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Perspective

Exploring the Ascorbate Requirement of the 2-Oxoglutarate-Dependent Dioxygenases

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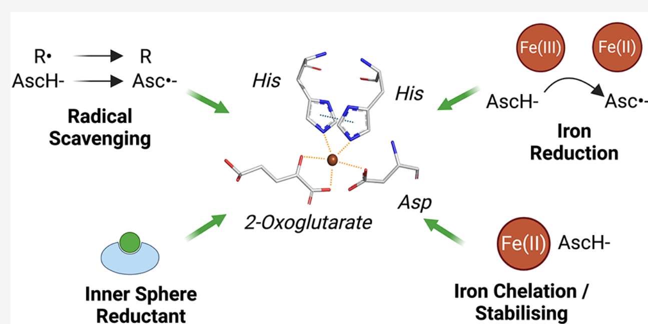
ABSTRACT: In humans, the 2-oxoglutarate-dependent dioxygenases (2-OGDDs) catalyze hydroxylation reactions involved in cell metabolism, the biosynthesis of small molecules, DNA and RNA demethylation, the hypoxic response and the formation of collagen. The reaction is catalyzed by a highly oxidizing ferryl-oxo species produced when the active site non-heme iron engages molecular oxygen. Enzyme activity is specifically stimulated by L-ascorbic acid (ascorbate, vitamin C), an effect not well mimicked by other reducing agents. In this perspective article we discuss the reliance of the 2-OGDDs on ascorbate availability. We draw upon findings from studies with different 2-OGDDs to piece together a comprehensive theory for the specific role of ascorbate in supporting enzyme activity. Our discussion centers on the capacity for ascorbate to act as an efficient radical scavenger and its propensity to reduce and chelate transition metals. In addition, we consider the evidence supporting stereospecific binding of ascorbate in the enzyme active site.



■ SIGNIFICANCE

The biochemical flexibility of ascorbate contributes to its capacity to support 2-oxoglutarate-dependent dioxygenase activity, in preference to other antioxidants. As a highly efficient radical scavenger, Fe(III) reductant and chelating agent, ascorbate can prevent enzyme inactivation by the highly oxidizing reaction cycle intermediates and help stabilize the iron in the active site. Hence, the maintenance of cellular ascorbate levels *in vivo* as well as in culture systems should be prioritized to support dioxygenase activity.

■ INTRODUCTION

Ascorbate (vitamin C) is a highly versatile reducing agent that acts as a critical cofactor for many Cu and Fe-containing enzymes. These enzymes utilize O_2 to catalyze innumerable biosynthetic and metabolic processes. One example involves hydroxylation reactions involving Fe and O_2, for which the involvement of ascorbate was described in the 1950s.[1] In the 1960s, the first identified 2-OGDD enzymes, the collagen hydroxylases, were shown to utilize 2-OG and O_2, with Fe and ascorbate being described as cofactors.[2−5] The discovery of the ascorbate dependency of the collagen hydroxylases led to the proposal that decreased collagen hydroxylase activity underpinned the disintegration of tissues in the vitamin C deficiency disease, scurvy.[2,6] Since this time, many 2-OGDDs have been identified in plants, fungi, microbes and animals.[7,8] While these enzymes show variable dependence on ascorbate, *in vitro* mechanistic studies typically include ascorbate in reaction buffers without fully exploring its specific contribution to the reaction mechanism.

Ascorbate is synthesized in most plants and animals, but its availability can vary significantly. This is particularly true for humans, one of a few species that are dependent on dietary vitamin C intake due to their inability to synthesize ascorbate.[9] The 2-OGDDs have fundamental roles in human health and disease and there is now a strong body of evidence suggesting that states of dietary vitamin C deficiency compromise 2-OGDD activity, with significant implications for development and cancer [reviewed in refs 10−12]. Hence, understanding the specific ascorbate dependency of different 2-OGDDs is crucial. This perspectives review aims to explore the mechanistic interaction between ascorbate and the human 2-OGDDs.

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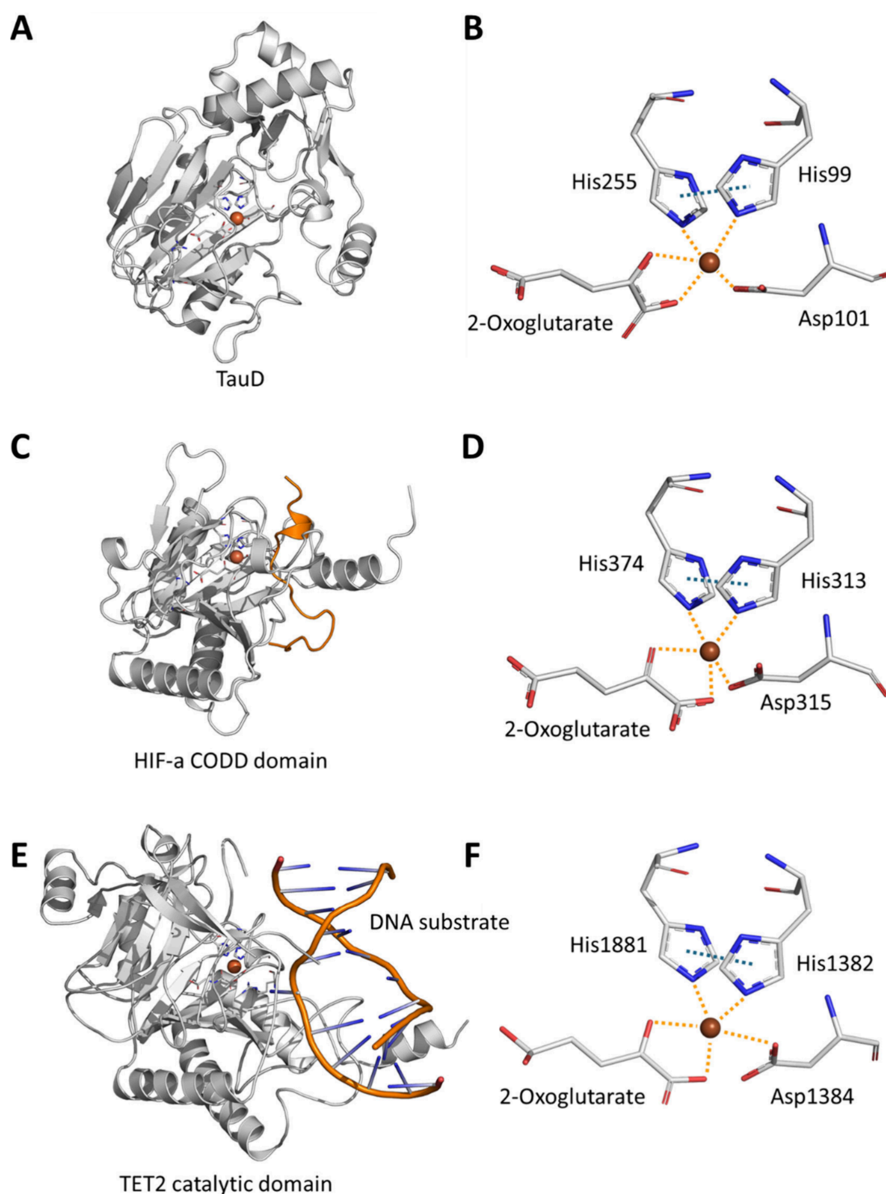

Figure 1. A comparison of the crystal structures of TauD, PHD2 and TET2. The crystal structures for the 2-OGDD enzymes (A,B) TauD [*E. coli*, PDB reference 1os7[19]], (C,D) PHD2 (*Homo sapiens*, PBD reference 5L9B[20]) and (E,F) TET2 [*Homo sapiens*, PDB reference 4nm6[21]] are depicted with some computational transformations (notably the transformation of N-oxalylglycine to 2-OG in TET2, Mn to Fe in PHD2). The TauD structure depicts one monomer from the oligomeric crystal structure and PHD2 one monomer from the dimeric crystal structure. These three enzymes are characterized by a conserved iron and 2-OG binding site. While the proteins are of vastly different size, have different substrates, and in the case of TauD, are from different organisms, they share a characteristic triad of amino acids (2 His and 1 Asp) which coordinate iron at the active site. Another key feature is the conserved 2-OG binding site, where 2-OG coordinates to iron in a bidentate manner with stabilizing interactions from a nearby arginine residue (not shown).

■ THE 2-OXOGLUTARATE-DEPENDENT DIOXYGENASES

The 2-OGDD non-heme iron-containing enzymes are found in plants, fungi, animals and microbes.[7,8] Many 2-OGDD are hydroxylases that split molecular oxygen, with one oxygen atom being inserted as a hydroxyl group into the substrate and the other incorporated into succinate following the decarboxylation of 2-oxoglutarate. In microorganisms and plants, the 2-OGDDs also catalyze additional reactions such as ring closure/opening, elimination, desaturation, halogenation, epimerization and epoxidation.[7,13,14] At least 70 genes encoding 2-OGDDs have been identified in humans.[7] These enzymes are responsible for the biosynthesis of small molecules, and protein and DNA hydroxylation.[7,15] These reactions are critical to a range of processes, including epigenetic regulation, collagen biosynthesis, the hypoxia response and the regulation of cell metabolism. Their dysregulation impacts broadly on development, cancer, neurological conditions, connective tissue disorders, and pulmonary and cardiac diseases.[15−17]

The 2-OGDDs share a conserved double-stranded β-helix (DSBH) fold, housing an active site with a triad of two histidines and a carboxyl group amino acid, typically His-Glu/Asp-Xn-His, which forms the iron-binding site, alongside a separate 2-OG binding site.[7,15,18] There are significant structural differences in the target substrate binding sites, reflecting the highly diverse functional nature of the

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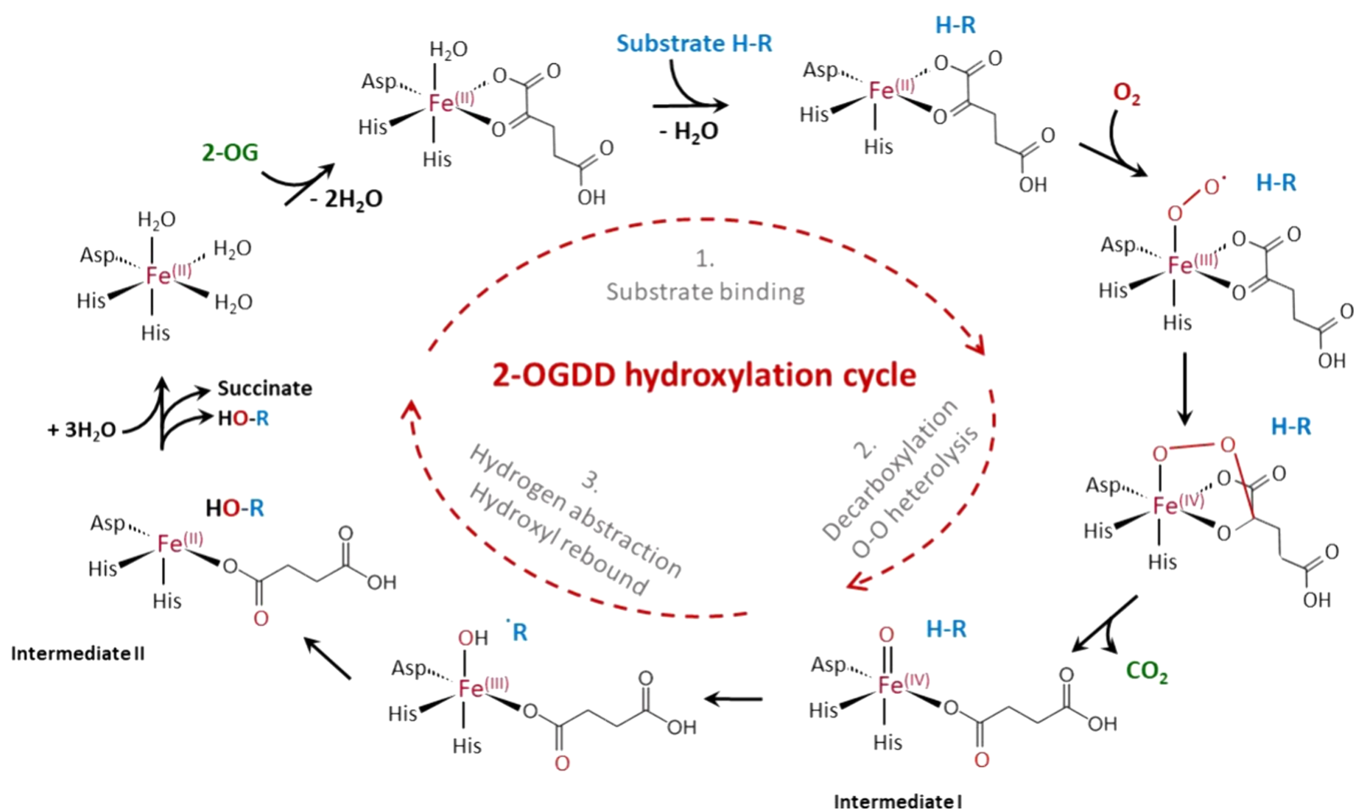

Figure 2. 2-OGDD hydroxylation consensus catalytic cycle. Human enzymes in the 2-OGDD family are proposed to employ the following hydroxylation mechanism. Step 1. In its ground state, enzyme bound Fe at the active site lies in an octahedral geometry, coordinating to three amino acid residues and three water molecules. Binding of the 2-OG substrate displaces two water molecules, generating the 2-OG-bound ligand configuration frequently observed in crystallographic structures. This process is followed by the binding of the principal substrate and the displacement of another water, generating a vacant binding site for molecular oxygen at the Fe center. Step 2. The resulting Fe(III)-superoxo species primes the enzyme for the decarboxylation of 2-OG, releasing CO_2 and creating a Fe(IV) ferryl intermediate with bound succinate. Step 3. The highly reactive ferryl intermediate hydroxylates the principal substrate via a two-step hydrogen abstraction/hydroxyl rebound mechanism.

enzymes.[7,18] To illustrate these similarities and differences, the crystal structures of the bacterial enzyme TauD (2-oxoglutarate-dependent taurine dioxygenase) and the human enzymes P4H2 prolyl hydroxylase (PHD2) and ten-eleven translocase (TET) 2 and their active sites are depicted in Figure 1.

THE HYDROXYLATION REACTION MECHANISM

The human 2-OGDDs carry out hydroxylation reactions via a conserved mechanism that was first proposed in 1982[22] and has subsequently been supported by numerous experimental and computational investigations.[8,14,23−28] The reaction cycle depicted in Figure 2 details the current consensus mechanism. In its initial state, the active site iron is in an octahedral Fe(II) state, coordinating to the His-Glu/Asp-X_n-His triad and three water molecules. The first step in the cycle involves the binding of 2-OG to displace two water molecules. This is followed by binding of the principal substrate at an adjacent site, with the displacement of another water molecule, thereby generating a vacant binding site for molecular oxygen at the Fe center. Specifically, it is the binding of the principal substrate that has been proposed to weaken the interaction between the residual water molecule and Fe, thus allowing for oxygen binding.[4,29−33] The formation of an Fe(III)-superoxo species is the critical step that primes the enzyme for subsequent reactions. This orchestrated substrate binding sequence appears to be fundamental to the efficiency of the enzymes by limiting the probability of a subsequent uncoupled reaction, in which the enzyme proceeds through the reaction cycle in the absence of a target substrate.[34,35] The potential consequences of the uncoupled reaction will be discussed below.

The 2-OGDD reaction cycle proceeds rapidly following O_2 binding. The Fe(III)-superoxo species triggers decarboxylation of 2-OG, generating succinate and carbon dioxide and creating an Fe(IV)═O species (the ferryl intermediate). One oxygen atom is incorporated into succinate. The highly reactive ferryl intermediate is then able to abstract a hydrogen from the target substrate, generating a C-centered radical and Fe(III)—OH, the latter participating in a hydroxyl rebound reaction that results in hydroxylation of the substrate, release of the reaction products and regeneration of Fe(II) at the active center.[14] Spectroscopic evidence in support of the Fe(IV)═O species (intermediate I) and the Fe(II)−product complex (intermediate II) comes from studies with TauD[36,37] and collagen prolyl-4-hydroxylase.[27] Detailed computational modeling suggests that enzymes in the TET and histone lysine demethylase families also employ a similar mechanism.[38,39] These similar observations in structurally distinct but functionally related enzymes support the conserved nature of the 2-OGDD hydroxylation mechanism. Other 2-OGGD-catalyzed reactions that occur in plants and microorganisms, such as halogenation, epoxidation and desaturation, involve variations

Table 1. Major Human Fe- and 2-Oxoglutarate-Dependent Dioxygenases and Their Ascorbate Requirement

Biological process	Enzyme	Biological function	Impact of ascorbate on enzyme activity and associated biological function	References
Collagen maturation	CP4H	Stabilizes collagen triple helix.	Highly specific requirement for ascorbate; reaction ceases after 15−30 cycles in the absence of ascorbate which reduces Fe(III) following the uncoupled reaction.	3, 32, 118, 129, 134
	CP3H	Uncertain; modification of collagen IV structure.	Ascorbate is required for reduction of lysyl hydroxylase enzyme bound Fe(III).	
	Lysyl hydroxylase (PLOD)	Stabilizes collagen triple helix.	Collagen synthesis is impaired in fibroblasts lacking ascorbate and in scorbutic animals.	
Regulation of the hypoxia inducible transcription factors (HIFs)	Prolyl hydroxylases (PHDs 1−3)	Hydroxylation of prolines on HIF-α, targeting the protein to the proteasome, preventing HIF transcription factor activation.	Ascorbate was shown to specifically boost HIF prolyl hydroxylases and FIH activity.	43, 51, 54, 130, 156−159
	Factor inhibiting HIF (FIH)	Asparagine hydroxylation on HIF-α, preventing the binding of HIF-α to CBP/p300 and formation of transcription complex.	Ascorbate availability in cultured cells moderates HIF activation and HIF-directed gene expression in response to hypoxia, metabolic disturbance, iron chelation and Ni(II) or Co(II) poisoning.	
		Asparagine hydroxylation in ankyrin-repeat domains. Stabilizes Ankyrin fold.	Normal and tumor tissue ascorbate levels are inversely correlated with HIF activation in animal and human tumor tissues.	
		Histidine hydroxylation in ankyrin-repeat domains—function unknown.		
Demethylation of DNA 5mC	TETs 1, 2, 3	Sequential oxidation of 5mC to generate 5hmC, 5fC and 5caC, leading to excision repair and effective demethylation of DNA cytosine.	Ascorbate specifically increases 5hmC levels in cultured cells. Ascorbate alters gene expression profiles.	13, 42, 44, 52, 53, 106, 116, 160−162
		Influence gene expression and epigenetics in growth and development.	Ascorbate supports the differentiation of leukemic bone marrow cells in a TET-dependent manner.	
			Evidence of a TET-dependent impact of ascorbate availability during pregnancy on fetal development in animal models.	
Demethylation of histones	Lysine demethylases (KDMs), members of the JmjC family (>16 enzymes)	Initial hydroxylation of Nε-methylated lysines and mono- and dimethyl arginines in histones as first steps in demethylation pathway.	Ascorbate requirement for histone demethylases has been demonstrated *in vitro*.	46, 145, 163−167
	Arginine demethylases	Extensive involvement in regulation of gene expression, with impact on epigenetics, development and cancer.	Ascorbate-mediated impact on histone demethylase-dependent gene expression in human embryonic stem cells and immune cells.	
DNA repair and modification	ALKB homologues 1−9	Hydroxylation of *N*-methyl nucleotides in DNA/RNA.	Ascorbate-mediated reduction of Fe(III) demonstrated in ALKBH3 *in vitro*.	46
		DNA repair. Removal of 1-mA and 3-mC with regeneration of unsubstituted bases.		
RNA modification	FTO (fat mass and obesity-associated protein)	Affects mRNA stability by oxidative demethylation and removal of N6,2′-O-dimethyladenosine and N6-methyladenosine.	Specific ascorbate requirement for FTO demonstrated *in vitro*.	46
		Variants in FTO are linked to metabolic syndrome and obesity.	Ascorbate status is associated with altered metabolism and obesity, but no direct associations with FTO function have yet been demonstrated.	
Carnitine synthesis	Trimethyl-lysine hydroxylase	Catalysis of the first and last steps step in the synthesis of carnitine.	Ascorbate depletion is associated with decreased γ-butyrobetaine hydroxylase and tissue carnitine levels in the guinea pig. Plasma ascorbate levels are associated with utilization of fats in energy metabolism in humans and animals.	40, 168−170
	γ-butyrobetaine hydroxylase	Carnitine transports fatty acids into mitochondria, and is obtained by synthesis and diet in humans.		

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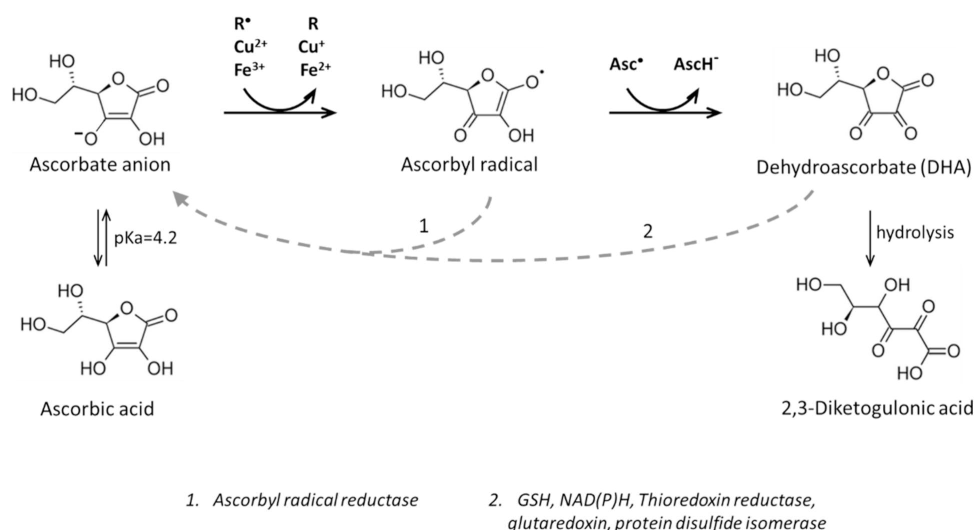

Figure 3. Reactions of ascorbate in mammalian systems. Ascorbate is a highly efficient reducing agent for biological free radicals and oxidized transition metals. Sequential oxidation of the ascorbate anion by single electron steps generates the ascorbyl radical and DHA. Both products can be recycled intracellularly but DHA is unstable at neutral pH and readily undergoes irreversible hydrolysis to generate inactive breakdown products.

in high valent Fe-oxo chemistry, discussed elsewhere in the literature.[25,34]

The 2-OGDD hydroxylation reaction cycle described in Figure 2 appears to have no fundamental requirement for ascorbate, and the involvement of ascorbate is rarely mentioned by those investigating 2-OGDD biochemistry. Despite this, there is a substantial volume of literature testifying to the capacity for ascorbate to enhance enzyme activity, dating back to the first described 2-OGDD prototypes, the collagen prolyl and lysyl hydroxylases[2,3,5] and now including many other 2-OGDDs[11,13,40−47] (Table 1). The ascorbate requirement has been shown to be specific, with only minimal activity being reported for other reducing agents such as dithiothreitol, glutathione, L-cysteine, NADPH, spermidine, tocopherol, and mercaptoethanol.[3,32,42,48−54] For example, even 2 mM DTT and 5 mM L-cysteine allowed the collagen hydroxylase reaction rate to proceed at maximally only 18% and 16% of the rate achieved with ascorbate, respectively, and other reducing agents were completely ineffective.[3] There is also a suggestion that the ascorbate requirement could be stereospecific for some enzymes, with numerous reports indicating variable efficacy with D-ascorbic acid, D-isoascorbate and isopropylidene-L-ascorbate.[51,54] To gain further insights into the role of ascorbate in the 2-OGDD reaction cycle, we should briefly revisit the unique redox properties of ascorbate that are relevant to the function and oxidative metabolism of these enzymes.

■ ASCORBATE BIOCHEMISTRY

Ascorbic acid ($AscH_2$) is a highly water-soluble compound with a pK_a of 4.2 (Figure 3). Hence the predominant species at neutral pH is the ascorbate anion ($AscH^-$).[55] Ascorbate is synthesized from glucose in the livers or kidneys of most animals, but humans, other primates and some other animal species are fully dependent on dietary intake due to having acquired inactivating mutations in gulonolactone oxidase, the terminal synthetic enzyme.[56,57] These species are therefore prone to deficiency. Regardless of its source of production, ascorbate is distributed to the tissues via the circulation,[9] with active transport across cell membranes and into intracellular compartments via the sodium-dependent vitamin C transporters SVCT1 and SVCT2, encoded by *SLC23A1* and *SLC23A2*, respectively.[58−61] Plasma levels are maintained at a concentration below ∼80−100 μM, with reuptake occurring in the kidney at concentrations below this.[62,63]

The SVCTs concentrate intracellular ascorbate to mM levels, far exceeding plasma levels.[9,63] These intracellular levels are in excess of the K_m values for ascorbate reported for the 2-OGDDs (150−300 μM).[3,64−67] Using the usual rule of thumb that substrate concentrations at approximately 10-fold the K_m are required to reach 90% of the V_{max}, it seems reasonable to suggest that mM intracellular ascorbate levels would be required to provide a buffer to ensure continued optimal 2-OGDD enzyme activity.[3,68,69] Tissue ascorbate levels are most concentrated in the brain, pituitary, adrenals and bone marrow stem cells, all locations of ascorbate-dependent enzyme activity.[9,44,58,63] Together, this information is consistent with the enzyme cofactor function being a major biological role for ascorbate.

Ascorbate reacts readily with biological free radicals or transition metals to generate the ascorbyl radical, a relatively stable entity that dismutates to regenerate ascorbate and produce dehydroascorbate (DHA) (Figure 3).[70] The ascorbyl radical can be reduced in mammalian cells by an NADH-dependent Cytochrome b_5 ascorbyl radical reductase found in some cell membranes[71] or by thioredoxin reductase.[72] DHA generated by further oxidation of the ascorbyl radical (Figure 3) is present only in very low concentrations in biological fluids,[57,73−75] due to its rapid reduction by glutaredoxin,[76] protein disulfide isomerase[77] and thioredoxin reductase,[78] using GSH, NADH and NADPH as electron donors.[79] In the absence of enzymatic reduction, DHA undergoes rapid hydrolysis (its half-life at neutral pH and 37 °C is ∼6 min) to form 2,3-diketogulonic acid, representing an irreversible loss of ascorbate to the system[80] (Figure 3).

Interaction with transition metals, particularly Cu and Fe ions, represents a major activity of ascorbate in biological systems. Ascorbate-mediated reduction of Fe(III) and Cu(II) maintains the activity of many metalloenzymes,[10,55,81] facilitates Fe storage in ferritin, promotes transferrin-mediated Fe transport[82,83] and contributes to the production of reactive

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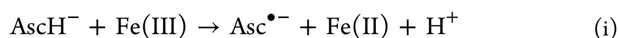
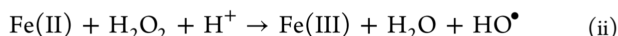
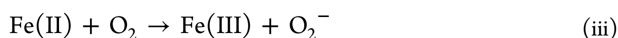
oxygen species.[57,84] The often-discussed pro-oxidant activity of ascorbate is in fact due to its efficient reduction of Fe(III) (Reaction i) thereby perpetuating the Fenton reaction with H_2O_2 to produce the highly reactive hydroxyl radical (Reaction ii). At neutral pH, the reaction with H_2O_2 is proposed to result in the formation of a ferryl-oxo compound.[85,86] In addition, ascorbate recycling of Fe(II) fuels the reaction with O_2 to generate superoxide radicals (O_2^-), leading to H_2O_2 production via dismutation (Reactions iii and iv).[10,57,87]



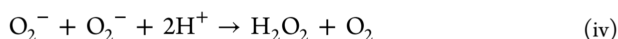
$$\mathrm{AscH^- + Fe(III) \rightarrow Asc^{\bullet -} + Fe(II) + H^+} \quad \text{(i)}$$



$$\mathrm{Fe(II) + H_2O_2 + H^+ \rightarrow Fe(III) + H_2O + HO^{\bullet}} \quad \text{(ii)}$$



$$\mathrm{Fe(II) + O_2 \rightarrow Fe(III) + O_2^-} \quad \text{(iii)}$$



$$\mathrm{O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2} \quad \text{(iv)}$$

The described reactions of ascorbate with transition metals result in the generation of DHA (Figure 3), contributing to the turnover of ascorbate in biological systems. In addition to its ready reaction with transition metals, ascorbate is also a weak chelating agent and can form complexes with Fe to facilitate the absorption of Fe from the diet[88] and intracellular iron transport.[82,83] We will consider all these properties of ascorbate with respect to their potential to impact on the 2-OGDD reaction mechanism. We propose that it is a combination of the possible interactions with Fe that accounts for the specialized requirement for ascorbate as a support partner to maintain optimal 2-OGDD enzyme activity.

■ THE NATURE OF THE ASCORBATE REQUIREMENT

Ascorbate in the Uncoupled Reaction. The 2-OGDD hydroxylation reaction mechanism has similarities to oxidation reactions catalyzed by other metalloenzymes.[25,89,90] Like the 2-OGDDs, the heme peroxidases and cytochrome P450 (CYP450) enzymes also generate high valency Fe-oxo species (Compounds I and II) using O_2 or H_2O_2 to form an Fe(IV)═O intermediate capable of hydrogen abstraction.[34,91−94] These heme enzymes target multiple substrates and are vital for oxidative metabolism.[91,92,95] However, unlike the 2-OGDDs, they are not dependent on ascorbate to maintain activity. This intriguing difference raises the question as to the nature of the 2-OGDDs that drives their specific requirement for ascorbate.

There is an extensive literature on the management of the highly reactive iron intermediates formed in the active sites of the metalloenzymes.[93,96,97] In the case of the heme-containing enzymes, the highly constrained hydrogen bonding environment in the active site and the participation of the porphyrin ring in the formation of iron(IV)−oxo porphyrin π-cation radicals are proposed to work together to facilitate well-targeted oxidation reactions, thereby minimizing the risk for off-target oxidative damage to the enzyme itself.[98] The Fe atom is also well-secured in the heme group, with only one coordination position available for O_2 binding.[92,95] In common with the 2-OGDDs, the reaction cycle of the heme-containing enzymes also involves the binding of the substrate before O_2 binding.[34,35] This "substrate triggering" is considered to help ensure that the highly reactive ferryl-oxo species forms only when there is a target substrate in place and limits the likelihood of an uncoupled reaction cycle.[34,35] In the CYP450s, the uncoupled reaction has been associated with the generation of reactive oxygen species—an undesirable outcome—although it is unclear as to how commonly this occurs.[99,100]

The 2-OGDDs are also prone to undergoing an uncoupled reaction cycle, and early studies with the prototype enzymes collagen prolyl-4-hydroxylase (CP4H) and lysyl hydroxylase indicated that, although ascorbate was absolutely required by the enzymes to maintain activity, it was consumed in a stoichiometric manner only during the uncoupled reaction.[4,101] The uncoupled reaction of CP4H is considered a sporadic process that occurs both in the presence and absence of the peptide substrate.[102−104] In the absence of ascorbate, CP4H was observed to catalyze around 15−30 reaction cycles over 5−10 s after which the reaction completely ceased unless it was rescued by the addition of ascorbate.[3,4,105] This interesting insight suggests that in 2-OGDDs, uncoupled reactions result in oxidized iron, thereby creating a role for ascorbate as a reducing agent.

Close reading of the literature suggests that pH may influence the measured ascorbate dependency of the 2-OGDDs. Iron oxidation occurs more readily at higher pH and changes in pH may affect the likelihood for the formation of Fe(III), increasing the need for ascorbate as a reducing agent. It is not uncommon for *in vitro* experiments with purified enzymes or catalytic domain constructs to be carried out close to pH 8 and ascorbate dependency has been demonstrated under these conditions.[3,4,52,65] In contrast, the presence of ascorbate did not affect *in vitro* TET catalytic domain activity at pH 6.8, where Fe(II) oxidation is slower, supporting a role for ascorbate as a reducing agent for Fe(III) that may not be required when Fe(II) is stabilized and not depleted in the reaction mixture.[53] However, ascorbate-mediated promotion of 2-OGDDs is observed at pH 7.4[51,104] and in cultured cells,[43,52,68,106,107] suggesting that ascorbate is a specific requirement under biological conditions.

Resolving or preventing the consequences of the uncoupled reaction may indeed provide a major rationale for the specific ascorbate requirement of the 2-OGDDs. The oxygen-sensing enzyme asparagine hydroxylase factor-inhibiting HIF (FIH) undergoes uncoupled reactions that can result in autohydroxylation and inactivation of the enzyme with the generation of a hydroxylated amino acid (Trp^{296}) or an oxidized methionine (Met^{275}).[108,109] This amino acid oxidation is potentially due to the reaction of the Fe(IV)═O species with a nearby internal residue in the absence of a target principal substrate.[108,109] Ascorbate stimulates FIH activity and it has been proposed that its role is in reducing Fe(III) formed in uncoupled reaction cycles.[109] However, there is a further layer of complexity: where autohydroxylation of FIH occurs, ascorbate is unable to rescue the enzyme, suggesting that ascorbate may play a radical scavenging role to prevent autohydroxylation in the first instance.[108] A similar phenomenon has been observed with the well-studied bacterial hydroxylase, TauD, in which uncoupled reactions generate an Fe(III) species coordinated to an aromatic alcohol that results in enzymatic inactivation, together with the autohydroxylation of an internal residue within the active site (Tyr^{73}).[110,111] TauD is inactivated more quickly in the absence of ascorbate[111] and ascorbate is able to partially restore activity following inactivation.[112] As with TauD and FIH-1, the α-ketoglutarate-dependent herbicide degrading enzyme 2,4-dichlorophenoxyacetate dioxygenase (TfdA) is also hydroxylated at an active site residue during the uncoupled reaction (Trp^{112})[113] and similarly, the autohydroxylated enzyme is inactive even after the addition of ascorbate.[113] These findings highlight the potential for some 2-OGDD to be permanently inactivated by autohydroxylation

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during uncoupled reactions. The prevention of enzyme inactivation could be due to effective reduction of Fe(III) by ascorbate or by its direct reaction with the reactive Fe-oxo intermediates or amino acid radicals such as tyrosine and tryptophan radicals, thereby preventing autohydroxylation. Interestingly, the reaction rate constants for ascorbate with biologically relevant radicals such as alkoxyl/peroxyl, tyrosyl and tryptophan radicals are $>10^5$ M^{-1} s^{-1}.[109,114] Hence the propensity for ascorbate to reduce transition metal ions and to act as a free radical scavenger could contribute to the protection of 2-OGDD activity during the uncoupled reaction cycle. Both Fe reduction and radical scavenging reactions would need to occur readily, and the combined redox properties of ascorbate lend themselves well to this function. Both reactions would result in DHA production and ascorbate turnover. These observations suggest that if the role of ascorbate is to intervene during uncoupled 2-OGDD reaction cycles, then there may only be a limited window of opportunity during which ascorbate will be effective.

Studies with the different recombinant HIF and collagen hydroxylases show that these enzymes share relatively high K_m values for ascorbate, with a range of 140−300 μM being reported.[65,66,103,115] Considering the consumption of ascorbate during the uncoupled reaction as discussed above, we propose that the propensity of the 2-OGDDs to undergo an uncoupled reaction is a major factor in their ascorbate dependency. As stated earlier, the managed substrate binding sequence generally limits the likelihood of an uncoupled cycle. Accordingly, the efficiency of substrate binding and the K_m for oxygen may both have an impact on this cycle. Variability in substrate binding has been shown to affect the rate of the uncoupled reaction for the HIF hydroxylases.[41] In addition, for the 2-OGDD enzymes in which these parameters have been measured, we note an intriguing inverse relationship between the K_m values for O_2 and ascorbate (Figure 4). While there is limited data available, this potential inverse relationship may indicate that enzymes with a higher oxygen binding affinity (and thus a lower K_m for O_2) increasingly undergo uncoupled reactions which would enhance their dependency on ascorbate. Hence the higher K_m for ascorbate reflects the increased ascorbate concentrations required to manage the consequences of the uncoupled reaction and thus achieve V_{max}. From the published data available,[15] we note that those enzymes with the lowest K_m values for O_2 include the epigenetic DNA and histone demethylases that, coincidentally, also exhibit a high requirement for ascorbate.[13,42,44,52,53,116,117] The K_m values for ascorbate have not been reported for these and many other 2-OGDD enzymes. However, mM levels of ascorbate are regularly added to *in vitro* assays. Further, certain cell populations primed for differentiation (and epigenetic reprogramming) such as hematopoietic stem cells have particularly high intracellular ascorbate concentrations.[44] This may be a reflection on the high ascorbate requirement for the epigenetic 2-OGDD enzymes.

Ascorbate and Fe(III) Reduction. Aside from having a specific role in mitigating the pro-oxidant effects of the uncoupled reaction cycle, numerous studies propose that the highly efficient Fe(III) reducing capacity of ascorbate is involved in maintaining 2-OGDD activity. The enediol moiety of ascorbate was shown to be required to support HIF hydroxylase enzyme activity, suggesting a key role for Fe(III) reduction.[51,118] A recent study also demonstrated that the ratio of Fe(III)/Fe(II) in TET2, the RNA demethylase FTO,

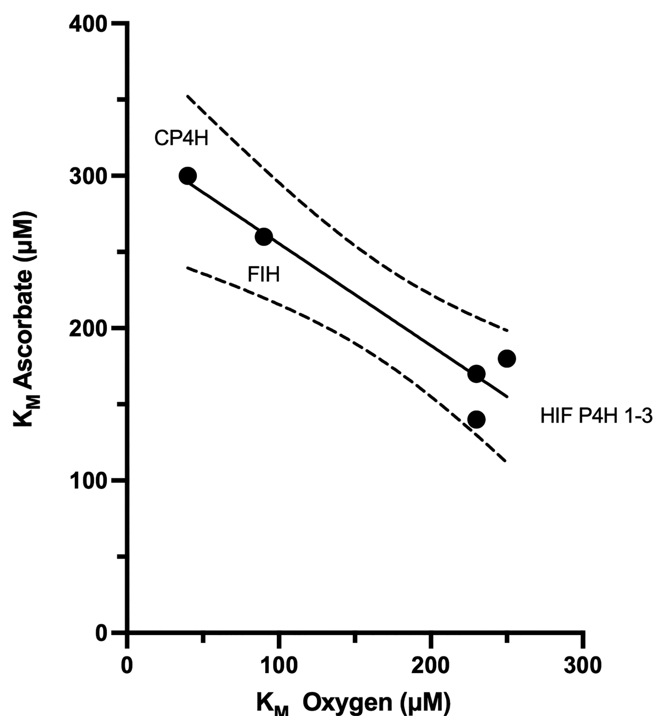

Figure 4. Relationship between the 2-OGDD K_m values for oxygen and ascorbate. This relationship was constructed from published data available for the collagen and HIF hydroxylases.[65,66,103] The values are as follows: CP4H1: Ascorbate K_m 300 μM, Oxygen K_m 40 μM. FIH: Ascorbate K_m 260 μM, Oxygen K_m 90 μM. HIF P4H 1−3: Ascorbate K_m 140−180 μM, Oxygen K_m 230−250 μM.

histone demethylases and in the DNA repair enzyme ALKBH3 decreased when ascorbate was present, suggesting that it was acting to ensure efficiency of the reactive cycle by reducing Fe(III).[46] Indeed, the catalytic efficiency (k_{cat}) of the enzymes was significantly greater in the presence of ascorbate.[46] A recent NMR study with the purified catalytic domain of TET2 indicated that enzymatic activity was exclusively dependent upon ascorbate to reduce Fe during the catalytic cycle in both the uncoupled and coupled reaction.[47] However, ascorbate can also act as a general reducing agent for extracellular Fe, and may not only be acting within the enzyme active site.[53] The potential role of "free" iron in the maintenance of 2-OGDD activity and iron-binding by the enzymes has received little attention. This will be further discussed in the following section.

Ascorbate as a Chelator to Stabilize Fe in the Active Site. The 2-OGDDs can be inhibited by transition metals, particularly Ni(II) and Co(II).[119] This inhibition is markedly affected by the presence of ascorbate, providing a line of evidence suggesting that the iron chelating capacity of ascorbate may form part of the 2-OGDD enzyme-supporting activity. The mechanism of Ni(II) and Co(II)-mediated inhibition is considered to be the substitution of the active site Fe with transition metals that are unable to undergo the redox chemistry necessary for the enzymatic reaction.[119−123] This substitution has been directly demonstrated in the histone demethylase JMJD1 and the DNA repair enzyme ABH2[120] as well as in the HIF hydroxylases.[119]

Ascorbate was found to be highly protective of intracellular HIF hydroxylase activity in the presence of Ni(II), Co(II) and the iron chelator desferal (DFO) (Figure 5).[60,68,119,122−124]

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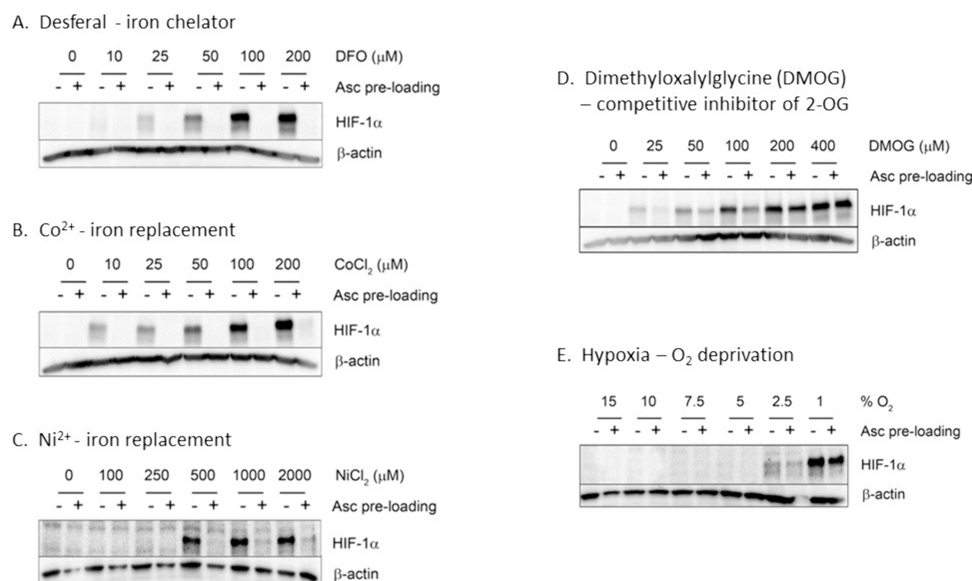

Figure 5. Impact of ascorbate on the inhibition of the HIF hydroxylases by iron chelation, Co(II) and Ni(II) exposure, competitive 2-OG inhibitor DMOG and hypoxia. HIF-1α protein accumulates when the regulatory HIF hydroxylases are inhibited. The Western blots shown demonstrate HIF-1α stabilization in ascorbate-deficient compared with ascorbate-loaded Jurkat cells (500 μM ascorbate overnight). Ascorbate completely prevented hydroxylase inhibition following induction with (A) the Fe chelator DFO, (B) Co(II) and (C) Ni(II). In contrast, ascorbate only partially blocked the effects of (D) DMOG, the competitive inhibitor of 2-OG and (E) O_2 deprivation. The data shown are representative of 2−3 independent experiments with Jurkat cells, showing HIF-1α band accumulation as a marker for HIF hydroxylase inhibition and β-actin as a loading control. The Figure has been adapted from previously published results.[125]

These data suggest that ascorbate can stabilize the active site Fe. In contrast, ascorbate only partially protected HIF hydroxylase-mediated stabilization of HIF-1α when cells were challenged with the 2-OG competitive inhibitor DMOG or by O_2 deprivation (Figure 5). Similar findings were reported in a study using a HIF-1 ODD-Luc reporter assay: ascorbate was highly effective at preventing inhibition of the HIF hydroxylases by compounds with iron chelating properties as opposed to competitive inhibitors of 2-OG binding.[54] Other reducing agents such as D-isoascorbic acid or N-acetic cysteine were much less effective, suggesting a stereospecific effect of ascorbate.[54] The authors proposed that L-ascorbate could be successfully docked into the prolyl hydroxylase binding pocket occupied by 2-oxoglutarate, and that the Tyr310 residue in this pocket might interfere with the binding of D-isoascorbate—thus explaining the stereospecificity of L-ascorbate for this enzyme.[54]

The 2-OGDD K_m values for Fe(II) are between 0.05 and 10 μM[15] and binding constants are ≪1 μM, suggesting tight binding.[104] However, these K_m and binding constant values refer to Fe(II) and not Fe(III) and it is possible that ascorbate prevents the loss of bound Fe(III) from the enzyme via rapid reduction to Fe(II). This suggestion is consistent with the ability of ascorbate to prevent enzyme inhibition by DFO, which specifically chelates Fe(III).[126] Indeed, DFO-mediated PHD inhibition and HIF-1 activation was thought to occur by chelation of the active site Fe(III) during the HIF hydroxylase enzymatic cycle.[127] Considering these combined observations, we propose that, in addition to facilitating Fe reduction, the chelating ability of ascorbate[128] may result in its being able to structurally stabilize the active site Fe during the reactive cycle transitions, preventing free iron loss from the active site. In contrast to its impact on iron competition, the much less dramatic effect of ascorbate on hypoxia- or DMOG-mediated HIF hydroxylase inhibition probably reflects the ability of ascorbate to mitigate the effects of the uncoupled reaction cycle and is therefore less dependent on the Fe-stabilizing function.

In addition to the prevention of Fe loss from the 2-OGDD active site, ascorbate has also been shown to promote Fe binding. Using an *in vitro* biochemical assay with the purified catalytic domain of TET2, the addition of L-ascorbic acid to the reaction buffer promoted an 8-fold increase in the rate of Tet2-mediated hydroxymethylation.[52] Other reducing agents such as spermidine, vitamin B1, vitamin E, glutathione, NADPH and L-cysteine all failed to promote TET activity. Although these *in vitro* data demonstrate an ascorbate dependency for TET2, it is unclear whether ascorbate is acting on Fe in the active site or in the medium. Highly efficient ascorbate-mediated reduction of Fe(III) in the medium was demonstrated using the recombinant murine TET1 catalytic domain (mTET1-CD) as a model system, with resultant restoration of enzyme activity.[53] Other reducing agents were much less efficient at reducing Fe(III), which is consistent with the known proficiency for ascorbate as an effective reducing agent for transition metals.[10,53,55]

Whether ascorbate is functioning to reduce Fe in the active center, or whether its role is to ensure a supply of Fe(II) in the medium remains a point for discussion. Notably, the observations of increased hydroxymethylation activity with the mTET1-CD were pH dependent. At pH 8, where Fe(II) is more readily oxidized, the enzyme required ascorbate to reduce Fe(III) to Fe(II), thereby restoring enzymatic activity.[53] In contrast, at pH 6.8, where Fe(II) is more stable, mTET1-CD remained fully active even without ascorbate in the reaction mixture. Promotion of the uncoupled reaction by incubating mTET1-CD with 2-OG and O_2 in the absence of the methylated DNA substrate did not result in loss of activity.[53] This suggests that, in this model system, ascorbate acts not as a bound cofactor to repair the catalytic center after

the uncoupled reaction, but rather by ensuring the supply of Fe(II) to maintain enzyme activity.[53] However, the extent to which maintenance of the active site Fe requires a source of cytosolic Fe(II) in an intracellular setting is uncertain. Ascorbate promotes 2-OGDD activity at pH 7.4 *in vitro,* intracellularly and in animal models when other readily available reducing agents that could reduce free iron, such as GSH and cysteine are ineffective.[42−44,51,106,129−131]

Proposing a role for ascorbate as a general reducing agent for Fe(III)/Fe(II) is dependent on the availability of a cytosolic iron pool. The metabolism, uptake and trafficking of iron is highly regulated *in vivo,* presumably to minimize the potential for redox cycling and the consequent production of damaging free radicals.[84,132,133] Iron is mostly sequestered within storage proteins such as ferritin and other metalloproteins and cellular uptake and transport is managed by transferrin.[132] Ascorbate is thought to participate in iron uptake and metabolism in numerous ways, including stimulating iron mobilization from ferritin, cellular uptake and intracellular homeostasis.[82] These ascorbate-mediated activities could contribute to iron availability for the 2-OGDDs *in vivo* and provide yet another, more general, mechanism whereby ascorbate promotes enzyme activity. The extent of Fe loss and turnover from the active site of the 2-OGDDs during the reactive cycle is unknown but, as noted above, the K_m values for Fe(II), 0.05−10 μM[15] and the low binding constants for Fe(II) suggest tight binding at the active site.[104] The low K_m values for Fe(II) suggest that the mM ascorbate concentrations added to *in vitro* assays in order to reach V_{max}[3,52] far exceed the amount of ascorbate required to ensure Fe reduction.[82] Considering all these factors, whether the extra-enzymatic reduction of Fe(III) adequately explains the unique efficacy of ascorbate in promoting 2-OGDD activity is unresolved. This is clearly a complex matter. Additional information on the formation of DHA in concert with 2-OGDD activity would help determine the extent to which ascorbate is required to maintain activity. To date, only one early study has investigated ascorbate turnover and reported that DHA was formed stoichiometrically with the uncoupled cycle.[4] It would be of value to investigate the kinetics of ascorbate dependency with more modern enzymatic systems.[84]

■ THE NATURE OF THE ASCORBATE-ENZYME INTERACTION

The principles considered above are strongly supportive of a role for ascorbate as an inner sphere reductant for the 2-OGDDs, in addition to, but quite separate from, its ability to reduce free iron in solution. The first evidence for this concept was the demonstration that CP4H activity was dependent on ascorbate specifically, even when only 1−2% of free iron in solution had been oxidized.[3] Studies with ascorbate analogues also revealed that the ascorbate lactone ring was essential for activity, as analogues with identical lactone ring components such as D-isoascorbate and 5,6-O-isopropylidene ascorbate were similarly effective.[118] However, modification of the ring atoms that abolished the chelating capacity of ascorbate rendered the selected analogue molecule ineffective as a cosubstrate.[118] These observations suggest that ascorbate interacts with a unique binding pocket at the active site of the CP4H that is at least partially identical to the 2-OG binding site and that includes two co-ordination sites for the Fe atom.[118] This site would appear to be inaccessible to other reducing agents due to steric or charge constraints, providing an explanation for their relative lack of efficacy. Interestingly, two small molecule ligands, 2,2′-bipyridine-4-carboxylate (bipy4C) and 2,2′-bipyridine-5-carboxylate (bipy5C), have been proposed to facilitate the ability of ascorbate to reduce iron during uncoupled CP4H reaction cycles.[134] The proposed mechanism involves the direct binding of bipy4C and bipy5C to Fe(III) with the displacement of coordinating ligands such as succinate.[134] The authors of this study did not elaborate on how the binding of these ligands might facilitate iron reduction by ascorbate. Broadly speaking, these data with the collagen hydroxylating enzymes are also consistent with early *in vivo* studies showing that guinea pigs deprived of dietary vitamin C but given large oral doses of D-isoascorbate did not develop scurvy and exhibited normal growth, tooth development and wound healing.[135]

More recently, L-ascorbate and D-isoascorbate, effective stimulants of TET2 activity, exhibited a binding constant of ~8.7 × 10^3 M^{-1} with the TET2 catalytic domain, whereas the ineffective L-ascorbic acid 2-phosphate and L-ascorbic acid 2-sulfate showed a much weaker binding interaction.[52] It was proposed that ascorbate was able to displace succinate from the active site of ALKBH3, FTO and TET2.[46] The possibility that ascorbate occupies a similar binding site to 2-OG and succinate is plausible given the structural similarities between these molecules. Indeed, a working theory is that ascorbate might conceivably displace succinate during the uncoupled reaction cycle and coordinate directly to Fe(III). Such an exchange with succinate was recently suggested to occur during the coupled reaction cycle.[46] The possibility for engagement of ascorbate in the active site at the end of the reaction cycle would ensure the reduction of Fe(III) present after the uncoupled reaction or following the coupled reaction if Fe(III) were stabilized by a nearby thiol as has previously been proposed.[118] In support of these data with the human 2-OGDDs, a possible ascorbate binding site within the active site, although not directly at the 2-OG binding site, was also reported for anthocyanidin synthase, a plant 2-OGDD family member.[136]

Fresh insights regarding the ascorbate-binding theory can be gleaned from recent studies with the algal TET homologue, CMD1, that catalyzes the conversion of 5mC to C5-glycerylmethylcytosine (5gmC). Ascorbate acts as a cosubstrate in this reaction, being the glycerol donor.[137,138] CMD1 shares key similarities with enzymes in the 2-OGDD family, such as the characteristic His-His-Asp iron binding motif, and undergoing a similar reaction cycle with the formation of an Fe(IV)═O intermediate. However, CMD1 utilizes ascorbate in the place of 2-OG. A crystal structure for CMD1 in complex with ascorbate and dsDNA reveals that ascorbate directly coordinates to the enzyme-bound iron via a monodentate coordination (Figure 6). This coordination differs from the bidentate coordination observed with 2-oxoglutarate in published 2-OGDD crystal structures (Figure 1). Although ascorbate is utilized as a cosubstrate in the CMD1 reaction rather than as an electron donor, this binding conformation should theoretically permit ascorbate to transfer electrons to the enzyme bound iron and act as an inner sphere reducing agent. It remains to be seen whether ascorbate binds in a similar manner to the active sites of human 2-OGDDs. Indeed, it may not, given the differences in 2-oxoglutarate bindings sites—notably CMD1 does not appear to bind 2-oxoglutarate. Many questions concerning the binding of ascorbate with the 2-OGDDs remain unanswered.

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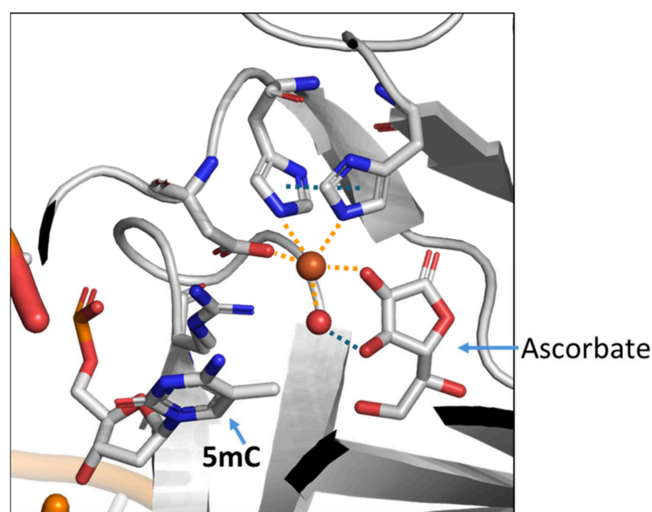

Figure 6. Ascorbate binding site within the TET homologue CMD1. The active site of TET homologue CMD1 (PDB code 7cy8) shows that iron is bound by a characteristic triad of amino acids (2 His and 1 Asp). Ascorbate also directly coordinates to the enzyme bound iron, with the 5-methylcytosine substrate sitting in a nearby pocket.[138]

BIOLOGICAL AND FUNCTIONAL IMPLICATIONS OF 2-OGDD ASCORBATE DEPENDENCY

There is mounting evidence that ascorbate availability impacts 2-OGDD activity in humans, with potentially profound effects on our biology. Table 1 contains a summary of known interactions between ascorbate and major human 2-OGDDs. In light of the growing number of human 2-OGDDs now identified and our absolute dependency on dietary vitamin C intake, it is worth considering the current evidence for the links between decreased vitamin C status and the many 2-OGDD-associated aspects of human health. Due to the extensive literature in this area, the reader is referred to relevant review articles where possible.

Collagen Synthesis, Skin Health and Wound Healing. The role of ascorbate as a cofactor for the collagen hydroxylases is widely accepted.[134] Indeed, the identification of this activity led to the first plausible link between ascorbate deficiency and the most notable symptoms of scurvy such as bleeding, gum disease and impaired wound healing, which can all be associated with defective collagen formation.[139] Collagen formation is the major function of skin fibroblasts and this process is well established to be dependent on ascorbate, with deficiency being associated with decreased collagen synthesis and defective cross-linking.[140,141] The skin contains high concentrations of ascorbate, reaching mM concentrations in intracellular compartments by active uptake from the circulation [reviewed in ref 142]. Both SVCT1 and SVCT2 transporters are expressed in the skin, with the specific localization of SVCT1 in the epidermis.[142,143] Interestingly, the epidermal layer of the skin contains very high ascorbate concentrations. As these cells do not synthesize collagen, their requirement for ascorbate remains a point for investigation. The 2-OGDD enzymes involved in the regulation of the hypoxic response and in epigenetics have been demonstrated to have an impact on the morphology, gene expression and differentiation of the skin epidermal cells, with ascorbate availability affecting these processes [reviewed in ref 142].

Immune Function. Ascorbate is widely considered to play an important role in the function of immune cells, an opinion driven largely by the high levels of ascorbate in these cells. Intracellular levels have long been known to be ∼1.5 mM, ∼3 mM and ∼3.5 mM in neutrophils, monocytes and lymphocytes, respectively,[63,144] but it is only more recently that the importance of 2-OGDD-mediated processes in immune cells has become apparent [reviewed in refs 145−148]. The HIF transcription factor is a major driver of immune cell function, influencing bacterial killing, cell metabolism and cell death pathways.[149−152] The ascorbate dependency of the HIF hydroxylases is well documented,[51,103] providing a major role for ascorbate as a moderator of HIF-dependent immune cell functions. Accordingly, decreased ascorbate levels are associated with impaired immune cell responses driven by the HIF transcription factor.[145,146]

The complexity of the immune system and the myriad of interactions requires a sophisticated network of immune cells that adapt and alter their function in response to challenges. Epigenetic regulation of gene expression by the TETs and Jumonji C domain-containing histone demethylases (JHDMs) has been shown to be crucial for immune cell development, expansion, differentiation and function.[153−155] Ascorbate has also been shown to affect immune cell maturation, differentiation and cytokine expression, frequently in association with activation of TET2 or histone demethylases [reviewed in refs 145, 146, and 148].

Cancer. The development and progression of cancers can be considered as a culmination of acquired mutations and the adaptation of the tumor to the metabolic pressures that lead cells to uncontrolled growth and inappropriate survival. Data from cancer studies illustrate the potential for the activity of important 2-OGDDs to be compromised in a low vitamin C context *in vivo*. It is now well recognized that cancers are dependent on epigenetic processes including those mediated by the TETs and histone demethylases.[148,171,172] The complex role of epigenetics in cancer will not be dealt with here, but it is notable that there is a growing awareness that by supporting the activity of epigenetic 2-OGDD enzymes, ascorbate may provide a means of targeting epigenetic dysregulation in cancers.[173−175]

The activation of the hypoxic response is fundamental to the survival of solid tumors, as it promotes an adaptive glycolytic response, increased angiogenesis and survival responses that promote tumor growth and metastasis.[176,177] An inverse correlation between ascorbate levels from patient tumor samples and HIF-1 activation in tumor tissue has been observed in papillary renal carcinoma,[178] colorectal,[157] breast,[159] endometrial,[158] thyroid cancer[179] and glioblastoma.[180] Notably, there was no relationship between HIF activation and tissue ascorbate levels in clear cell renal carcinoma, where HIF is constitutively active and thus is not under the regulatory control of the HIF hydroxylases.[178] These studies highlight the potential for low tissue levels of ascorbate to promote the hypoxic response in cancer.

Low ascorbate levels are common in patients with cancer, presumably due to disease burden[174,181] and human tumor tissue has been shown to contain less ascorbate than adjacent normal tissue.[157,158] These factors should be considered when determining a need for ascorbate supplementation in cancer treatments.

Development. Epigenetic programming during early development directs cellular differentiation and influences phenotypic differences and this process can be influenced by maternal nutrition.[182,183] The TET enzymes play an important

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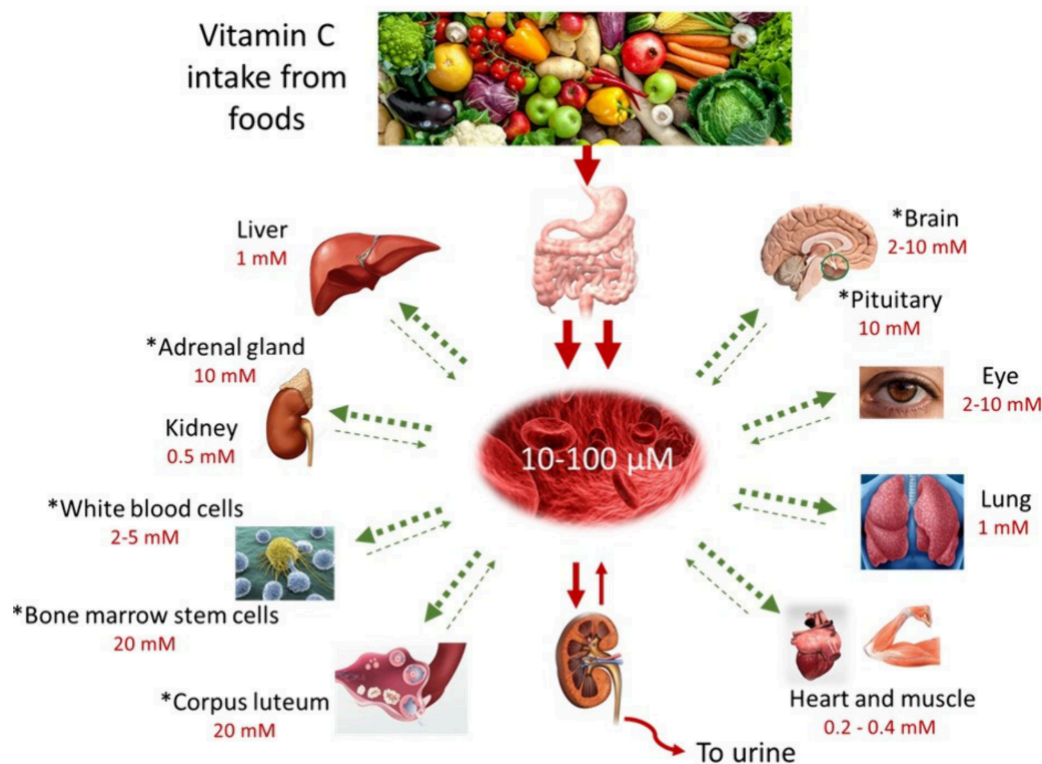

Figure 7. Uptake of ascorbate in humans and distribution to the tissues. Humans are dependent on dietary sources of ascorbate, mostly from fresh fruit and vegetables in which it is not destroyed by cooking. Following uptake in the bowel, ascorbate is transported via the circulation into tissues around the body. Plasma levels are maintained below 100 μM by filtration and reabsorption through the kidneys. Accumulation in tissues is controlled by active transport via the SVCTs, and tissue saturation levels vary across a wide range, as shown. The highest levels are recorded in those tissues that are sites of 2-OGDD activity or Cu enzyme-dependent hormone synthesis (marked with an asterisk).

role in the establishment of the epigenome during early embryonic development when DNA methylation patterns are subject to dynamic change [reviewed in ref 12]. For example, TET1 and TET3 are highly expressed during early development and the disruption of their expression or activity affects neurogenesis and differentiation.[184,185] Coincidentally, low maternal plasma ascorbate during pregnancy has been shown to affect offspring development and pregnancy outcomes in humans and in animals, including growth retardation and effects on hippocampal and cerebellar neurogenesis.[160,183,186,187] Maternal vitamin C deficiency in the ascorbate-dependent Gulo mouse was shown to affect germline development in a Tet-dependent manner and to impact on the reproductive health of subsequent generations, which is strongly suggestive of epigenetic dysregulation.[162] In support of this theory, perturbations in methylation were observed in germ cells from female embryos at genomic regions associated with the regulation of meiosis.[162] The irreversibility of many of the phenotype changes reported in these studies is suggestive of an epigenetic effect of ascorbate in development, with important public health implications.[12,160]

■ THE BIOLOGICAL IMPACT OF ASCORBATE AVAILABILITY ON 2-OGDD ENZYME FUNCTION—HOW MUCH IS ENOUGH?

While its importance to sustain 2-OGDD function is often not discussed, ascorbate is typically included in buffers at mM concentrations for *in vitro* work with these enzymes. As mentioned earlier, the reported K_m values for the limited number of 2-OGDDs investigated are between 140 and 300 μM: for CP4H it is 300 μM, for FIH it is 260 μM and for the HIF-P4Hs it is 140−180 μM.[66,103] Hence, ascorbate concentrations above 1 mM, which are observed in most body tissues[9] (Figure 7) could be necessary to sustain 2-OGDD activity at levels close to the V_{max}. These concentrations are consistent with the mM ascorbate levels required for C4PH to achieve its V_{max}.[3,4]

Ascorbate is transported around the body via the circulation, and renal filtration and reuptake restricts basal plasma ascorbate to concentrations below 100 μM (Figure 7).[9] Tissue ascorbate concentrations are highly variable. In humans, intracellular ascorbate varies from around 0.2 mM in skeletal and heart muscle cells, to as high as ∼10 mM in the adrenal glands, white blood cells and the pituary.[9] The heterogeneity in tissue ascorbate concentrations likely represents different tissue specific requirements for ascorbate. Tissues that are locations of amidated peptide hormone synthesis by the ascorbate-dependent Cu-containing peptidylglycine α-amidating monooxygenase (PAM) include the adrenals, pituitary, corpus luteum, testes and neurons. Ascorbate levels in these tissues are very high: for example, ascorbate levels in the corpus luteum have been reported to be around 20 mM. They also cycle with maturation of this organelle, presumably to support hormone synthesis as required.[188]

Ascorbate levels are also high in cells that are functionally dependent on 2-OGDD activity. Skin fibroblasts that synthesize most of the collagen in the body, contain high ascorbate concentrations.[141] Undifferentiated hematopoietic stem cells (HSC) were reported to express levels of SVCT2 and to contain ascorbate concentrations approximately 2−20-

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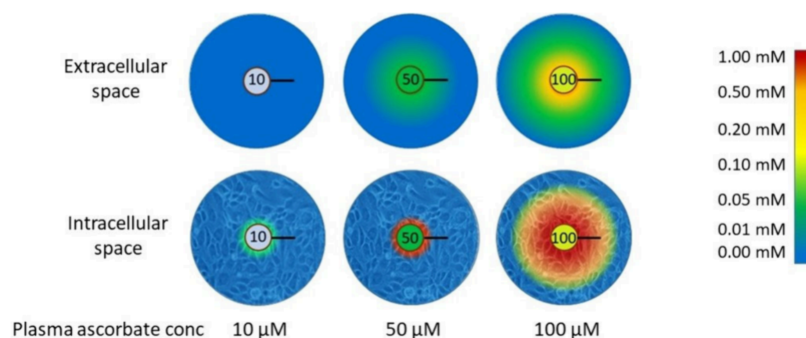

Figure 8. Modeling penetration of ascorbate from plasma into the tissues and accompanying cellular uptake. This schematic illustrates the pharmacokinetic modeling of the relationship between ascorbate diffusion from the circulation, penetration into the tissues, and cellular uptake by tissue cells. The central circle represents a blood vessel with 10, 50, or 100 μM ascorbate, with a heat map showing the corresponding diffusion distance and cellular uptake. The black bars indicate 100 μm: intervessel distance in normal tissues is 50 μm. The image shows that plasma saturation levels of ∼100 μM ascorbate are required to ensure intracellular ascorbate levels can reach the mM range required to support 2-OGDD activity. Average levels in the human population are regularly reported to be ∼50 μM[194] which, according to this model, would compromise ascorbate delivery at short distances form the blood vessels, even in normal tissues. Figure drawn to summarize data from study modeling ascorbate penetration through multicellular layers.[195]

fold higher than those measured in their differentiated counterparts.[44] Given the 2−5 mM ascorbate levels in mature immune cells, this could equate to ascorbate concentrations in HSCs being as high as 10−20 mM. Ascorbate levels declined in HSCs as they differentiated, as did SVCT2 expression.[44] In the case of the HSCs, one theory is that undifferentiated cells poised for epigenetic reprogramming require high levels of ascorbate in order to maintain activity of ascorbate-dependent TETs and histone lysine demethylases.[189] This theory is supported by the observation that ascorbate treatment of IDH1 mutant bone marrow cells resulted in methylation changes at enhancers implicated in myeloid differentiation, with corresponding phenotypic changes mirroring differentiation.[190] Studies showing that ascorbate supplementation promotes demethylation, catalyzed by the TETs, across the epigenome of human embryonic stem cells also lend weight to this argument.[166] Other cells with heightened epigenetic activity include neurons, which undergo constant TET-mediated regional methylation to regulate neuronal processes.[12] This phenomenon again coincides with high tissue levels of ascorbate in the brain. Despite these observations, the precise amount of ascorbate required *in vivo* for optimal TET activity (or for that matter 2-OGDD activity in general) is unknown. We have observed that leukemia cells grown *in vitro* with ascorbate supplementation to the media acquired 2−4 fmoles ascorbate/cell, which is equivalent to 8.3−12.5 mM, based on the approximate volume of these cells.[131] This accumulation coincided with a significant increase in global 5hmC.[131] Whether HSC can maintain optimal TET activity at lower intracellular ascorbate concentrations is unclear, since the K_m of the TET enzymes for ascorbate has not been reported.

The ascorbate concentrations required to maintain optimal 2-OGDD activity will also be influenced by the rate of ascorbate consumption. There is some evidence that cells with high 2-OGDD activity exhibit accelerated ascorbate turnover, driving the need for a higher concentration to buffer this loss and maintain a sufficient concentration to ensure ongoing enzyme function. Hence patients with acute myeloid leukemia have been observed to have extremely low vitamin C levels,[174] which may reflect the high demand for ascorbate in the large HSC population in their bone marrow. It should also be remembered that many cells will be dependent on multiple 2-OGDDs. Hence, high ascorbate consumption by one group of enzymes may limit the ascorbate availability for a second group of enzymes. One such scenario was described for pluripotent and cancer stem cells: increasing CP4H activity in these cells by administering L-proline led to a decrease in 5hmC that was not observed in CP4H KO embryonic stem cells.[191] The authors of this study therefore proposed that intracellular ascorbate depletion due to increased consumption by CP4H limited ascorbate availability for the TET enzymes, causing the reduction in 5hmC. Based on these considerations it is likely that tissue ascorbate levels in the mM range are necessary to support optimal 2-OGDD function.

The relationship between plasma and tissue ascorbate levels is well understood on a macro level. It is accepted that ascorbate deficiency, defined as plasma concentrations below 11 μM, compromises tissue ascorbate levels which are known to closely reflect plasma concentrations.[9,192−194] However, whether there is a biological advantage to achieving plasma saturation, rather than simply avoiding deficiency, remains a point of debate. Insight into this discussion can be gained from a study that modeled the pharmacokinetics of ascorbate diffusion from the circulation and penetration into the tissues. By monitoring ascorbate diffusion through multicellular layers, in combination with ascorbate stability data and cellular uptake, this study determined that mM intracellular levels would be achievable at distances up to 100 μm from blood vessels when plasma ascorbate is 100 μM[195] (Figure 8). Lower plasma ascorbate concentrations of 50 μM, which reflect healthy but not saturated plasma ascorbate intake,[63] would result in compromised intracellular availability within the 50−100 μm intervessel distance in normal tissues. This scenario would be exacerbated in tumor tissues, where poor vascularization increases the average distance between microvessels to ∼300 μm.[196] Given the involvement of the 2-OGDDs in cancer cell biology, both as epigenetic modulators and regulators of the hypoxic response,[148,171,172,176,177] effective delivery of ascorbate to tumor cells should be considered. When measured, tumor tissues have been shown to contain less ascorbate than adjacent normal tissues.[69,157−159,179,180] Modeling ascorbate diffusion into tissues suggests that this is a consequence of impaired delivery.

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Studies such as these, combined with improved understanding of the specificity and K_m of the 2-OGDDs for ascorbate, will underpin future public health initiatives and the setting of dietary ascorbate requirement targets.

■ PERSPECTIVES AND POTENTIAL FUTURE DIRECTIONS

The family of identified human 2-OGDDs has expanded enormously in the past two decades. Our current knowledge highlights the many vital roles for the 2-OGDDs in human health and disease, where they are recognized as being essential for metabolic regulation, energy production, protein synthesis and epigenetic regulation of gene expression. An accessible active site allows the 2-OGDDs to engage with a wide variety of target substrates, ranging from small molecules to large protein and nucleic acid structures. It appears that catering for the catalytic flexibility required to accommodate this diversity of substrates can result in a propensity for the 2-OGDDs to undergo uncoupled reactive cycles, to be prone to inactivation by incomplete reduction of the Fe at the end of the catalytic sequence or autohydroxylation or to lose iron from the active site. In this way they differ from the CYP450s and heme peroxidases in which the porphyrin ring stabilizes the Fe-binding and limits the likelihood of off-target oxidations. Hence controlling the redox chemistry of the highly reactive ferryl iron-oxo reactive intermediate and restoration of the Fe(II) is essential to maintain 2-OGDD enzyme activity. As an efficient radical scavenger, reducer of transition metals and weak iron chelator, the capacity for ascorbate to sustain optimal 2-OGDD activity is unparalleled. In addition, the identification of potential stereospecific ascorbate binding sites within or close to the enzyme active site supports its role as a superior partner for the 2-OGDDs. Further studies to provide more comprehensive K_m and enzyme kinetics data with the more recently discovered 2-OGDDs would improve our understanding of the specific nature of the ascorbate requirement.

The need for ascorbate to support the hydroxylase-mediated formation of hydroxyproline in collagen maturation was well established in the 1980s and is widely accepted as being critical for the maintenance of connective tissues. We propose that the biological impact of the ascorbate requirement of the other 2-OGDDs should also be more widely appreciated. Confirmation of the ascorbate requirement will have knock-on effects on our understanding of the human nutritional requirement for ascorbate and dietary recommendations for daily intake, in both health and disease states. To support this proposal, better information on the K_m for ascorbate for different hydroxylases should be obtained to inform the pharmacokinetics for ascorbate uptake and distribution around the body.

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Biographies

Carlos C. Smith-Díaz graduated with a PhD in epigenetics from the University of Otago, New Zealand in 2024 as well as a first-class honours degree in law from Victoria University of Wellington, New Zealand in 2018. His PhD explored the capacity for vitamin C deficiency to alter the TET-mediated regulation of methylation during early mammalian development as well as investigating how vitamin C supplementation might alter the epigenetic properties of leukaemia cells grown *in vitro*. Carlos' research interests include the regulation of methylation, the regulation of the TET enzymes, cancer cell transcriptomics and the bioinformatics analysis of RNAseq and bisulphite sequencing datasets.

Andrew B. Das completed a combined MBChB/PhD degree at the University of Otago in 2017. His thesis explored how various proteins are damaged by metabolic oxidants and how this impacts a rate limiting step in the synthesis of critical nucleotides. His research interests include how metabolism and epigenetics intersect to determine cell phenotype, which he explored during his postdoctoral work at Mātai Hāora − Centre for Redox Biology and Medicine (Christchurch, NZ), and the Peter MacCallum Cancer Centre in Melbourne. Across several different projects, he developed proficiency in stem cell differentiation, single molecule tracking, and the design and analysis of CRISPR screens for probing cell fate decisions. His current work leverages this expertise to investigate how heterogeneity can arise during cell fate decisions.

Tomasz P. Jurkowski completed his PhD at Jacobs University Bremen in 2008. After postdoctoral training, he became a Junior Professor in Biochemistry and Molecular Epigenetics at the University of Stuttgart. In 2019, he joined Cardiff University as a Senior Lecturer in Mammalian Systems and was promoted to Reader in 2022. His research focuses on epigenetic regulation, specifically investigating the mechanisms of DNA methylation and demethylation. Jurkowski's group employs an interdisciplinary approach, integrating biochemistry, synthetic biology, and multiomics techniques to dissect chromatin networks and epigenetic control mechanisms. By combining these methodologies, his team aims to understand how cellular epigenetic patterns are established and maintained, and to develop synthetic approaches to modulate these processes in various biological contexts.

Timothy A. Hore completed his PhD at the Australian National University in Canberra in 2008, and continued postdoctoral research studies at Cambridge, England. He established his own research group

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at the University of Otago, New Zealand in 2015 where he is now Associate Professor. Hore's research interest is in understanding the links between DNA methylation and development. He is currently investigating the impact of DNA demethylation on the successful creation of pluripotent stem cells. His team is also actively researching the consequences of epigenetic reprogramming and DNA demethylation in the germlines of divergent vertebrate models, and the consequences this has upon transgenerational epigenetic inheritance.

Margreet C. M. Vissers graduated with a PhD in Clinical Biochemistry from the University of Otago, New Zealand in 1985 and completed postdoctoral training at the University of Michigan. She is now a Research Professor in the Department of Pathology and Biomedical Science at the University of Otago, Christchurch, New Zealand. Her research interests include immune cell function, oxidative stress in chronic disease and antioxidant protection. She has published >160 peer-reviewed papers, with an h-Index of 58. A focus of her interest is the role of ascorbate in the regulation of the 2-oxoglutarate-dependent dioxygenases. She has investigated the impact of ascorbate availability on the hydroxylases controlling the hypoxic response and the epigenetic demethylases in cancer and development and in general health maintenance.

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■ ABBREVIATIONS USED

ALKBH3, AlkB homologue 3; $AscH^-$, anionic ascorbate; bipy4C, 2,2′-bipyridine-4-carboxylate; bipy5C, 2,2′-bipyridine-5-carboxylate; CP4H, collagen prolyl-4-hydroxylase; CYP450, cytochrome P450; DHA, dehydroascorbate; DFO, desferal; ESC, embryonic stem cells; FIH, factor-inhibiting HIF; FTO, fat mass and obesity-associated protein; HIF, hypoxia inducible factor; HSC, hemapoietic stem cell; JMHDs, Jumonji C domain-containing histone demethylases; 5-mC, 5-methylcytosine; mTET1-CD, recombinant murine TET1 catalytic domain; 2-OG, 2-oxoglutarate; 2-OGDD, 2-oxoglutarate-dependent dioxygenase; PHD, prolyl hydroxylase; SVCT, sodium-dependent vitamin C transporter; TauD, 2-oxoglutarate-dependent taurine dioxygenase; TET, ten-eleven translocase enzyme; TfdA, 2,4-dichlorophenoxyacetate dioxygenase

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