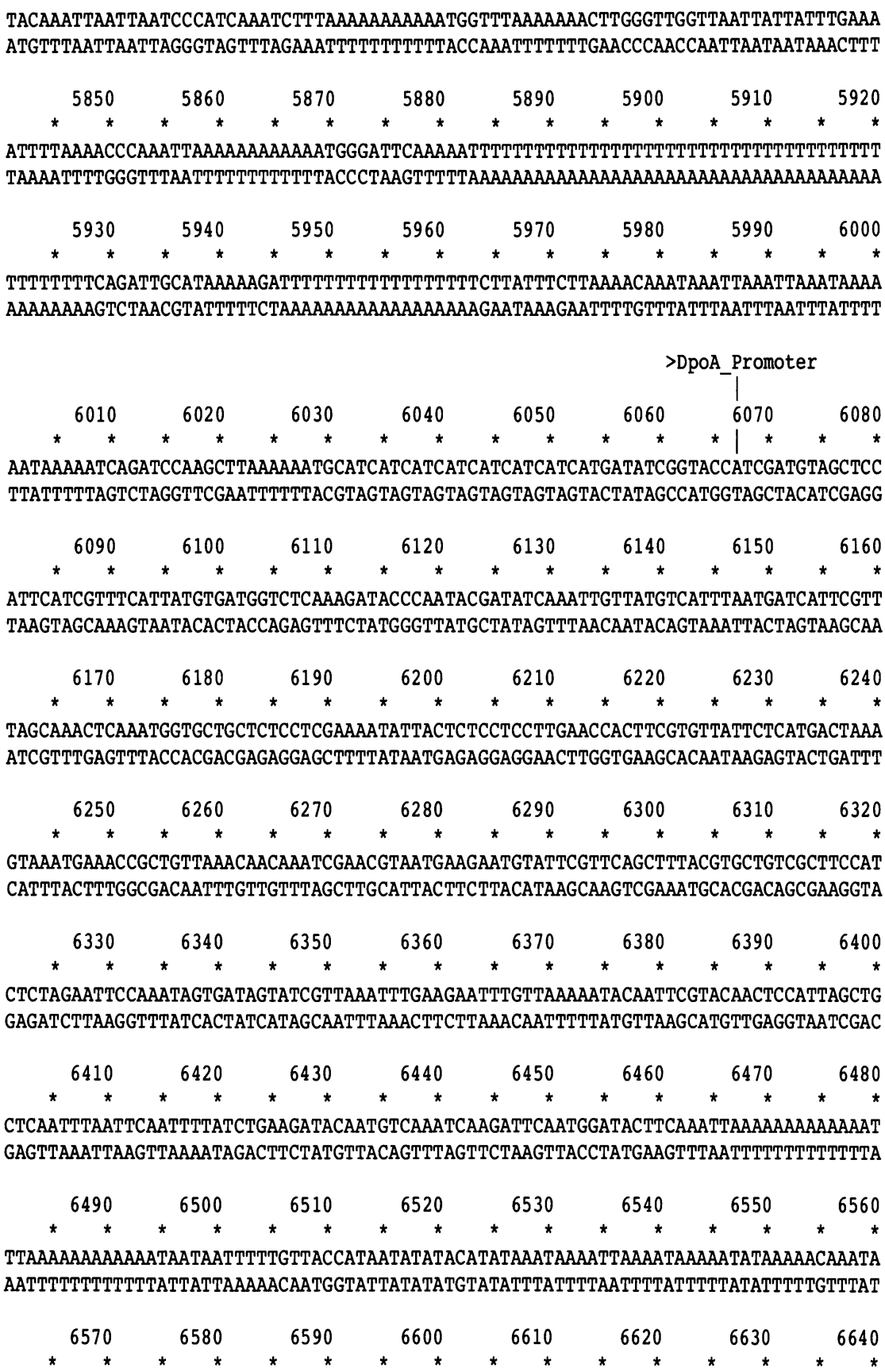
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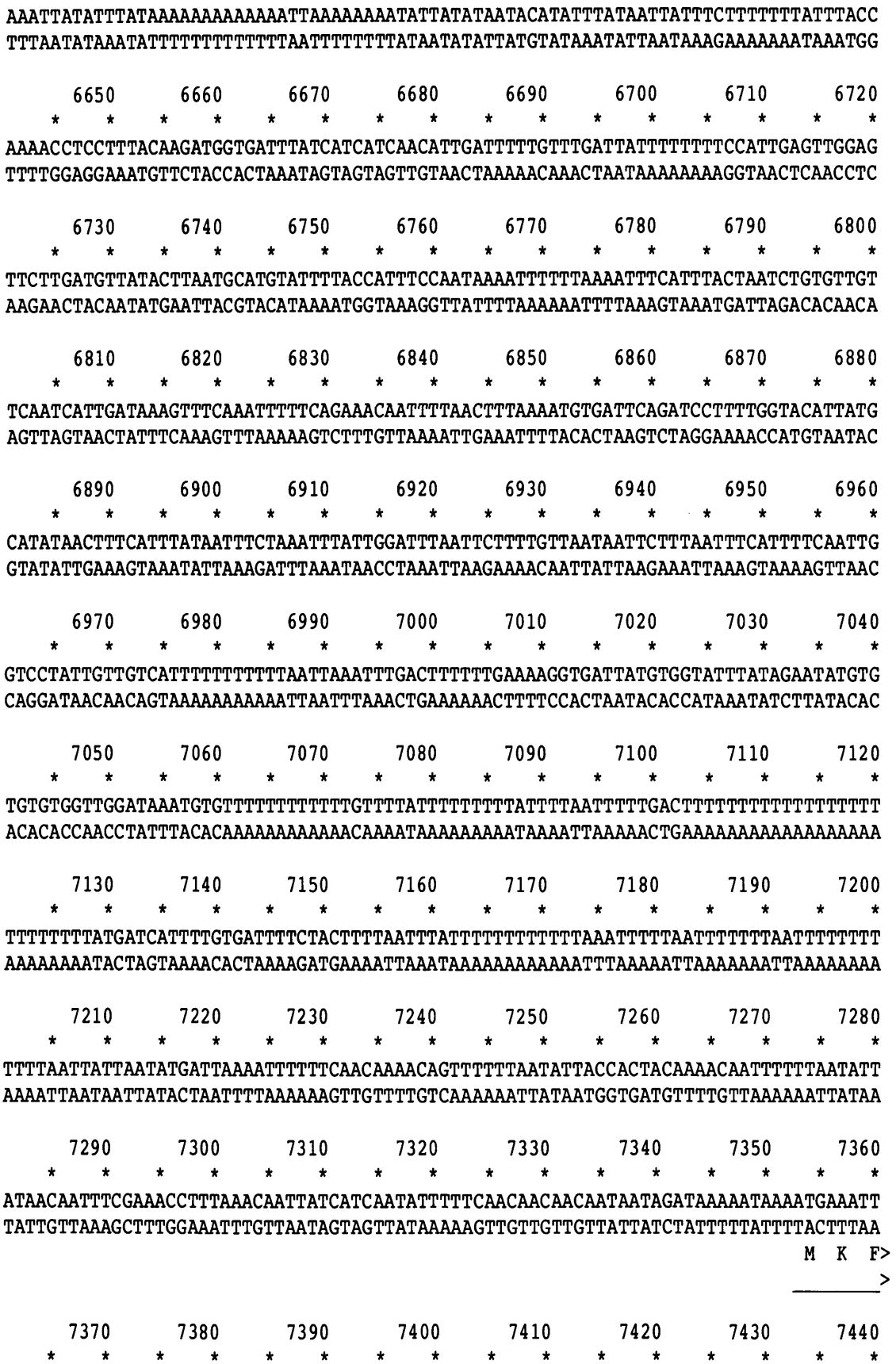
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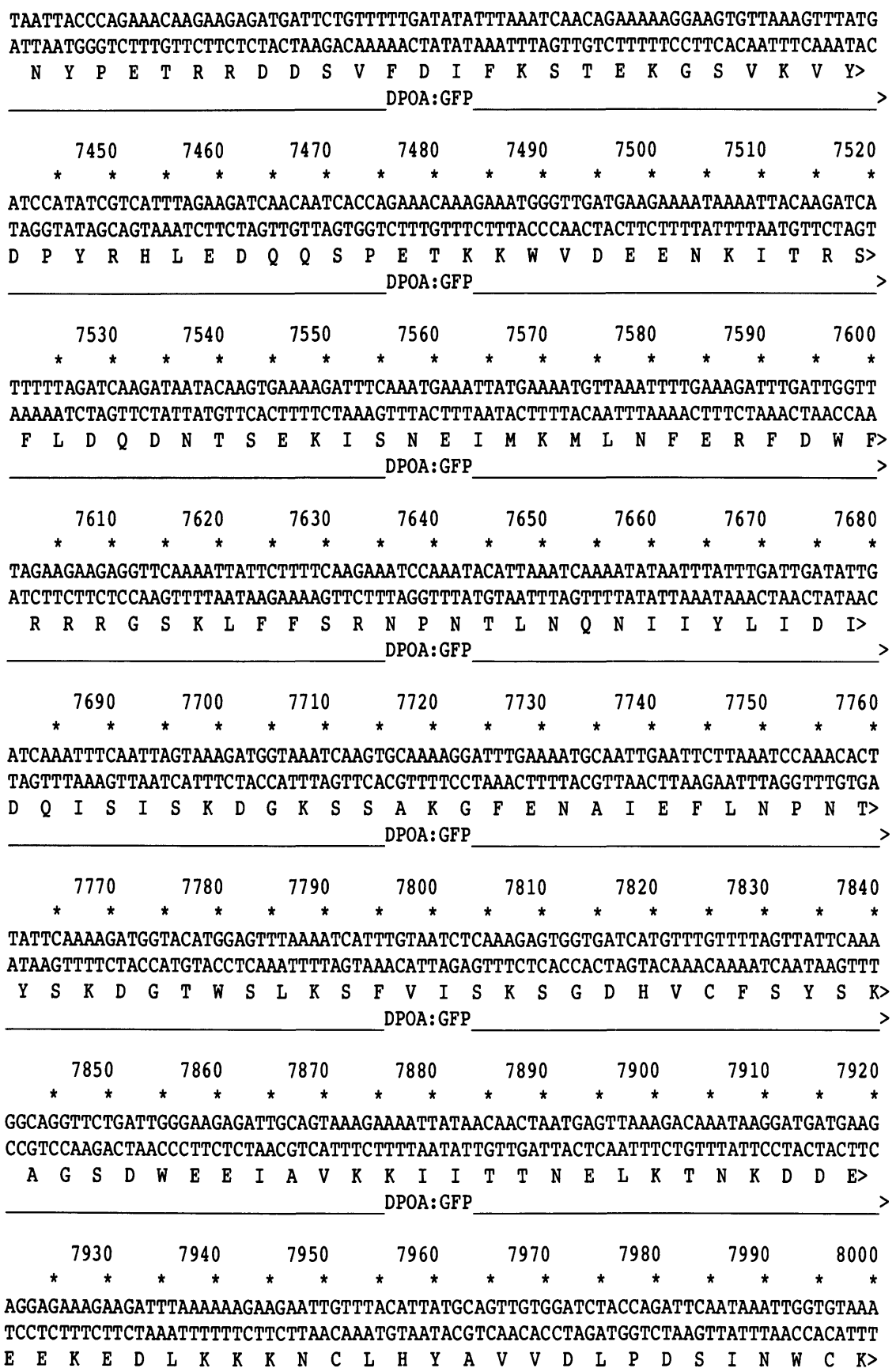
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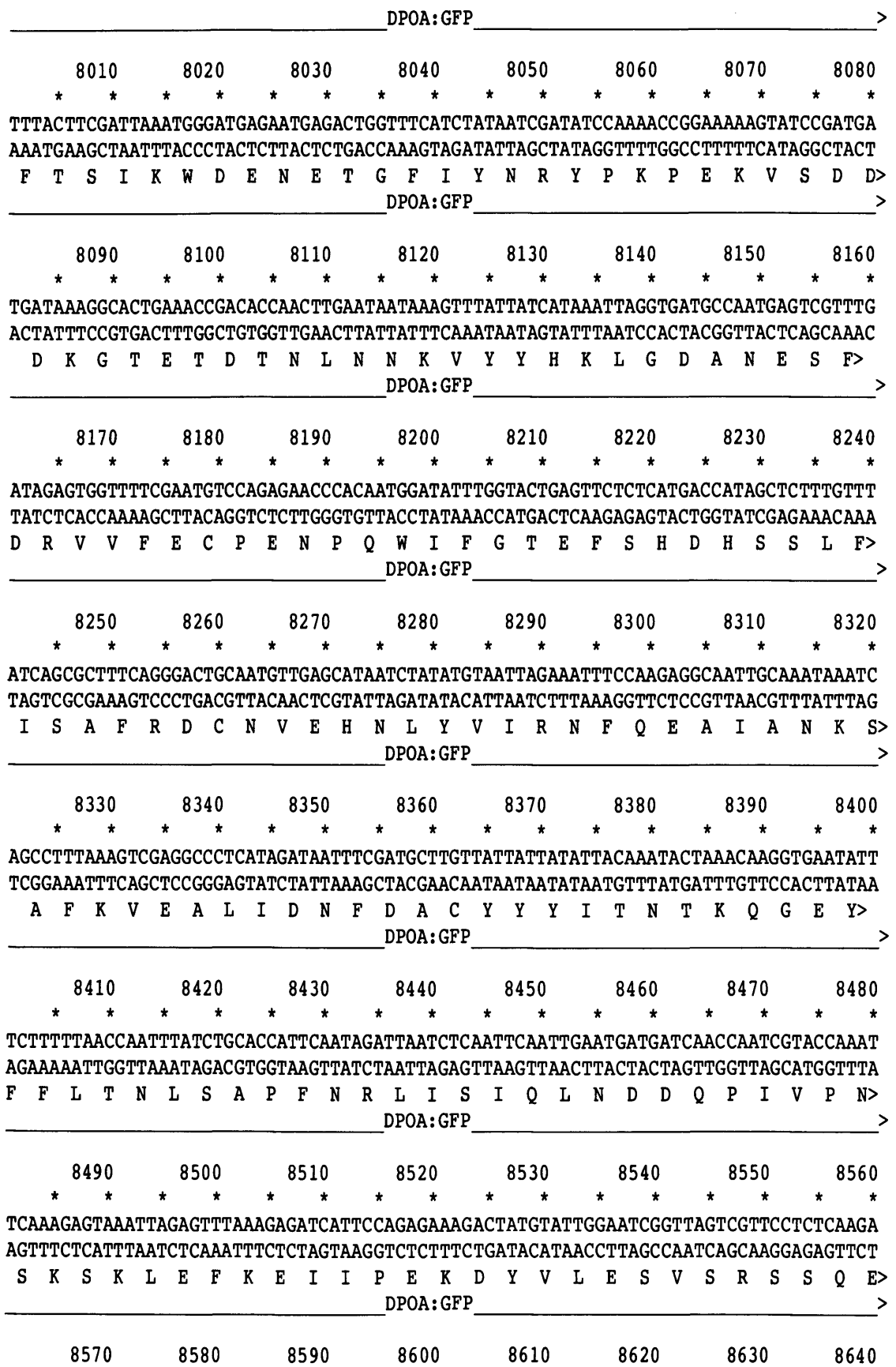
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_____________________________________DPOA:GFP_____________________________________>

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_____________________________________DPOA:GFP_____________________________________>

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_____________________________________DPOA:GFP_____________________________________>

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_____________________________________DPOA:GFP_____________________________________>

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_____________________________________DPOA:GFP_____________________________________>

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_____________________________________DPOA:GFP_____________________________________>

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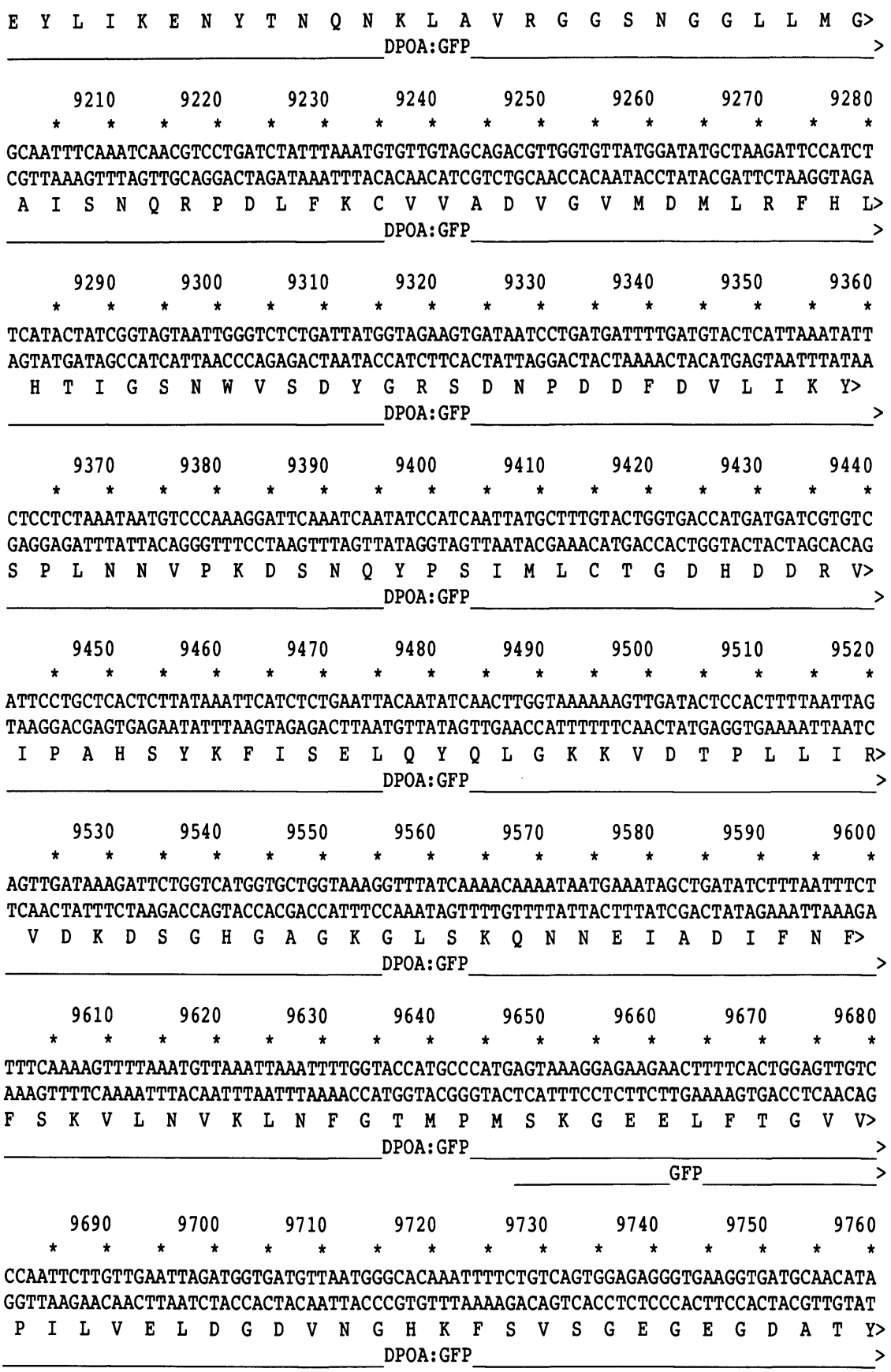
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V D K D S G H G A G K G L S K Q N N E I A D I F N F>
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DPOA:GFP
GFP
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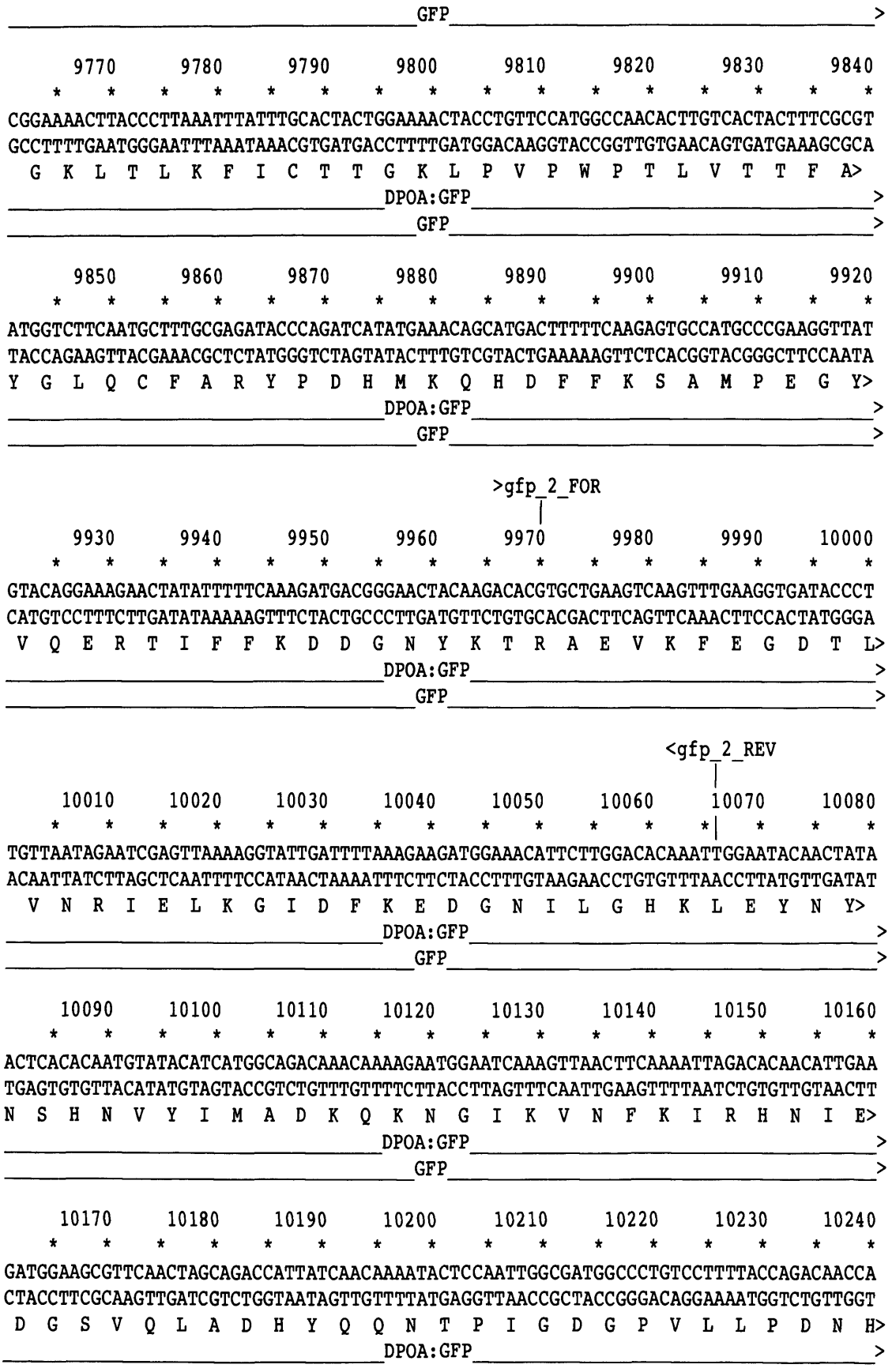
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DPOA:GFP
GFP
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GFP
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GFP
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D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H>
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  Y  L  S  T  Q  S  A  L  S  K  D  P  N  E  K  R  D  H  M  V  L  L  E  F  V  T>
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                                        GFP                                    >

    10330     10340     10350     10360     10370     10380     10390     10400
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                                        GFP                                    >
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