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Substrate Activation by Iron Superoxo Intermediates

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Abstract

A growing number of non-heme-iron oxygenases and oxidases catalyze reactions for which the well-established mechanistic paradigm involving a single C-H-bond cleaving intermediate of the Fe(IV)-oxo (ferryl) type [1] is insufficient to explain the chemistry. It is becoming clear that, in several of these cases, Fe(III)-superoxide complexes formed by simple addition of O_2 to the reduced [Fe(II)] cofactor initiate substrate oxidation by abstracting hydrogen [2]. This substrate-oxidizing entry route into high-valent-iron intermediates makes possible an array of complex and elegant oxidation reactions without consumption of valuable reducing equivalents. Examples of this novel mechanistic strategy are discussed with the goal of bringing forth unifying principles.

Introduction

Non-heme iron enzymes catalyze a remarkably diverse repertoire of oxidation reactions [3] using an equally remarkable breadth of reaction mechanisms [4]. In transformations of atoms lacking π or non-bonding electrons, one well-described mechanistic strategy involves input of electrons to O_2 by the reduced iron(II) cofactor and a reducing co-substrate (e.g., α-ketoglutarate, tetrahydropterin, reduced nicotinamide), resulting in O-O-bond scission and generation of an Fe(IV)-oxo (ferryl) complex [5]. This intermediate then abstracts hydrogen from the substrate to initiate its oxidation. However, the members of a small subset of these enzymes have been found to oxidize electron-poor aliphatic substrates without need of reducing co-substrates. With no obvious, substrate-independent pathway to the potently oxidizing ferryl complex, these enzymes have hinted at a surprising potency and versatility of superoxo or peroxo intermediates [1,6,7] that are merely precursors to ferryl species ("pre-ferryls") in the better-studied systems. This review will cover recent developments in this area that highlight the mechanistic plasticity of these proteins.

Isopenicillin N Synthase

Recognition of the "pre-ferryl" type of hydrogen abstraction originated with the classic experiments of Baldwin and co-workers on isopenicillin *N* synthase (IPNS) [8]. This non-heme-iron oxidase forms β-lactam and thiazolidine rings from a linear tripeptide substrate (ACV; Figure 1A) to make the penicillin nucleus. In this process, two C-H bonds must be cleaved along with one N-H and one S-H bond. Substrate deuterium kinetic isotope effects revealed that cleavage of the β C-H bond of the L-cysteine residue initiating closure of the β-lactam occurs before cleavage of the β C-H bond of D-valine that initiates formation of the thiazolidine [8]. From this result, analysis of alternative reactions occurring with chemically and isotopically modified substrates, and X-ray crystal structures of numerous enzyme forms, the mechanism shown in Figure 1B was formulated [9]. By direct analogy to mechanisms previously put forth for related enzymes, a ferryl complex was proposed to cleave the D-valine C-H bond. This proposal required that the $C_{\beta,Cys}$-H cleavage be effected by an intermediate at the same oxidation state as the initial Fe(II)-O_2 adduct, an $\{FeOO\}^8$ species in the Enemark-Feltham notation [10]. Computational studies suggest that this complex should have an Fe^{III}-O_2^- (ferric superoxide) electronic structure **I** [11,12]. However, neither this intermediate nor the presumptive ferryl complex **IV** has been directly characterized yet, an important remaining goal for the field.

The IPNS reaction and proposed mechanism exemplify at least three functional themes exhibited by these pre-ferryl-utilizing non-heme-iron oxidases and oxygenases. First, the reaction is a four-electron oxidation of the substrate by a single equivalent of dioxygen [8], a redox economy shared by several other members, including *myo*-inositol oxygenase (MIOX) [13], 2-hydroxyethylphosphonate dioxygenase (HEPD) [14] and one of the two reactions catalyzed by the enzyme CloR [15] (see below). Second, a heteroatom (for IPNS, the L-cysteine sulfur) on the oxidation target is coordinated by the iron cofactor [16] (which acts as Lewis acid) thereby facilitating abstraction of a hydrogen from the adjacent carbon by the $\{FeOO\}^8$ complex [17]. Third, subsequent to this hydrogen abstraction, another electron is harvested from the substrate (generating the thiocarbonyl in **III**,Figure 1B, in the case of IPNS) to permit progression to a ferryl complex. Either its distinct geometry or motion of the substrate relative to the cofactor (or both) permits the ferryl complex to target a different position of the substrate (the D-valine methine) for oxidation. Although, for IPNS, the mode of reactivity is the same for both intermediates (hydrogen abstraction), it appears that fundamentally different modes of reactivity for the pre-ferryl and ferryl intermediates are utilized in other members (e.g. HEPD; see below). The ability to wield two oxidizing intermediates to target different positions for distinct oxidation outcomes endows the pre-ferryl-utilizing enzymes with even greater catalytic versatility than the more conventional, exclusively ferryl-utilizing oxygenases and oxidases. It also leads to a bewildering array of alternative reactions on chemically or isotopically modified substrates. For example, in IPNS, substitution of the D-valine moiety of ACV with D-α-hydroxyisovalerate (converting amide to ester) reroutes the second two-electron oxidation to the L-cysteine β-carbon, resulting in production of the acyclic thiocarboxylate derivative [18] (Figure 1C). Similarly, the ACV analogue with 3,3-$[^2H]_2$-D-aminobutyrate in place of D-valine is converted largely to the corresponding cepham (containing a six-membered ring), presumably because a large intramolecular selection effect against abstraction of deuterium from the 3-methylene redirects the ferryl intermediate to the protium-containing 4-methyl (Figure 1C) [8]. Such product analyses of reactions with altered substrates can be extremely informative (and have been in the case of IPNS [8]), but, particularly for this class of enzymes, must be interpreted with caution. The flexibility required to target multiple positions, in some cases for different outcomes, makes the point of mechanistic divergence of the altered reaction from the natural reaction ambiguous. In other words, the reaction with the substrate analog can follow a

profoundly different pathway, making inferences with respect to the natural pathway somewhat perilous (see discussion of HEPD below).

Myo-Inositol Oxygenase

The only known pathway in mammals for degradation of *myo*-inositol (cyclohexan-1,2,3,5/4,6-*hexa*-ol, MI, Figure 2A), the carbon backbone of cell-signaling inositol (poly)phosphates, begins with its oxidation to D-glucuronate catalyzed by *myo*-inositol oxygenase (MIOX) [13]. MIOX is a unique non-heme diiron monooxygenase, structurally and mechanistically unrelated to the well-studied bacterial multicomponent monooxygenases (e.g., soluble methane monooxygenase [19]), which activates MI and O_2 at a mixed-valent ($Fe_2^{II/III}$) diiron cluster. Mechanistic and structural studies have suggested that the Fe^{III} site coordinates the substrate via its 1 and 6 hydroxyl groups and that the Fe^{II} site activates O_2 [13]. An intermediate, designated **G** and assigned on the basis of its EPR-spectroscopic properties as a superoxo-$Fe_2^{III/III}$ complex (Figure 2B), accumulates to ~ 0.5 equiv only when abstraction of hydrogen from C1 is slowed by deuterium substitution [20]. The deuterium kinetic isotope effect on its decay establishes that **G**, or a complex with which **G** reversibly interconverts, cleaves the C1-H/D bond. To date, MIOX remains the only enzyme for which a pre-ferryl intermediate has been directly detected and shown to abstract hydrogen [20]. Similarly direct evidence should be (and is being) sought for the other systems.

Events subsequent to hydrogen atom abstraction from C1 leading to formation of D-glucuronate are still largely unknown. Pathways involving transfer of either a hydroxyl radical (pathway A, Figure 2B) or hydroperoxyl radical (pathway B) from the hydroperoxo-$Fe_2^{III/III}$ complex to C1 of the *myo*-inositol radical have been envisaged [13]. The proposed hydroxylation step would produce a ferryl-like $Fe_2^{III/IV}$ state of the cofactor (pathway A), which would act as electrophile toward the formal carbanion formed during C1-C6 cleavage. As discussed below, this pathway has some analogy (albeit imperfect) to one of the two mechanisms proposed for HEPD. In pathway B, the hydroperoxide **I** is set up for β-scission to provide the product. A third pathway not previously discussed would involve a second molecule of O_2 (the first reacting with the cofactor to form **G**) adding to the C1 radical (Figure 2C), analogous to the oxygenation step in lipoxygenases [21]. Hydrogen transfer from the cofactor-bound hydroperoxide to the substrate-bound peroxyl radical would again give hydroperoxide **I** prepared to undergo β-scission. The reversibility of O_2 addition to the cofactor in the formation of **G** [20] creates a major challenge for testing the idea that O_2 could be involved both as cofactor (in mediating the initial 1-H abstraction) and as co-substrate (adding to the C1 radical).

MIOX largely conforms to the functional themes outlined above for IPNS. The reaction is a four-electron oxidation by a single equivalent of dioxygen. As with IPNS, this redox economy is made possible by obviating reductive cleavage of O_2 prior to hydrogen abstraction, the modus operandi of the ferryl-utilizing enzymes. In addition, coordination of the hydroxyl at C1 to the Fe^{III} site almost certainly leads to deprotonation, activating the C1-H bond for cleavage [17]. Whether extraction of a second electron from the formal ketyl radical allows progression to a high-valent, ferryl-like state (pathway A), analogous to the mechanisms proposed above for IPNS and below for HEPD, remains an open question.

2-Hydroxyethyl Phosphonate Dioxygenase

During the biosynthesis of phosphinothricin, the active ingredient in various herbicides that have found widespread use in combination with transgenic crops, 2-hydroxyethyl phosphonate dioxygenase (HEPD) converts 2-hydroxyethyl phosphonate (2-HEP) to hydroxymethylphosphonate (HMP) (Figure 3A). As discussed for IPNS and MIOX, HEPD

does not require external reductants or cofactors: all four electrons required for reduction of O_2 are provided by 2-HEP. During the transformation, the excised carbon is converted to formate and the hydrogens on C1 of HEP are retained in HMP [14]. Experiments using $^{18}O_2$ demonstrated that both HMP and formate contained label, but surprisingly, the label in HMP was sub-stoichiometric indicating exchange with solvent during catalysis. In support of this hypothesis, conducting the reaction in $H_2{}^{18}O$ also resulted in incorporation of ^{18}O into HMP [14].

The structure of a Cd^{2+}-substituted form of HEPD was recently solved [14] and is remarkably similar to the previously reported structure of 2-hydroxypropylphosphonate epoxidase, HppE, which is discussed below [22]. The overall fold of HEPD consists of tandem repeats of a bi-domain architecture with each repeat consisting of an all α-helical domain linked to a β-barrel fold that is characteristic of the cupin superfamily. The amino acid metal ligands, His129, Glu176, and His182, make up the usual "facial triad" characteristic of the enzyme family [23], but the spacing between the first two metal ligands is unusually long: ($HX_{46}E$) in HEPD, compared to ($HX_{1–4}E/D$) in other facial triad enzymes [24], including the structurally similar HppE. The substrate, 2-HEP, coordinates to the Cd^{2+} via its C2 hydroxyl and a phosphonate oxygen. Similarly to the situation for IPNS and MIOX, C2-O coordination and resultant deprotonation should activate the substrate for abstraction of a hydrogen from C2 [17], a necessary step in its conversion to formate; this coordination may also activate the metal for reaction with O_2.

Two mechanisms have been proposed for the HEPD reaction based on the X-ray structure and isotope labeling studies [14]. After bidentate binding of 2-HEP to Fe(II), O_2 addition would result in the $\{FeOO\}^8$ species, most likely a superoxo-Fe^{III} intermediate (**I**,Figure 3B). **I** may abstract a hydrogen atom from C2 of HEP to generate intermediate **II**, similar to the mechanisms discussed above for isopenicillin N synthase (IPNS) and MIOX [9,25]. Two different pathways can then be envisioned to complete catalysis and account for the labeling studies. The substrate radical could attack the hydroperoxide generating a ferryl intermediate and a hemiacetal (Figure 3B). The latter can undergo a retro-Claisen type C-C bond scission with the incipient negative charge on C1 attacking the electrophilic ferryl, either in concerted or stepwise fashion with a carbanion intermediate stabilized by metal coordination as drawn. Such C-C bond cleavage is unprecedented, but the intermediacy of an Fe(IV)-oxo species explains the observed exchange with solvent.

In the second mechanism the substrate radical is converted to an alkyl hydroperoxide, either by attack of the radical directly on the hydroperoxo-Fe^{III} complex, or alternatively by reaction of the alkyl radical with a second molecule of O_2 and hydrogen atom transfer between the resulting alkylperoxy radical and the hydroperoxo-Fe^{III} complex (as suggested above in pathway C for the MIOX reaction). Regardless of the details of the formation of the alkylhydroperoxide, this intermediate could undergo a Criegee-type rearrangement to yield the formate ester of HMP. Hydrolysis of this ester would normally be expected to take place by attack at the carbonyl carbon, but this would not account for the incorporation of oxygen derived from solvent into HMP. The solvent exchange can be explained if hydrolysis were to take place at C1 as shown in Figure 3B.

Experiments with substrate analogs provided support for the hydroperoxylation mechanism (Figure 3C). Incubation of HEPD with O_2 and 1-hydroxyethylphosphonate (1-HEP) resulted in the formation of acetylphosphate [26], the direct product of a precedented Criegee rearrangement in which the phosphorus atom migrates [27] (Figure 3C). It is difficult to envision a mechanism involving hydroxylation that would result in the formation of this product. To provide further support for the hydroperoxylation mechanism, 2-HEP stereospecifically deuterium-labeled at C1 was prepared. The hydroperoxylation mechanism

in Figure 3B predicts retention at C1 during the Criegee rearrangement but inversion during the hydrolysis at C1. Surprisingly, however, the experimental outcome showed racemization at C1, which cannot be rationalized by the mechanisms in Figure 3B and requires further experimentation [28]. Possibly, the mechanisms for 2-HEP and 1-HEP are different with hydroperoxylation for the latter but not the natural substrate [29]. If so, it would be yet another demonstration of the plasticity of this type of enzyme. Also in keeping with the other enzymes discussed here, use of substrate analogs result in very different types of chemistry (Figure 3C).

CloR

The bifunctional non-heme iron oxygenase CloR catalyzes two consecutive oxidative decarboxylations during the biosynthesis of the aminocoumarin antibiotic clorobiocin [15]. The enzyme first converts 3-dimethylallyl-4-hydroxyphenylpyruvate (3-DMA-4-HPP) into 3-dimethylallyl-4-hydroxymandelic acid (3-DMA-4-HMA), and subsequently oxidizes the latter product to 3-dimethylallyl-4-hydroxybenzoate (3-DMA-4-HB) (Figure 4A). As with most of the other enzymes discussed in this review, CloR does not have any sequence similarity with known oxygenases. Instead the protein shows similarity to class II aldolases. The oxidative decarboxylations catalyzed by CloR do not require α-ketoglutarate, but the assays were performed with excess Fe^{II} and ascorbate, which may have provided additional electrons. Experiments with $^{18}O_2$ demonstrated incorporation of the two oxygen atoms into 3-DMA-4-HMA with one oxygen residing in the carboxyl moiety and one in the new hydroxyl group. Conversely, only one oxygen in 3-DMA-4HB is derived from O_2 when unlabeled 3-DMA-4-HMA was incubated with CloR and $^{18}O_2$ (Figure 4A) [15]. Collectively, these studies suggest that the substrates for CloR have the roles of α-ketoacid co-substrate and hydroxylation substrate combined in a single compound. A proposed mechanism for the transformation of 3-DMA-4-HMA to 3-DMA-4-HB is shown in Figure 4B [15,30]. The first few steps, in which the superoxo-Fe^{III} pre-ferryl complex oxidizes the α-hydroxyl moiety to a carbonyl and the resulting hydroperoxo-Fe^{II} complex **I** then attacks the carbonyl as nucleophile, are strikingly analogous to the mechanism proposed by Baldwin and co-workers for thiocarboxylate formation in the IPNS reaction with the AC-$_D$-α-hydroxyisovaleric acid substrate analogue [31] (Figure 1C). The energetic accessibility of this pathway was recently supported by DFT calculations [32]. Moreover, recent model studies have succeeded in reproducing such oxidative decarboxylation of an α-hydroxy-carboxylate substrate by an Fe(II) catalyst in the presence of O_2 [30]. Mechanistic studies supported the catalytic cycle shown in Figure 4B. A hypothetical mechanism for the first reaction catalyzed by CloR based on the understanding of α-ketoglutarate dependent hydroxylations [33–36] is shown in Figure 4C.

2-Hydroxypropylphosphonate Epoxidase

In the last step of the biosynthesis of fosfomycin, an FDA-approved drug for the treatment of uncomplicated acute cystitis (urinary tract infections), (*S*)-2-hydroxypropylphosphonate (2-HPP) is converted to the mature antibiotic (Figure 5A). Feeding studies with isotopically labeled precursors demonstrated that the epoxide oxygen is not derived from molecular oxygen but from the hydroxyl group in 2-HPP [37]. These studies suggested an unprecedented mechanism of epoxide formation involving dehydrogenation of a secondary alcohol (Figure 5A). Liu and coworkers reconstituted the activity of the HPP epoxidase (HppE) in vitro [38] and showed that the enzyme requires Fe(II), O_2, an electron carrier (either a general reductase or catalytic amounts of FMN) and NADH as reductant, but not α-ketoglutarate. Subsequent spectroscopic, mechanistic, and structural investigations established that the enzyme is a mononuclear non-heme-iron-dependent oxidase [22,39–41].

A series of crystal structures of the enzyme have provided a solid foundation for mechanistic analysis. As noted above, HppE is a member of the cupin superfamily with its characteristic jelly roll domain that contains the typical 2 His/1 Glu facial triad iron ligand set [22]. A structurally unique smaller domain made up of 5 short α-helices completes the structure. The mode of substrate binding in these structures varies from monodentate coordination of 2-HPP through one of the phosphonate oxygens to bidentate ligation of both the phosphonate and hydroxyl groups. The latter binding mode is associated with a more closed conformation that appears to shield the active site from solvent and may help protect reactive intermediates. Further support for productive bidentate HPP binding has been provided by EPR studies with ^{17}O-labeled substrate and using NO as mimic of oxygen [41]. The bidentate binding of HPP resembles the binding geometry of α-ketoglutarate (α-KG) in other members of the cupin family [42,43], and analogously the bidentate binding by HPP may serve to facilitate the normally unfavorable reaction of ferrous iron with molecular oxygen [22].

The two most likely mechanisms for the HppE reaction are shown in Figure 5B [41]. They differ in the timing of the entry of the two electrons required for the overall reaction, and therefore in whether either a (pre-ferryl) Fe(III)-superoxide complex (**I**) or a ferryl complex (**II**) abstracts the hydrogen atom from C1 to permit epoxide formation; alternatively, this removal of the hydrogen at C1 may occur via proton-coupled electron transfer (not shown). In both mechanisms, the resulting substrate radical at C1 subsequently carries out an unprecedented intramolecular rebound-like reaction. Recent kinetic isotope effects studies on $k_{cat}/K_m(O_2)$ using $^{16}O_2$ or $^{18}O_2$ suggest the formation of an Fe^{III}–OOH species in the rate-limiting step of O_2 activation, either by hydrogen atom abstraction or H^+-coupled ET from the substrate or by electron transfer from the presumptive reductase coupled to local proton transfer [44]. The former possibility is, from the perspective of this review, quite provocative, as it would include HppE among the small group of pre-ferryl-utilizing oxygenases and oxidases, despite the fact that it deviates from two of the general themes to which the other enzymes conform. First, the substrate lacks a coordinating heteroatom α to the carbon targeted for H-abstraction. Thus, it is unclear whether the C1-H bond can be sufficiently activated to make abstraction by a pre-ferryl complex (**I**) thermodynamically feasible. Second, the reaction is only a two-electron oxidation, requiring two exogenous electrons. A conventional pathway to an H-abstracting ferryl complex (**II**), involving sequential delivery of two electrons first to the initial Fe^{III}-O_2^- adduct and then to the resulting Fe^{III}–OO(H) complex by the reductase (Figure 5B), seems completely plausible. It is possible that the unique rebound-like reaction, in which the epoxide forms by formal transfer of a coordinated alkoxyl radical to the substrate radical, precludes such a conventional ferryl mechanism. Perhaps the alternative, pre-ferryl pathway, in which the alkoxyl radical transfer occurs from the ferryl rather than the ferric state of the cofactor, provides a lower, more surmountable activation barrier to the strained ring. Alternatively, it is possible that the need to avoid a hydroxylation outcome by the conventional hydroxyl-radical rebound provides the rationale for use of the pre-ferryl pathway. These intriguing mechanistic issues make resolution of the pathway in HppE an important goal for the field. Transient state kinetic experiments may be capable of distinguishing the two main possibilities by resolving the timing of electron delivery and hydrogen abstraction. However, the fact that neither the presumptive reductase protein nor a facile chemical reductant has been identified has, to date, thwarted these efforts. Similar to the observations with IPNS and HEPD, the use of substrate analogs results in various types of diverted chemistry [39,41] (Figure 5C).

Summary and outlook

The ability of this small set of non-heme-iron oxygenases and oxidases to use "pre-ferryl" complexes to abstract hydrogen from their substrates allows them to obtain the electrons needed to cleave the O-O bond of O_2, providing the thermodynamic driving force for their difficult oxidation reactions, while retaining all four oxidizing equivalents for conferral to their substrates. Their ability to target two adjacent or distal positions of the substrate with the pre-ferryl and subsequent ferryl complexes, often for fundamentally different types of oxidation, confers remarkable catalytic versatility that undoubtedly includes reaction types yet to be discovered. It appears that the key underlying chemical principle permitting this novel mode of O_2 and C-H activation is heteroatom coordination and deprotonation to activate the substrate C-H bond and probably also tune the cofactor for facile reaction with O_2 (Figure 6). In many cases, a second group on the substrate (e.g., hydroxyl or phosphonate oxygen) also coordinates, possibly to anchor the crucial heteroatom to the metal and ensure its deprotonation. Whether some enzymes are capable of using pre-ferryl complexes for H-abstraction without this means of substrate activation (e.g., HppE) remains to be determined. An important objective for the immediate future, to date achieved only for MIOX, is the detection and characterization of the proposed pre-ferryl complexes and direct demonstration that they are, in fact, the H-abstracting intermediates.

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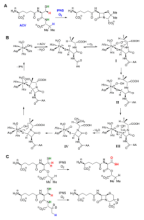

Figure 1.
(A) Reaction catalyzed by IPNS in the formation of the penicillin nucleus. (B) Mechanism proposed by Baldwin and co-workers for the IPNS reaction [9]. (C) Aberrant reactions occurring with ACV derivatives containing either D-α-hydroxyisovalerate [18] or 3,3-$[^2H]_2$-D-aminobutyrate in place of D-valine [8].

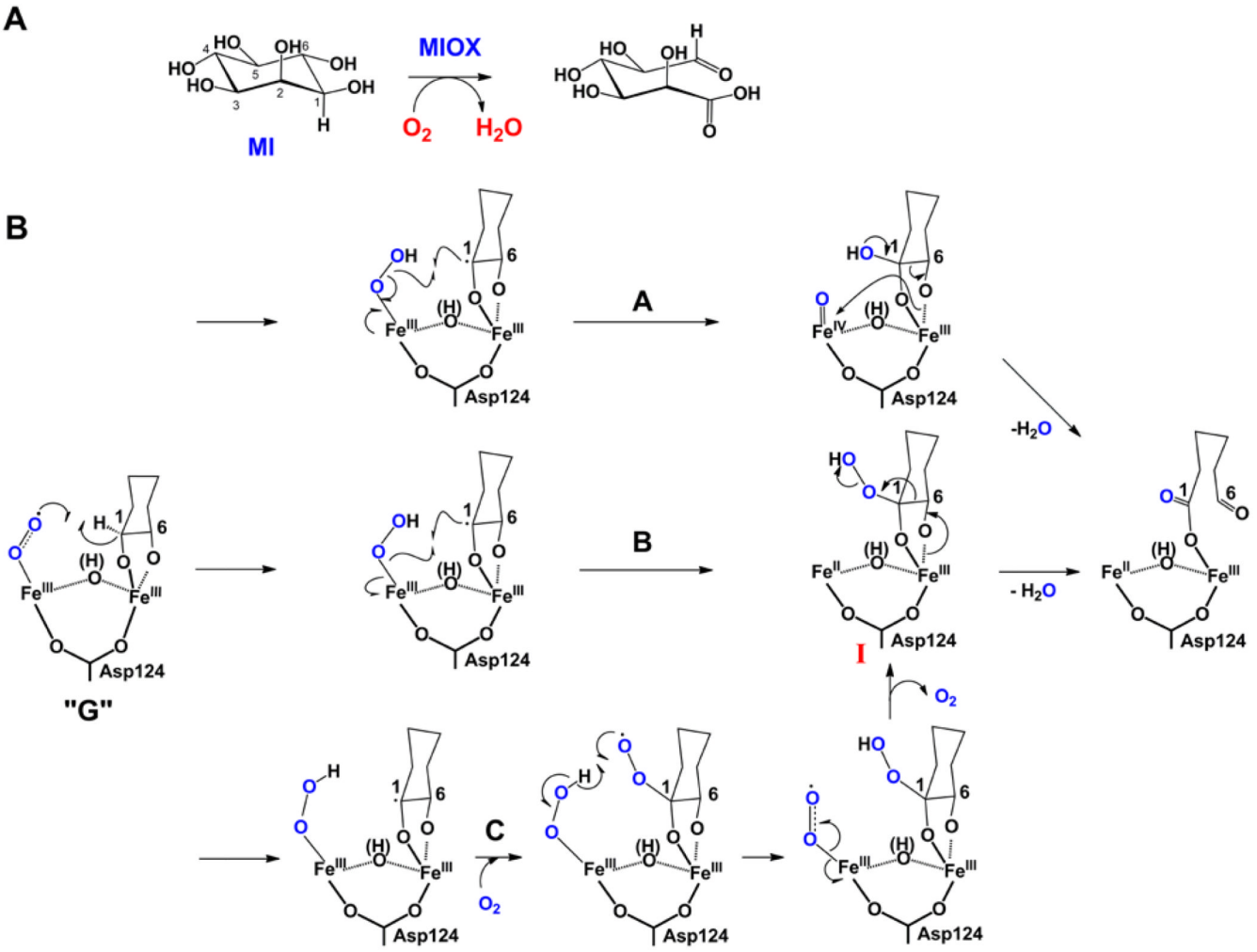

Figure 2.

(A) Reaction catalyzed by MIOX. B) Possible mechanisms for conversion of the superoxo-$Fe_2^{III/III}$ complex, **G**, to the D-glucuronate product complex involving C1 hydroxylation coupled to peroxide O-O cleavage (pathway A), C1 hydroperoxylation by peroxyl-radical rebound (pathway B), or C1 hydroperoxylation by addition of a second molecule of O_2 to the C1 radical (pathway C). In pathway A, C-C bond cleavage with the electrons of the incipient anion directly attacking the ferryl complex is also feasible, but this would lead to incorporation of the ferryl oxygen into the resulting hydrated aldehyde. Oxygen labeling experiments did not detect any oxygen derived from O_2 in the aldehyde [45], requiring that for this mechanism to hold, either the oxygen of the ferryl intermediate underwent complete exchange, or the conversion of the initial hydrated product to the aldehyde occurred stereospecific and enzyme catalyzed.

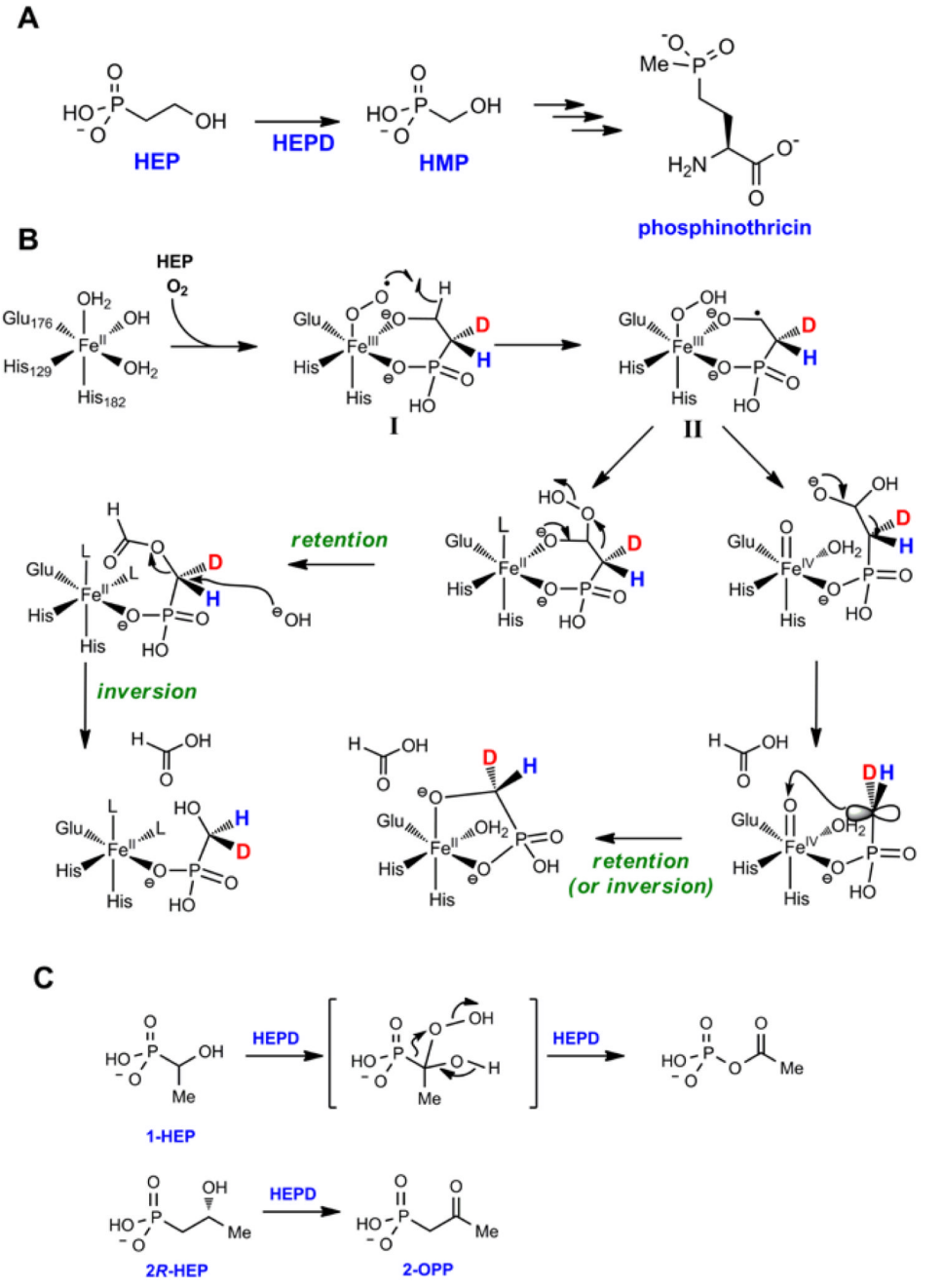

Figure 3.

(A) Reaction catalyzed by HEPD in the biosynthesis of phosphinothricin. (B) Two proposed mechanisms for the conversion of 2-HEP to HMP by HEPD. The intermediates are shown with monoprotonated phosphonates but they may be fully deprotonated. The predicted stereochemical outcome at C1 is also shown. The retro-Claisen-like reaction could occur with retention of inversion. (C) Conversion of substrate analogs to various products by HEPD.

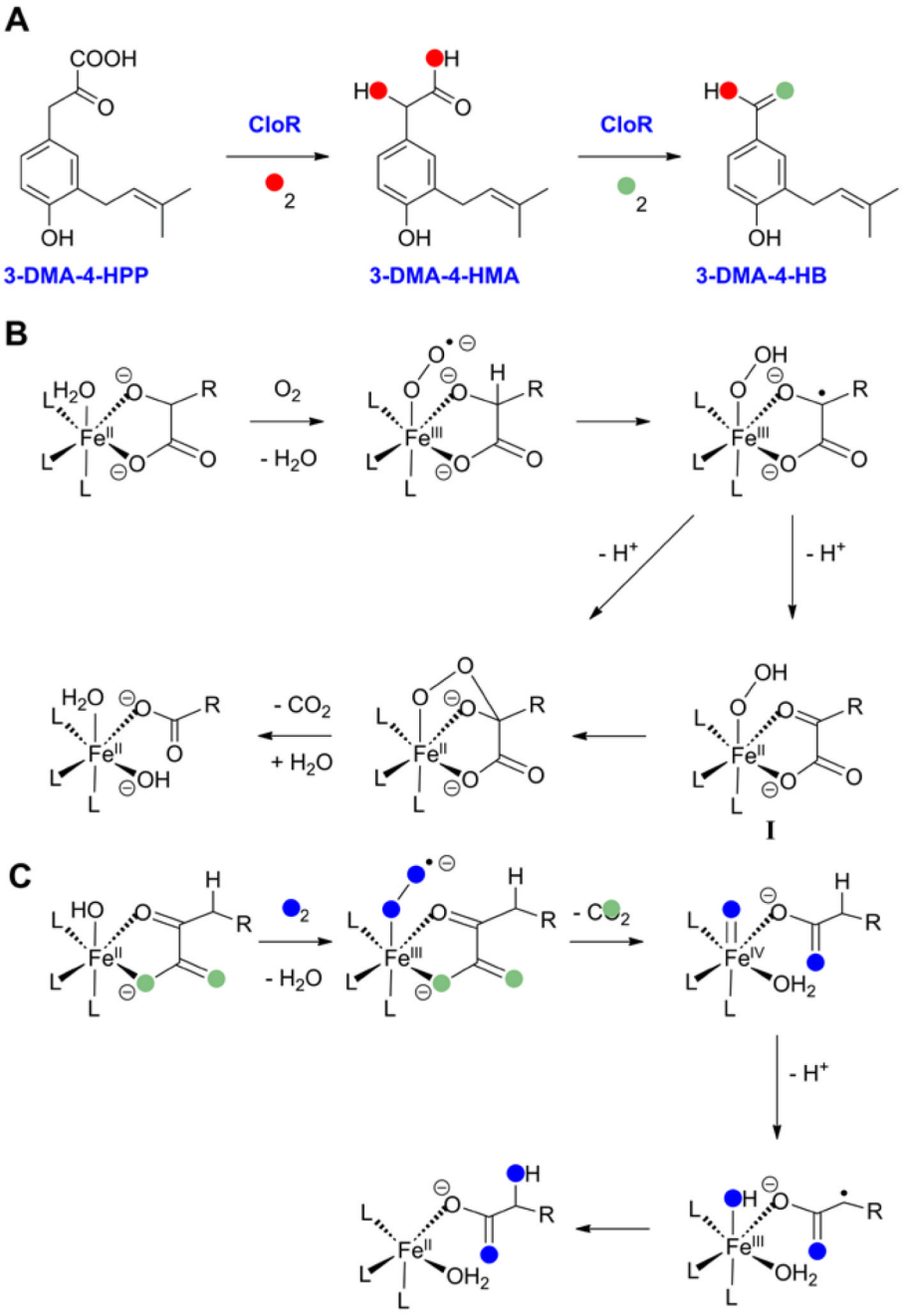

Figure 4.

(A) Reactions catalyzed by CloR. The results of oxygen labeling studies are indicated. (B) Proposed mechanism for the second reaction catalyzed by CloR. (C) Proposed mechanism for the first reaction catalyzed by CloR.

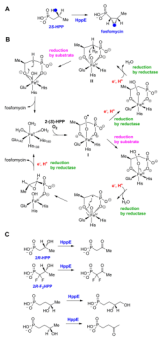

Figure 5.
(A) Reaction catalyzed by HppE illustrating retention of the hydroxyl oxygen in the epoxide of fosfomycin. (B) Two proposed mechanisms for the conversion of 2*S*-HPP to fosfomycin. (C) Conversion of substrate analogs to various products by HppE.

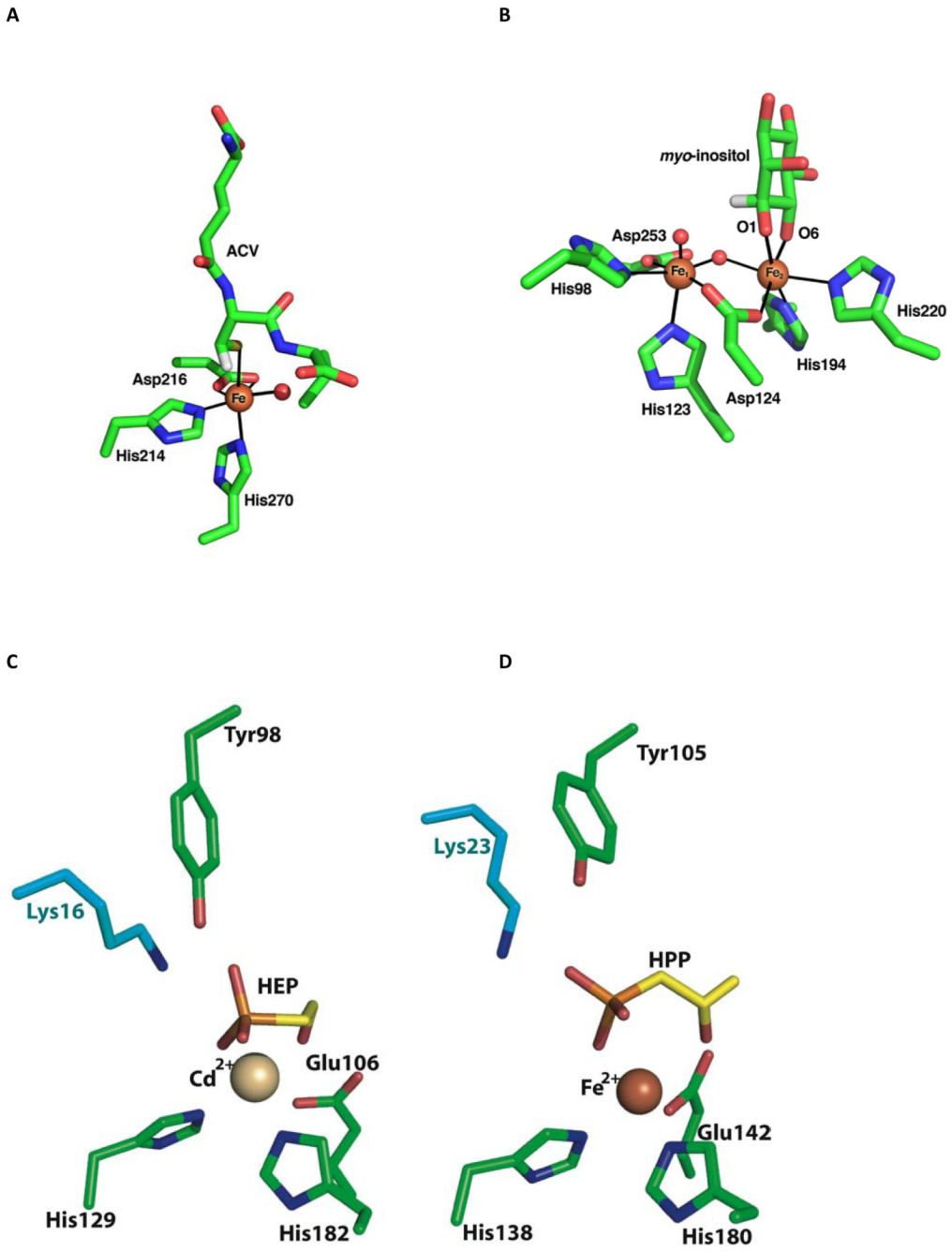

Figure 6.
Active sites of co-crystal structures of (A) IPNS, (B) MIOX, (C) HEPD, and (D) HppE with their substrates.