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NOTES

Kinetic Characterization of VIM-7, a Divergent Member of the VIM Metallo-β-Lactamase Family▽

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Purified recombinant VIM-7 possesses efficient penicillinase and carbapenemase activities comparable to those of VIM-2. Cephalosporinase activity was variable and generally lower than those of VIM-1 and VIM-2. A homology model suggests that the VIM-7 Tyr-218 Phe substitution may be responsible for the reduced catalytic efficiency against certain cephalosporins, including ceftazidime and cefepime.

Metallo-β-lactamases (MBLs) effectively hydrolyze most β-lactams and are not inhibited by clinical β-lactamase inhibitors (13, 16). Five clinically important MBLs (IMP, VIM, SPM, GIM, and SIM) are reported to be encoded by genes located on plasmids or associated with mobile genetic elements (13, 16). So far, 18 distinct VIM enzymes from a variety of gram-negative opportunist pathogens have been described (www.lahey.org/Studies). We have previously identified VIM-7, the most divergent of all reported VIM subtypes (77% amino acid identity with VIM-1) and the first MBL to be reported from the United States (14). Here we report the overexpression of VIM-7 in *Escherichia coli* and the kinetic characterization of the purified enzyme.

The $bla_{\text{VIM-7}}$ gene was amplified from *Pseudomonas aeruginosa* 07-406 (14) by PCR using the primers VIM-7F 5′-GAATTCCATATGTTTCAAATTCGCAGCTTTCTGGTTG-3′ and VIM-7R 5′-CGCGGATCCTTACTCGGCCACCGGGCGTACTTTG-3′ to introduce NdeI and BamHI restriction sites, respectively (underlined). The PCR product was cloned into the T7 expression vector pET-26b (Novagen) and transformed into *E. coli* BL21 (DE3) (Novagen). Protein expression was induced in Terrific broth (Sigma-Aldrich) at 37°C by using 1 mM isopropyl-1-thio-β-D-galactopyranoside.VIM-7 was purified from the periplasm according to the method of Avison et al. (1) with modifications including 50 mM Tris (pH 7.5), 100 μM $ZnCl_2$, 0.02% (wt/vol) sodium azide used as the buffer system, and Q-Sepharose and Superdex 75 matrices (GE Healthcare) for the ion-exchange and gel filtration steps, respectively. In addition, $bla_{\text{VIM-2}}$ was amplified from *P. aeruginosa* 81-11963 (15) by using primers VIM-2F 5′-GGAATTCCATATGTTCAAACTTTTGAGTAAGTTATTGG and VIM-2R 5′-CGCGGATCCCTACTCAACGACTGAGCGATTTGTG (NdeI and BamHI restriction sites underlined), and cloned, expressed and purified as described for VIM-7. The concentrations of VIM-7 and VIM-2 were determined from the absorbance at 280 nm by using extinction coefficients of 26,930 M^{-1} cm^{-1} (calculated from the amino acid sequence [www.expasy.ch]) and 28,500 M^{-1} cm^{-1} (2), respectively. Protein preparations were estimated to be >99% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Steady-state kinetic measurements were performed at 25°C in UV-transparent 96-well plates (BD Biosciences) in a SpectraMax 190 spectrophotometer (Molecular Devices) in 50 mM sodium cacodylate (pH 7.0), 100 μM $ZnCl_2$, and 0.1 mg/ml bovine serum albumin. A parallel investigation of nitrocefin hydrolysis by VIM-7 in 1-ml cuvettes yielded kinetic parameters identical to those obtained with 96-well plates (data not shown). The wavelengths and extinction coefficients used were those previously reported (7, 12) excepting those for ertapenem (Δε, −6,920 M^{-1} cm^{-1}; λ, 300 nm). For 96-well plates, extinction coefficients were recalculated from standard curves constructed from absorbance measurements of serially diluted unhydrolyzed and completely hydrolyzed substrates. K_m (μM) and k_{cat} (s^{-1}) were determined from plots of initial velocity of hydrolysis against substrate concentration. For penicillin G, ampicillin, carbenicillin, piperacillin, aztreonam, and clavulanic acid, the K_m was determined as the K_i by measuring inhibition of hydrolysis of the reporter substrate nitrocefin across a range of substrate and nitrocefin concentrations. K_i values were determined by a global (shared-parameter) fit of these multiple data sets to a competitive inhibition model. All kinetic data were fitted by nonlinear regression as implemented in the Prism program (GraphPad Software, San Diego, CA).

Table 1 lists the steady-state kinetic parameters k_{cat}, K_m,

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TABLE 1. Steady-state kinetic parameters of the purified VIM-7 in comparison with those of the other variants, VIM-1 and VIM-2[a]

Substrate	K_m (μM)			k_{cat} (s^{-1})			k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)		
	VIM-7	VIM-1[b]	VIM-2[b]	VIM-7	VIM-1[b]	VIM-2[b]	VIM-7	VIM-1[b]	VIM-2[b]
Penicillins									
Benzylpenicillin	17 ± 2[c]	840	70	430 ± 2	30	280	25	0.036	4
Ampicillin	15 ± 2[c]	920	90	190 ± 17	35	125	13	0.038	1.4
Carbenicillin	84 ± 10[c]	75	205	1,200 ± 74	170	185	14	2.3	0.9
Piperacillin	26 ± 3[c]	3,500	125	140 ± 13	1,900	300	5.4	0.54	2.4
Azlocillin	66 ± 16	120	200	78 ± 4	1,500	200	1.2	12.5	1.0
Cloxacillin	860 ± 310	ND	250 ± 47	2,500 ± 400	ND	350 ± 18	2.9	ND	1.4
Cephalosporins									
Cephalothin	45 ± 5	55	11	180 ± 6	280	130	4.0	5.1	12
Cephaloridine	250 ± 21	30	50	180 ± 6	315	140	0.72	10.5	2.8
Cefoxitin	68 ± 7	130	13	10 ± 0.3	26	15	0.15	0.2	1.2
Cefuroxime	29 ± 4	42	20	16 ± 0.5	325	8	0.55	7.7	0.4
Cefotaxime	22 ± 2	250	12	56 ± 2	170	70	2.6	0.68	5.8
Ceftazidime	120 ± 25	800	72	1.4 ± 0.1	60	3.6	0.012	0.075	0.05
Cefepime	580 ± 61	150	>400	5.3 ± 0.2	550	>40	0.0091	3.7	0.1
Nitrocefin	58 ± 3	17	18	1,500 ± 29	95	770	26	5.6	42.8
Moxalactam	75 ± 15	450	55	230 ± 13	43	90	3.1	0.096	1.6
Carbapenems									
Imipenem	27 ± 2	1.5	9	100 ± 2	0.2	34	3.7	0.13	3.8
Meropenem	38 ± 4	50	2	42 ± 0.8	13	5	1.1	0.26	2.5
Ertapenem	28 ± 3	ND	9 ± 0.6	8 ± 0.2	ND	0.2 ± 0.01	0.29	ND	0.022
Monobactam (aztreonam)	2,700 ± 630[c]	>1,000	>1,000	NH	<0.01	<0.01	ND	$<1.0 \times 10^2$	$<1.0 \times 10^2$
Inhibitors									
Tazobactam	3,500 ± 360	340	875	68 ± 3	5.3	28	0.019	0.016	0.032
Sulbactam	740 ± 90	200	320	110 ± 6	10	23	0.15	0.05	0.072
Clavulanic acid	940 ± 320[c]	ND	>5,000[c]	2.3 ± 0.4[d]	ND	5.4 ± 2.1[d]	0.0025	ND	0.0011

[a] ND, data not determined; NH, no measurable hydrolysis.
[b] Kinetic constants for VIM-1 are from Franceschini et al. (3), and those for VIM-2 are from Docquier et al. (2) except for those for cloxacillin, ertapenem, and clavulanic acid (this study).
[c] K_m values were measured as inhibition constants (K_i) in a competitive model using nitrocefin as the reporter substrate.
[d] k_{cat} values were calculated from fits of initial velocity against the clavulanate concentration assuming the K_m equals the K_i (from the experiment).

and k_{cat}/K_m for hydrolysis of a series of substrates by VIM-7 and compares these data with published values for VIM-1 and VIM-2 (2, 3) and data from our own investigations of cloxacillin, ertapenem, and clavulanic acid hydrolysis by VIM-2 (this study). Note that we also investigated hydrolysis of nitrocefin and cephaloridine by VIM-2 under our conditions and found no major discrepancies with previously published values (2). VIM-7 hydrolyzed all the tested β-lactams except aztreonam, for which only very weak interaction with enzyme was observed. VIM-7 efficiently hydrolyzes all penicillins and carbapenems ($k_{cat}/K_m > 10^6$ M^{-1} s^{-1}) with the exception of ertapenem, while activity against cephalosporins varies by almost four orders of magnitude (activity against cefepime, 9.1×10^3 M^{-1} s^{-1}; activity against nitrocefin, 2.6×10^7 M^{-1} s^{-1}). The β-lactamase inhibitors tazobactam, sulbactam, and clavulanic acid are hydrolyzed with lower efficiencies, due primarily to high K_m values. Activity against penicillins, and to some extent carbapenems, arises both from high k_{cat} and, with the exception of that for cloxacillin, relatively tight (10 to 100 μM) K_m values. Comparison with VIM-1 and VIM-2 (Table 1) suggests three general trends in overall catalytic efficiency. That for penicillins is VIM-7 > VIM-2 > VIM-1, that for cephalosporins is VIM-1 ≈ VIM-2 > VIM-7, and that for carbapenems is VIM-7 ≈ VIM-2 > VIM-1. The trend in k_{cat}/K_m values for VIM-7 broadly follows that observed for MICs determined with *E. coli* DH5α transformed with the pMATVIM-7 plasmid (14) in that penicillin MICs are generally high, the value for imipenem is higher than that for meropenem, and there is little effect on aztreonam susceptibility.

Experiments in which VIM-7 (2 nM) was incubated with EDTA (200 μM) at set time points and activity assessed by nitrocefin (100 μM) hydrolysis rates revealed loss of more than 85% of activity in an incubation period of 2 h. The inactivation process was dominated by an exponential process with a rate (0.014 min^{-1}) comparable to the slower of the two phases (0.012 min^{-1}) observed in the equivalent experiment performed with *P. aeruginosa* SPM-1 (9). However, the data also suggest a second, faster phase (0.48 min^{-1}) accounting for approximately one-eighth of the activity lost. Although this might be considered indicative of the presence of two metal sites of significantly differing affinities, as has been proposed for SPM-1 (8), further experiments are necessary to resolve this point.

A homology model of VIM-7 (Fig. 1) constructed using the EsyPred3D server (www.fundp.ac.be/urbm/bioinfo/esypred) (6) based on the structure of reduced VIM-2 (5) suggests that four residues (positions 68, 218, 224, and 228) might have an impact upon substrate binding and hydrolysis. The Pro-68 Ser and Tyr-218 Phe substitutions are unique to VIM-7, while VIM-7 shares the Tyr-224 His substitution with VIM-1,

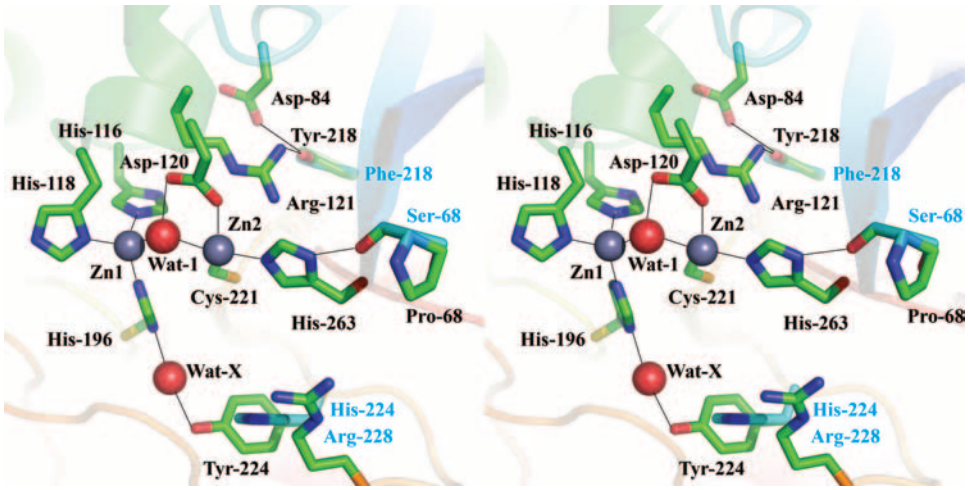

FIG. 1. Stereo view of VIM active sites. The figure shows VIM-2 active site (atom colors as standard [carbon green, oxygen red, nitrogen blue, sulfur yellow] excepting alpha-carbon rainbow ramped by sequence position; zinc ions are rendered as gray, and water molecules are rendered as red spheres). Residues are numbered according to the BBL standard numbering scheme (4). Metal-ligand and hydrogen bonds are shown as thin gray lines. Positions showing variation between VIM-1, VIM-2, and VIM-7 (68, 218, 224, and 228) are labeled in cyan. Side chains of Ser-68, Phe-218, and His-224 of the VIM-7 homology model are shown with carbon atoms rendered in cyan. This figure was rendered using Pymol (http://www.pymol.org).

VIM-4, and VIM-12 only. Arg-228 is present in all VIM enzymes other than VIM-1 and VIM-12. Pro-68, a well-conserved residue in subclass B1 MBLs, forms an H bond to the Zn2 ligand His-263 through its main-chain carbonyl oxygen, and although the replacement of this residue by Ser will likely increase flexibility in this region of the protein, our model does not suggest that this interaction will be abolished. VIM-2 Tyr-218 participates in an H-bonding network, involving Asp-84, that is present in some form in all MBLs of known structure and connects the two Zn^{2+} sites (8). In the related IMP enzymes, enhancing these interactions through the Phe-218 Tyr mutation promotes hydrolysis of so-called type II β-lactam substrates with neutral or charged substituents at the C-2/C-3 (R2) position (10, 11). Our model suggests that in VIM-7, replacement of Tyr-218 by Phe will disrupt this network (Fig. 1). Consistent with this hypothesis, VIM-7 indeed displays a loss of activity against type II cephalosporins bearing charged cyclic substituents (ceftazidime, cefepime, and cephaloridine).

For VIM-2 (2), the Tyr-224 hydroxyl is suggested to interact with the charged C-2/C-3 (R2) substituents of some β-lactams, while Arg-228 is proposed to make effective H bonds to the invariant β-lactam C-3/C-4 carboxylate. The generally tight K_m values observed with VIM-7 (Arg-228), compared with those reported for VIM-1 (Ser-228), would support the latter conclusion. The structure suggests that the Tyr-224 hydroxyl moiety makes a second-shell metal-ligand interaction by H bonding via a water molecule (Wat-X) (Fig. 1) to the distal nitrogen of the Zn1 ligand His-196. Substitution of Tyr-224 for His might be expected to disrupt this interaction. Nevertheless, the efficient hydrolysis of many substrates by VIM-7 suggests either that this interaction is of marginal importance to the function of the VIM active site or that compensating structural rearrangements are possible.

In summary, our kinetic data show that, despite possessing a number of alterations close to the active site, VIM-7 effectively hydrolyzes both penicillin and carbapenem substrates but displays some diminished activity against cephalosporins, particularly those with bulky, charged C-3 substituents. Analysis of a VIM-7 homology model suggests that these effects might arise primarily from the Tyr-218 Phe substitution that distinguishes this enzyme from other family members. Further structural and mutagenic studies will be required to confirm this hypothesis.

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