MECHANISTIC STUDIES ON THREE ORGANOPHOSPHONATE-PROCESSING ENZYMES, HppE, HEPD AND MPnS

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ABSTRACT

Naturally occurring phosphonates and phosphinates have bioactivities (e.g., herbicidal, antibiotic) that are useful in agriculture and medicine. Phosphonate and phosphinate compounds can potently inhibit enzymes in various metabolic pathways by functioning as stable mimics of phosphate esters and carboxylic acids. Biosynthetic pathways to phosphonate and phosphinate compounds have proven to be treasure troves for the discovery of unusual enzymatic reactions. The investigation of these conserved pathways has revealed three unprecedented biochemical steps catalyzed by the non-heme-iron(II) enzymes, HppE [(S)-2-hydroxypropyl-1-phosphonate epoxidase], HEPD (2-hydroxyethylphosphonate dioxygenase) and MPnS (methylphosphonate synthase). The work described herein focused on understanding both the mechanisms of the individual reactions and the structural/functional features of each enzyme important in specifying its reaction and pathway.

The iron-dependent epoxidase, HppE, converts (S)-2-hydroxypropyl-1-phosphonate (S-HPP) to the antibiotic, fosfomycin [(1R, 2S)-epoxypropylphosphonate], in an unusual 1,3-dehydrogenation of a secondary alcohol to an epoxide. HppE had been classified as an oxidase, with proposed mechanisms differing primarily in the identity of the O_2-derived iron complex that abstracts hydrogen (H•) from C1 of S-HPP to initiate epoxide ring closure. In my work, we showed that the preferred co-substrate is actually H_2O_2 and that HppE therefore almost certainly employs an iron(IV)-oxo complex as the H• abstractor. Reaction with H_2O_2 is accelerated by bound substrate and produces fosfomycin catalytically with a stoichiometry of unity. The ability of catalase to suppress the HppE activity previously attributed to its direct utilization of O_2 showed that reduction of O_2 and utilization of the resultant H_2O_2 were actually operant.

The mechanism of the conversion of 2-hydroxyethylphosphonate to hydroxymethylphosphonate (2-HEP) catalyzed by iron-dependent enzyme, HEPD during the
biosynthesis of the commercial herbicide, phosphinothricin, had been enigmatic. By using rapid-kinetic and spectroscopic methods, we detected an iron(IV)-oxo (ferryl) intermediate in the HEPD reaction. Kinetic analysis suggested that the intermediate is kinetically competent to be on the productive pathway. The accumulation of this intermediate only with substrate having deuterium in the abstracted pro-S position of C2 of 2-HEP implied that the ferryl intermediate abstracts this hydrogen, but the increased accumulation of the ferryl complex in $^2$H$_2$O solvent implied that the hydrogen becomes solvent-exchangeable before the ferryl abstracts it. To account for these unanticipated results, a mechanism involving initial abstraction of the pro-S hydrogen by an Fe(III)-superoxo precursor to the ferryl complex, transfer of a hydroxyl group containing the originally abstracted hydrogen to C2 concomitant with formation of the ferryl complex, and an unprecedented abstraction of H• from the newly installed C2 OH group by the ferryl complex was proposed.

Like HEPD, the iron-dependent oxygenase, MPnS, also catalyzes the 4e-oxidative C-C cleavage of 2-HEP, but generates different products, methylphosphonate and CO$_2$. MPnS, HEPD, and HppE have quite striking structural similarity and utilize identical or similar phosphonate substrates, but they employ different oxidants, O$_2$ or H$_2$O$_2$, to effect three completely different reactions. By using H$_2$O$_2$, HppE effects a 2e-oxidation (epoxide installing 1,3-dehydrogenation) of S-HPP. HEPD and MPnS catalyze distinct 4e-oxidative C1-C2-cleaving transformations of 2-HEP, in each case with O$_2$ as the oxidant. We explored the distinct but potentially overlapping catalytic capabilities of the three enzymes on the two different phosphonate substrates with the two different oxidants. As expected from its reassignment as a peroxidase, HppE fails to catalyze a 4e-oxidative C-C cleavage reaction with O$_2$ as oxidant. However, we found that both MPnS and HEPD can catalyze the 2e dehydrogenation of 2-HEP with H$_2$O$_2$ rather than O$_2$ as the oxidant. HEPD catalyzed only a fraction of a turnover under the conditions examined, consistent
with the fact that it is an oxygenase. By contrast, MPnS, also a known oxygenase, surprisingly catalyzed up to 25 turnovers of 2-HEP to the corresponding aldehyde with $\text{H}_2\text{O}_2$ as the oxidizing co-substrate. The physiological relevance of this activity is unknown. In sum, all three enzymes possess some peroxidase activity, with HppE being by far the most efficient, but only HEPD and MPnS exhibit the 4e⁻-oxidative C-C-cleavage activity.
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<td>(1R, 2S)-3</td>
<td>(1R, 2S)-1-hydroxyl-2-aminopropyl phosphonate</td>
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Chapter 1 Introduction to Phosphonate-Processing Enzymes, HppE, HEPD and MPnS

1.1 Phosphonate and phosphinate natural products and their biosynthetic pathways

Phosphonate and phosphinate natural products, an underexploited group of bioactive compounds possessing one or two carbon-phosphorous bonds, have been attractive because they are stable mimics of phosphate esters and carboxylic acids (1-2). Thus, phosphonate and phosphinate compounds can potently inhibit enzymes in various metabolic pathways. Although, phosphonate and phosphinate possess only one or two C-P bonds, the C-P bond is more resistant to chemical hydrolysis, thermal decomposition, enzymatic degradation, and photolysis than congeneric compounds containing an O-P linkage (1). Therefore, phosphonate and phosphinate display diverse applications in medicine and agriculture as antibiotics, insecticides, herbicides and fungicides by mimicking phosphate esters and carboxylic acids (1). The phosphonate and phosphinate natural products involving in this study include fosfomycin [(1R, 2S)-epoxypropylphosphonate], an antibiotic, phosphinothricin (PT), the active component in commercial herbicides, and methylphosphonate (MP), the precursor of methane in the catabolism of methane in the aerobic ocean. In spite of the diversity of natural phosphonate and phosphinate compounds, their biosynthetic pathways are well conserved. The investigation of these pathways has revealed three unprecedented biochemical steps catalyzed by non-heme-iron(II) enzymes. These enzymes include HppE [(2S)-hydroxypropylphosphonic acid epoxidase], HEPD (2-
hydroxyethylphosphonate dioxygenase) and MPnS (methylphosphonate synthase). HppE effects the epoxidation of (2S)-hydroxypropylphosphonate (S-HPP) to yield fosfomycin (3). HEPD catalyzes the C-C bond cleavage of 2-hydroxyethylphosphonate (2-HEP) to produce formate and hydroxymethylphosphonate (HMP), and such a C-C cleavage step is involved in the biosynthesis of PT (4). MPnS effects the C-C bond cleavage of 2-HEP to generate MP and CO₂ (5). This dissertation will focus on the mechanistic study of these three phosphonate-processing non-heme-iron-dependent enzymes, HppE, HEPD and MPnS.

1.1.1 Fosfomycin, a clinically useful antibiotic, and its biosynthesis

Fosfomycin, a natural phosphonate compound, is an antibiotic produced by Pseudomonas and Streptomyces species, showing a powerful antimicrobial activity against a wide range of enteric Gram-negative bacteria and Gram-positive cocci. Since its approval by the FDA in 1996, fosfomycin (Monural®) has been widely used for the treatment of lower urinary tract infections in US (6). In clinic, fosfomycin has long been used in certain countries for the treatment of soft tissue and diabetic foot infection, central nervous system infection and gastrointestinal infection (7). Furthermore, it was demonstrated that fosfomycin is a powerful bactericidal activity against antibiotic-resistant Enterbacteriaceae isolates, methicillin-resistant and vancomycin-resistant strains of Staphylococcus aureus (8). The epoxide ring of fosfomycin is installed by a non-heme-iron(II) dependent enzyme, HppE, in the last step of the biosynthetic pathway of fosfomycin. The increasing microbial resistance to antibiotics has drawn more attention to the research of this old antibiotic, fosfomycin (7, 9).
1.1.1.1 The antibiotic function of fosfomycin

Since Alexander Fleming discovered the first antibacterial compound, penicillin, from the mold *Penicillium notatum* in 1928, numerous antibiotics have been identified and been extensively used in the treatment of various bacterial and fungal infections. The mechanisms of action of most antibiotics include inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleotide synthesis, and/or inhibition of metabolism (10). Fosfomycin functions by inhibiting cell wall synthesis by targeting at essential enzyme, uridine diphosphate (UDP)-N-acetylglucosamine enolpyruvyl transferase (MurA), involved in this process (11).

The biosynthesis of bacterial cell wall includes the synthesis of a crucial component of both Gram positive and Gram negative bacterial cell wall, peptidoglycan. Peptidoglycan is a polymer consisting of polysaccharides and polypeptides and forms a mesh-like layer outside of the bacterial plasma membrane. The polysaccharide chains comprise of alternating residues of β-(1,4) linked N-acetylglucosamine (NAG) and N-acetlymuramic acid (NAM). These polysaccharide chains are linked to the polypeptides by an enolpyruvate ether linker at the C3 position of UDP-N-acetylmuramic acid (UDP-GlcNAc) introduced by a key enzyme named UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) (12). A subsequent NADPH (Nicotinamide Adenine Dinucleotide Phosphate)-dependent reduction reaction converts UDP-N-acetylglucosamine-enolpyruvate (UDP-GlcNAc-enolpyruvate) to UDP-GlcNAc, the material of peptidoglycan biosynthesis (Figure 1-1) (13).

MurA catalyzes the transfer of the enolpyruvate group from phosphoenolpyruvate (PEP) to the C3’-OH of UDP-GlcNAc, which is the first committed step of peptidoglycan biosynthesis (12). The MurA catalysis employs an addition-elimination mechanism and processes through a tetrahedral ketal intermediate, both of which are facilitated by a general acid Cys 115 and a
general base Asp 305 in the active site (Figure 1-2 A) \((11, 14-15)\). MurA is conserved among Gram positive and Gram negative bacteria and its deletion leads to a detrimental effect on bacteria. The antibiotic function of fosfomycin is due to the inactivation of the key enzyme, MurA. The chemical "warhead" of fosfomycin is its strained epoxide ring, which is attacked by the active-site Cys 115, and the attack results in an irreversible covalent alkylation of Cys115 and the formation of a covalent phospholactyl-enzyme adduct derived from substrate PEP (Figure 1-2B) \((11)\).
Figure 1-1. The biosynthetic pathways of UDP-\(N\)-acetylglucosamine (UDP-GlcNAc) and UDP-\(N\)-acetylglucosamuramic acid in bacteria. Adapted and modified from ref. (13). Fructose-6-P denotes fructose-6-phosphate. Glucose-1-P denotes glucose-1-phosphate. \(N\)-acetylglucose-6-P denotes \(N\)-acetylglucose-6-phosphate. \(N\)-acetylglucose-1-P denotes \(N\)-acetylglucose-1-phosphate.
Figure 1-2. (A) The mechanism of MurA catalysis; (B) the mechanism of the inhibition of MurA by fosfomycin. Adapted and modified from ref. (13).
1.1.1.2 The biosynthetic pathways of fosfomycin

Previous studies have suggested that various *Streptomyces* and *Pseudomonas* strains, including *Streptomyces fradiae*, *Streptomyces wedmorensis*, *Streptomyces viridochromogenes*, *Pseudomonas syringae*, *Pseudomonas fluorescens* and *Pseudomonas viridiflava*, are able to produce fosfomycin (16). Since 1990s, the studies in the Seto laboratory using the combination of molecular biology techniques and feeding experiment with isotopically labeled precursors, have led to a proposed fosfomycin biosynthetic pathway in *S. fradiae* and *S. wedmorensis* (Figure 1-3A) (17-21). Later, in 2012, a study in van der Donk group suggested an alternative fosfomycin biosynthesis process in *P. syringae* (Figure 1-3B) (16). The enzymes involved in the two different fosfomycin biosynthetic pathways will be discussed in detail in the following section.
Figure 1-3. (A) Proposed biosynthetic pathway of fosfomycin in *Streptomyces fradiae* and *Streptomyces wedmorensis*; (B) proposed biosynthetic pathway of fosfomycin in *Pseudomonas syringae*. The steps, which have been confirmed by experimental data with isolated enzymes, are shown with solid arrows, whereas putative conversions are shown with dashed arrows. MeCbl denotes methylcobalamin. All the abbreviations refer to the list of abbreviations. Adapted and modified from ref. (16).
Fosfomycin biosynthesis in S. wedmorensis

The first two steps of fosfomycin biosynthesis in S. fradiae and S. wedmorensis are catalyzed by enzymes phosphoenolpyruvate mutase (Fom1) and phosphoenolpyruvate decarboxylase (Fom2) (Figure 1-3A). These two steps are common among natural products with C-P bonds, such as phosphonopyruvic acid, bialaphos, 2-aminoethyl-phosphonic acid and 2-HEP and MP, to name a few (2, 21). The conversion of PEP to phosphonopyruvate (PnPy) catalyzed by Fom1 is not thermodynamically favored, due to the relatively higher energy of the C-P bond of PnPy compared to the O-P bond of PEP (18). Interestingly, Fom1 has also been shown to catalyze the reverse reaction from PnPy to PEP based on the characterization and activity studies on a number of isolated phosphoenolpyruvate mutases (18). It has been understood that the subsequent decarboxylation step catalyzed by Fom2 is the driving force for Fom1 to effect the thermodynamically unfavorable forward reaction. Fom2 belongs to the α-ketodecarboxylase family, employing thiamine diaphosphate and a Mg$^{2+}$ ion to catalyze the conversion from PnPy to phosphonoacetaldehyde (PnAA) (22).

The third step of fosfomycin biosynthesis is catalyzed by phosphonoacetaldehyde reductase (FomC), a member of the alcohol dehydrogenase family that requires an Fe$^{2+}$ and a NADH as cofactor (23). The ensuing methylation step involves a radical S-adenosyl-L-methionine (SAM) enzyme, Fom3. The feeding experiment with $[^{14}C]$methylcobalamin and the characterization of isolated enzyme suggested that Fom3 is responsible for transferring a methyl group from methylcobalamin (MeCbl) to 2-HEP, facilitated by a [4Fe-4S] cluster and SAM (19, 24). However, due to the relatively low activity of Fom3 in vitro, further investigation is required for an unambiguous functional assignment of Fom3.
An alternative biosynthetic pathway of fosfomycin in *P. syringae*

In 2012, van der Donk and coworkers discovered that a fosfomycin producer, *P. syringae* PB-5123, lacks the gene encoding phosphoenolpyruvate decarboxylase, which catalyzes the reaction from PnPy to PnAA in fosfomycin biosynthesis of *Streptomyces* strains (16). However, *P. syringae* PB-5123 contains a gene coding for a citrate synthase-like enzyme, Psf2, homologous to the enzymes that transfer an acetyl group to PnPy in the biosynthesis of PT and FR-900098 (16). Heterogeneous expression, purification and activity detection of Psf2 confirmed its acetyltransfer activity (16). Furthermore, the production of fosfomycin was found in *Pseudomonas aeruginosa* strain carrying the fosfomycin biosynthesis gene cluster of *P. syringae* PB-5123 (25). This finding indicated that the gene cluster is functional (16). Thus, a novel fosfomycin biosynthetic pathway was proposed (Figure 1-3B). The first and the last steps, involving the conversion of PEP to PnPy and conversion of S-HPP to fosfomycin, have been established by experimental evidence; however, other steps, including hydroxylation, decarboxylation & elimination, tautomerization, decarboxylation and reduction, have not been fully resolved.

Although *P. syringae* and *S. wedmorensis* employs different biosynthetic pathways to produce S-HPP, the two biosynthetic pathways do share the first and last steps. In the following section, I will discuss the last epoxidation step in detail.
1.1.2 Phosphinothrinicin and its biosynthetic pathway

1.1.2.1 Phosphinothrinicin, an active component of herbicide

Phosphinothrinicin (PT), a non-protein amino acid found in lots of peptide antibiotics, is the only known phosphinate natural product (Figure 1-4). PT was found as a component of a tripeptide antibiotic (PT-Ala-Ala, Phosphinothrinicin tripeptide, PTT) produced by *Streptomyces viridochromogenes* (26) and *Streptomyces hygroscopicus* (27), of phosalacine, a PT-Ala-Leu tripeptide, and of trialaphos (PT-Ala-Ala-Ala), a tetrapeptide (28). PT is a structural mimic of glutamate and a potent inhibitor of glutamine synthetase (28). The free amino acid form of PT has relatively weak antibiotic activity, but the peptide versions of PT, like PTT, exhibit a better antibacterial activity. Many organisms readily take up the peptide forms of PT and release the active component with the hydrolysis carried out by cytoplasmic peptidases. However, the antibiotic activity of PTT is easily to be counteracted by glutamine *in vivo* (28). The cytosolic glutamine synthetase plays an essential role in the control of normal plant growth and pH homeostasis in plants (29-30). Thus, as the active component in commercial herbicides (e.g. Liberty, Basta, and Ignite), PT is widely used in combination with transgenic crop, like soybean, corn, cotton and canola (31). The investigation into PT biosynthesis revealed a unique C-C bond cleaving transformation in the conversion of 2-HEP to HMP and formate. This transformation is catalyzed by a previously uncharacterized non-heme-iron(II) enzyme, HEPD (31). Chapter 2 will focus on the mechanistic study of HEPD.
Figure 1-4. The proposed biosynthetic pathway of phosphinothricin (PT). Adapted and modified from ref. (2).
1.1.2.2 Biosynthesis of Phosphinothricin

Due to the unique two-C-P bond structure and the wide applications of PT in agriculture, extensive studies of the biosynthesis of PT have been conducted (28, 32). The PTT biosynthetic pathway of *Streptomyces hygroscopicus* was mostly solved by the Seto group (30) using a combination of *in vivo* feeding experiments, *in vitro* biochemical characterization and genetics. Subsequent studies using *Streptomyces viridochromogenes* provided further evidence to the PTT biosynthesis (29, 33). The complete PTT gene clusters from *S. viridochromogenes* and that from *S. hygroscopicus* have been sequenced, and they are nearly identical (33-34). Taken together, these investigations led to a proposed biosynthesis, as shown in Figure 1-4. The proposed pathway involves the synthesis of 2-HEP with PEP mutase, PnPy decarboxylase, and PnAA reductase. These enzymes are homologous to those involved in the biosynthesis of fosfomycin (Figure 1-4). 2-HEP is then converted to HMP by a non-heme-iron(II) enzyme, HEPD, which is of significant interest for this study. HMP is further converted to CPEP (carboxyphosphoenolpyruvate), and the second C-P bond is installed by CPEP mutase with CPEP as the starting material (30). Following the transformation from CPEP to CPnPy (carboxyphosphopyruvate), CPnPy is converted to PT with several biochemical reactions.

1.1.3 Methylphosphonate and its biosynthetic pathway in *Nitrosopumilus maritimus*

Methane, a potent greenhouse gas, is supersaturating the aerobic oceans, which is surprising because the biological methane production is an anaerobic process (5). Recently, Karl *et al.* suggested that methane might be generated in the aerobic ocean from methylphosphonate (MP) by the action of carbon-phosphorus (C-P) lyase, which has been proved to be able to produce methane with MP (35-36). Furthermore, the gene encoding C-P lyase has been found in a
number of marine microbes (35) and the incubation of marine microorganisms with MP results in the production of methane (37). However, MP has never been found in the ocean ecosystem, nor is it a known naturally occurring compound. Recently, van der Donk and coworkers discovered a phosphonate pathway in the archaea *Nitrosopumilus maritimus*, which provides a plausible explanation for this methane paradox in aerobic oceans (5). Genome mining for the phosphonate biosynthesis gene clusters from *N. maritimus* suggested that *N. maritimus* possessed the canonical pathway for the production of 2-HEP (5). The biosynthetic pathways of fosfomycin and PT also involve the similar process for the production of 2-HEP (2). One gene in this gene cluster encoding a protein homologous to HEPD was detected (5). After this protein was over-expressed in *E. coli* and reconstituted with Fe(II), the aerobic incubation of this protein with 2-HEP led to the production of MP, rather than HMP, the product of HEPD (5). This protein named methylphosphonate synthase (MPnS) is proved to be a novel non-heme-iron(II) dependent oxygenase, catalyzing the transformation of 2-HEP to MP (5). Its catalytic activity and mechanism will be discussed in detail in Chapter 3.

Furthermore, the analysis of cell extracts of *N. maritimus* indicated that MP is likely to be converted to methylphosphonate esters. The abundance of the gene encoding MPnS in this biosynthetic pathway in the metagenomic database indicates that MP biosynthetic pathway is a relatively common metabolic pathway in marine microorganisms, which provides a reasonable explanation for the methane production in the aerobic ocean.
1.2 The phosphonate-processing non-heme-iron(II) enzymes, HppE, HEPD and MPnS

1.2.1 (S)-2-hydroxypropylphosphonic acid epoxidase (HppE) is a unique epoxidase

1.2.1.1 HppE is a non-heme-iron(II) dependent epoxidase

The special epoxide ring of fosfomycin is installed in the last step of the fosfomycin biosynthetic pathway, which is catalyzed by HppE encoded by genes fom4 and psf4 from S. wedmorensis and P. syringae PB-5123, respectively (21, 38). Previously, by using heterologously expressed HppE, Liu and coworkers have established that HppE is an iron-dependent enzyme and both NAD(P)H and a flavin or flavin-dependent reductase are required for its activity (39-42). Feeding experiments together with activity assay using $^{18}$O labeled S-HPP have demonstrated that the ring oxygen of fosfomycin is derived from the hydroxyl group of S-HPP, rather than O$_2$ (39-40). Therefore, the formation of the epoxide ring illustrated in Figure 1-5 formally undergoes a dehydrogenation process, rather than an oxygenation process (39-40).
Figure 1-5. Previously proposed HppE catalyzed oxidation reaction.
The assigned identity of HppE, a non-hem-iron(II) enzyme, was further confirmed by its X-ray crystal structure. The X-ray crystal structure revealed that HppE exists as a homotetramer containing one ferrous ion in each monomer (43). Same as other members of cupin family, each HppE monomer is composed of an α-domain that is a whole α-helix, and a β-domain consisting of anti-parallel β-strands in a jellyroll β-barrel motif (Figure 1-6) (43). The majority of non-heme mononuclear iron enzymes employ a 2-His-1-carboxylate (2H1C) facial triad as the coordination for the ferrous ion (44-45). Combined evidence from sequence alignment, site-directed mutagenesis, spectroscopic studies and X-ray crystallography suggests that the “2H1C” motif is also conserved in HppE, in which the “2H1C” motif is consisted of His 138, Glu 142 and His 180 and is housed within its β-barrel (Figure 1-6) (40, 43, 46). The substrate S-HPP has been shown to interact with the active site iron in a bidentate mode via the 2-hydroxyl oxygen and the oxygen from the phosphonate group, and at the same time replaces water ligands and leaves an open coordinate for the subsequent binding of oxidant (Figure 1-6) (43). During the process of substrate binding, amino acid residues Tyr 102, Tyr 105 and Arg 97 move towards the iron atom, leading to alignment of the three tyrosine residues in the vicinity of the active site (43). It is believed that these tyrosine residues may facilitate the transfer of electrons from an unknown source to the active site iron.
Figure 1-6. The structure of HppE-Fe(II) and HppE-Fe(II)-S-HPP-NO. (A) A HppE-Fe(II) form tetramer; (B) the active site structure of HppE-Fe(II)-S-HPP-NO enzyme complex. Adapted and modified from ref. (47).
1.2.1.2 HppE is a peroxidase, rather than an oxidase

It has previously been established that HppE catalyzes a dehydrogenation of the secondary alcohol, S-HPP, rather than oxygen atom insertion to the substrate (39-40). Meanwhile, Liu and coworkers found that no fosfomycin production was detected under anaerobic conditions, while no H$_2$O$_2$ formation was detected during reaction with O$_2$ (39-40). Additionally, their research suggested that NAD(P)H and a flavin or flavin-dependent reductase are essential for HppE activity (39-41). It is worth noting that the common co-substrates, namely α-ketoglutarate and tetrahydropterin, serving as two-electron donors to various well-studied O$_2$-activating non-heme-iron enzymes, cannot reconstitute HppE activity (40, 48-51). Consequently, it was proposed that HppE catalyzes a four-electron, instead of two-electron, redox reaction and utilizes dioxygen as electron acceptor. As the dehydrogenation of S-HPP can only provide two electrons, thus two more electrons are required from NAD(P)H to balance the four-electron reduction of dioxygen to water (Figure 1-4). This also distinguishes HppE from other known non-heme-iron epoxidases (52-53), which will be discussed in the section 1.2.1.4.

**Does HppE catalyze a four-electron reaction?**

In addition to electron donor NAD(P)H, it has been found that electron mediator flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) can enhance fosfomycin production, leading to the hypothesis that a flavin-binding pocket may be present in HppE (39). However, several lines of evidence have argued against such proposal. Firstly, although FMN has a high binding affinity for the HppE-Fe(II) binary complex, it barely binds to the HppE-Fe(II)-S-HPP ternary complex (41). The observed mutually exclusive binding of FMN and S-HPP to HppE casts doubt on the proposal that there is a specific flavin-binding pocket in HppE (41). Furthermore, the electron mediator riboflavin, an effective surrogate for FMN, shows no affinity
for HppE-Fe(II) complex; however, it is capable to support HppE catalyzed epoxidation reaction with a rate comparable to that of reaction with FMN as electron mediator (41). Thereby, the possibility of a defined flavin binding site in HppE is clearly quite low, which is also supported by the fact that no flavin is observed in HppE X-ray crystal structure(43).

As shown in Table 1-1, a protein electron mediator, reductase E3, supports a more efficient epoxidation reaction, compared with other electron mediators (41). E3 is a NADH-dependent [2Fe-2S]-containing flavoenzyme from Yersinia pseudotuberculosis (54). It relays electrons from NADH to final electron acceptor with a chain of redox-active cofactors including FAD and Fe-S cluster in E3 (41). Such finding implied that in the physiological condition, the electron mediator of HppE is likely a protein reductase, rather than a cofactor (41). However, the well-established structures of HppE do not reveal a reductase domain, and attempts to search for a reductase gene in the gene clusters specifying fosfomycin biosynthesis have failed (41, 55). Therefore, it is very unlikely that the electron mediation of HppE catalyzed reaction relies on a specific reductase in vivo.

In addition to the NADH/electron mediator combination, HppE activity could be reconstituted with chemical electron donors, like [Ru^{II}(NH_3)_6]Cl_2 and L-ascorbic acid, and this process does not require any exogenous electron mediator (Table 1-1). Thus, the argument that NADH is essential for HppE activity needs to be re-considered in light of new evidence. However, as shown in Table 1-1, regardless of the nature of electron donors or electron donor/electron mediator combinations, the observed rates for the epoxidation reaction are noticeably slow, compared to the usual catalytic rate constant of O_2-activating oxygenases and oxidases, which ranges from 1 to 100 s^{-1} (56-57).
To summarize, NADH and FMN or other electron mediators are not essential for the HppE catalysis. Upon initial examination, a reductant considerably more efficient than all of the reported electron donors or electron donor/electron mediator systems might be required to provide the two electrons for the HppE catalyzed process. Alternatively, it is possible that O$_2$ may not be the oxidant of HppE catalyzed reaction. In other words, HppE may be catalyze a two-electron reaction, rather than a four-electron reaction.
<table>
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<th>Electron donor</th>
<th>Ru&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Asc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NAD(P)H</th>
</tr>
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<tbody>
<tr>
<td>Electron mediator</td>
<td>N/A</td>
<td>FAD</td>
<td>FMN</td>
</tr>
<tr>
<td>$k_{obs}$&lt;sup&gt;-1&lt;/sup&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.67</td>
<td>0.36</td>
<td>N/A</td>
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**Table 1-1.** Rates of HppE catalyzed epoxidation reaction by using different electron donors and electron mediators. *a represents [Ru<sup>II</sup>(NH<sub>3</sub>)<sub>6</sub>Cl<sub>2</sub>; b represents L-ascorbic acid. Partially adapted from ref. (41).
**HppE catalyzes a two-electron peroxidation reaction**

In searching for a more efficient reductant, we discovered that sodium dithionite (Na$_2$S$_2$O$_4$) can support multiple turnovers at a rate more than a 1000-time greater (15 s$^{-1}$) than those supported by the NADH-based reducing systems (Table 1-1) (55). However, dithionite can reactive with O$_2$ on the same time scale, creating a puzzling question as to how it could deliver electrons to HppE without first being oxidized by O$_2$ (55). The closer examination of this reaction led to a hypothesis that dithionite may actually reduce O$_2$ directly to H$_2$O$_2$ rather than donate electrons to HppE during an O$_2$-initiated catalysis (55). Therefore, to assess whether the O$_2$-reduction product, H$_2$O$_2$, is able to serve as the oxidant for fosfomycin production in HppE catalysis, a series of biochemical, kinetic and spectroscopic studies were performed. It was found out that H$_2$O$_2$ is the preferred cosubstrate of HppE and that HppE thus almost certainly uses an iron(IV)-oxo intermediate to abstract a hydrogen from C1 of S-HPP (Figure 1-7) (55). Furthermore, the reaction with H$_2$O$_2$ is accelerated by bound S-HPP and the stoichiometry of the production of fosfomycin and the consumption of H$_2$O$_2$ is a tight 1:1 (55). The fact that catalase can effectively suppress the HppE activity previously attributed to its direct use of O$_2$, implies that the reduction of O$_2$ and the utilization of the O$_2$-reduction product, H$_2$O$_2$, were actually operant (55). In appendix A, the peroxidase character of HppE is proved and a reformulated catalytic mechanism of HppE is proposed (Figure 1-7).
Figure 1-7. The proposed mechanism of HppE catalyzed peroxidation reaction. Adapted and modified from ref. (55).
1.2.1.3 The discussion on the epoxide ring closure of HppE-catalyzed epoxidation of $S$-HPP

As an peroxidase, HppE is almost certainly established to use an iron(IV)-oxo intermediate to abstract a hydrogen from C1 of $S$-HPP (Figure 1-7). However, after the stereo-specific C1-pro-R-H abstraction, the fate of the resulting C1 centered substrate radical and the formation of a new C-O bond are still not known. The existence of the postulated C1 centered substrate radical intermediate was supported by the studies on HppE catalyzed conversion of a series of substrate and substrate analogues.

**HppE-catalyzed reaction is a substrate radical-mediated process**

HppE possesses high substrate flexibility, which is indicated by the fact that HppE is able to catalyze the dehydrogenation reactions of a number of chemicals containing both hydroxyl and phosphonate groups, besides the natural substrate $S$-HPP (Figure 1-8 and Figure 1-9B) (58-59). In 2002, Liu and coworkers synthesized three compounds and tested HppE activity with them (58). These chemicals includes $R$-2-HPP and the C1 fluoro-substituted substrate analogues, ($S$)-[1,2-F$_2$]-HPP and ($R$)-[1,2-F$_2$]-HPP, as shown in Figure 1-8 (58). HppE converted $R$-2-HPP to a completely different product 2-oxopropylphosphonate (2-OPP), rather than epoxide (Figure 1-8) (58). Similarly, ($R$)-[1,2-F$_2$]-HPP was transformed into corresponded ketone product by dehydrogenation (Figure 1-8) (58). By contrast ($S$)-[1,2-F$_2$]-HPP is an inhibitor of HppE (Figure 1-8) (58). This study leads to the hypothesis that the initial hydrogen abstraction in each reaction is regiospecific, namely, C1-hydrogen abstraction in the reaction of HppE with $S$-HPP, C2-hydrogen abstraction in the reaction of HppE with $R$-HPP and C2-hydrogen abstraction in the reaction of HppE with ($R$)-[1,2-F$_2$]-HPP (Figure 1-8). This hypothesis is consistent with the study of the X-ray structures of HppE•Fe(II)•$R$-HPP and HppE•Fe(II)$S$-HPP•NO, which suggests that either C2-H or C1-H is positioned towards the active site Fe(II) (60). Thus, as shown in Figure 1-
substrate radical intermediates may account for the stereo-specific hydrogen abstraction carried out by HppE, when HppE reacts with different substrates and substrate analogues.
Figure 1-8. The dehydrogenation reactions catalyzed by HppE with substrate and substrate analogues.
To further probe the radical-mediated catalysis of HppE, four cyclopropyl- or methylencyclopropyl-containing compounds were synthesized and studied as radical clock probe. The structure of each compound was shown in figure 1-9 (59). When these analogues react with HppE, radical intermediates are expected to be trapped. The radical-triggered ring-opening of cyclopropylcarbinyl or methylenecyclopropylcarbinyl radicals are well-known in the study of chemical reactions and enzymatic reactions (61-64). Thus, the observation of ring-opened products or radical-induced enzyme inactivation would provide evidence for the existence of radical intermediates (59).

Enzyme activity assays indicated that the (S)- and (R)-isomers of the cyclopropyl-containing substrate analogues were efficiently converted to epoxide and ketone by HppE, respectively (Figure 1-9) (59). Intriguingly, although the (S)-isomer of methylencyclopropyl-containing compounds was converted to the corresponding epoxide, the (R)-isomer irreversibly inactivated HppE (Figure 1-9) (59). The fact that the ring-opening rate constant of (methylencyclopropyl) carbonyl radical (6.0 × 10⁹ s⁻¹ at 0 °C) is almost two orders of magnitude greater than that of cyclopropylcarbinyl radical (8.6 × 10⁷ s⁻¹ at 0°C), which explained why methylencyclopropyl-containing compound, rather than cyclopropyl-containing compound could serve as a sensitive radical probe to inactivate HppE (59). This study also suggested that the rate of the subsequent electron transfer step of HppE reaction is estimated to be between 8.6 × 10⁷ and 6.0 × 10⁹ s⁻¹ (59). Therefore, the reaction of HppE with radical clock compounds established the radical nature of HppE catalysis.
Figure 1-9. The reactions or inhibition of HppE with radical probes. (A) HppE reaction with (S)-1; (B) HppE reaction with (R)-1; (C) HppE reaction with (S)-2; (D) HppE reaction with (R)-2. Adapted and modified from ref. (59).
The epoxide ring closure of HppE involves a substrate-derived carbocation intermediate

In 2013, Chang et al. reported that HppE could catalyze an unprecedented 1,2-phosphono migration reaction with the substrate analogue (R)-1-hydroxypropylphosphonate ((R)-1-HPP) (Figure 1-10A), for which there is no enzymatic precedent (65). The investigation of this unique migration reaction provided strong evidence to that a C2-centered carbocation of (R)-1-HPP is involved in the migration of phosphono group from C1 to C2 (65).

In the HppE-catalyzed 1,2-phosphono migration reaction, the C2-pro-R-hydrogen was abstracted, which is suggested by the evidence of 1H-NMR spectra, 13C-NMR spectra and X-ray crystal structure of HppE-Fe(II)-(R)-1-HPP complex(65). On the basis of the fact that non-enzymatic 1,2-phosphono migrations are generally thought to proceed with carbocationic intermediates, a similar carbocation intermediate was thought to be involved in the HppE catalyzed 1,2-phosphono migration (route a, Figure 1-10B). Meanwhile, it is also possible that this HppE catalyzed migration reaction may employ a substrate radical intermediate to trigger the migration (route b, Figure 1-10B) (65).

Chang et al. showed that among non-enzymatic reactions, only the carbocation-mediated reaction could generate phosphono migration product, and the radical-mediated reaction with the same starting material fails to do that (65). Meanwhile, as shown in figure 1-10C, they showed that both (1R, 2R)- and (1R, 2S)-1-hydroxy-2-aminoethylphosphonate ((1R,2S)-3 and (1R, 2R)-3) could be converted into the same ketone product 4 (1-hydroxy-2-oxo-propylphosphonate) by HppE, without any detectable migration or other products shown in NMR spectra (65). Such a ketone product could be thought as forming through C2-hydrogen abstraction and then generating an α-aminoalkyl radical. The α-aminoalkyl radical is further oxidized to the stable C2 iminium ion, which could employ a spontaneous hydrolysis to yield 4, as indicated in Figure 1-10C. Further, HppE failed to convert 2-aminopropyl phosphonate (5) to any product, because
compound 5 cannot bind bidentately to the iron center of HppE. Combined with all these evidence, in the HppE catalyzed conversion from (1R,2S)-3 or (1R,2R)-3 to 4, the C2-centered radical species could be oxidized to corresponding C2-centered carbocation species, as depicted in Figure 1-10C.

Taken together, Chang et al. provided strong evidence to an enzymatic carbocation intermediate and demonstrated its catalytic competence in HppE catalyzed migration reaction. On the basis of this unprecedented finding and the DFT computation favored Fe(IV)-oxo hydrogen-abstraction intermediate, a new mechanism of HppE reaction with natural substrate S-HPP was proposed, as demonstrated in figure 1-11 (66). In the route A of figure 1-11, a C1-centered radical intermediate was thought to attack the C2-hydroxyl group directly to close the epoxide ring. However, the resulting product has a different stereochemistry on C1 compared with that of fosfomycin. This finding indicated that after the C1-hydrogen abstraction by ferryl species, C1 undergoes an inversion to generate the product with correct stereochemistry. Interestingly, the route B involving a carbocation intermediate will perfectly explain the C1 inversion. In route B, after forming the C1-radical, one electron from C1 is transferred to the Fe(IV)-oxo species, yielding a C1-center carbocation, and then the epoxide ring is closed and the product, fosfomycin, is generated. This mechanism involved in both substrate radical and substrate carbocation intermediates, which is further supported by the study of HppE catalyzed reactions with four stereo isomers of 3-methylenecyclopropyl-containing substrate analogues (67).
Figure 1-10. The summary of HppE catalyzed 1,2-phosphono-migration reaction with (R)-1-HPP and the proposed catalytic mechanisms of this migration reaction. (A) HppE catalyzed 1,2-phosphono-migration reaction; (B) Hypothetical mechanisms of HppE-catalyzed 1,2-phosphono-migration involving carbocation-mediated (route a, red) and radical-mediated (route b, blue) rearrangement; (C) HppE catalyzed reaction of (1R,2S)-3 or (1R, 2R)-3. Adapted and modified from ref. (65).
Figure 1-11. Proposed ring-closure mechanism of HppE catalyzed epoxidation of (S)-HPP involving C1 carbocation formation (route A, blue) or oxygen atom rebound (route B, red). Adapted and modified from ref. (65).
1.2.1.4 Other enzyme-catalyzed epoxidation reactions and their catalytic mechanisms

Epoxide, the strained three-member ring structure, has been found in a large number of natural products and these natural chemicals exhibit versatile pharmacological activities, which range from antibiotic activity to antiviral, anti-obesity, antineoplastic and anesthetic activities (68-69). The strained epoxide ring is usually the functional moiety for its biological activities by alkylating the target compounds (11). The specific enzymes responsible for the epoxide biosynthesis have been identified to be a large group of enzymes from various families. Most of the epoxide-forming enzymes are oxygenases or oxidases, like EpoK, allene oxide synthase (AOS), DdaC and Hyoscyamine 6β-hydroxylase (H6H), which require O₂ as a source for the epoxide ring oxygen or as an oxidant, respectively (69). However, the triplet ground state of O₂ presents a significant kinetics barrier to react with most biological molecules, in that a vast majority of biological molecule is in singlet ground state. Therefore, the dioxygen is required to be converted into the reactive singlet state to react with most of biological molecules. Usually, in enzymatic systems, the dioxygen activation is achieved by electron transfer from redox active transition metals, like Fe, Cu and Mn, or from organic cofactors or even substrates possessing stable radical states (69). Based on the dioxygen activation strategies employed by different epoxide-forming enzymes, these enzymes are assigned to several different enzyme families. The epoxide-forming enzymes include P450-dependent epoxidase, flavin-dependent epoxidase, cofactor-independent epoxidase and a unique group of epoxidases, non-heme-iron dependent epoxidase, which is of significant interest for this study. In this section, special attention will be focused on the prototype of these epoxidation reactions and the mechanistic details of theses enzymes involved.
**P450-dependent epoxidase**

**P450-dependent Monooxygenase**

Until now, the best-characterized group of epoxidases is P450 monooxygenase, which utilizes a heme as prosthetic group to activate dioxygen. With respecting to the P450-dependent epoxidase catalyzed reaction, this enzyme inserts an oxygen atom into the double bond of an alkene substrate and reduces the second oxygen to a water molecular, using two electron provided by NAD(P)H via NAD(P)H-dependent P450 reductase protein, as demonstrated in Figure 1-12A (70).

As shown in Figure 1-12B, in a typical P450 catalyzed reaction, the alkene substrate binding displaces one molecule of water, which results in the conversion of six-coordinate, ferric form resting state \((S = 3/2)\) to a five-coordinate, high spin \((S = 5/2)\) ferric state (69-70). This substrate binding process triggers one electron transfer from the reductase protein (usually an NAD(P)H-dependent P450 reductase) (70). Following the binding of \(O_2\) to ferrous ion, one electron is transferred from the heme iron, which results in the formation of a ferric superoxo complex. The following proton couple electron transfer leads to the formation of an Fe(III)-hydroperoxo specie, namely compound 0 (Cpd 0). The proton is believed to be supplied by the Thr-252 around active site (70). The second reduction step is a rate-determining step in many P450s (70). The protonation of Cpd 0 contributes to the O-O bond cleavage, which generates one molecule of water and an electrophilic Fe(IV)-oxo porphyrin cation radical specie, known as compound I (Cpd I). This protonation is probably assisted by the negative charge accumulated on the proximal cysteine residue in the first electron transfer step, which has been proven to be the driving forces of heterolytic cleavage of the O-O bond (70). Cpd I is believed to be the active species responsible for the oxygen insertion step. After the formation of Cpd I, two-electron oxidation of alkene substrate leads to the formation of final product, epoxide, in a concerted way.
(directly from Cpd 1 to product bound form enzyme) or a stepwise way (from Cpd I to Cpd II to product-enzyme complex). However, there is no clear indication that the converted way or the stepwise way is the real pathway P450 epoxidase may use. Finally, the epoxide product dissociates from the enzyme and one molecule of water rebinds to the ferric form enzyme, the resting from enzyme.

To date, several P450 enzymes with epoxidase activity have been identified biochemically or genetically in the biosynthesis pathway of natural products. These enzymes include EpoK, which catalyzes the epoxide ring formation of the anticancer agent epothilone, MycG, which installs an epoxide moiety of the antibiotic mycinamicin, GfsF, which involves in the biosynthesis of FD-891, a macrolide antibiotic, and PimD, which catalyzes the epoxide formation step in the biosynthesis of potent antifungal agent pimaricin (69).
Figure 1-12. (A) The typical epoxidation reaction catalyzed by the P450 monooxygenase; (B) The typical epoxidation mechanism of P450 monooxygenase. Adapted and modified from ref. (69).
Allene Oxide Synthase (AOS)

Besides the P450-dependent monooxygenase, there is another type of P450-dependent epoxidase, allene oxide synthase (AOS). AOS belongs to the fatty acid hydroperoxide-metabolizing P450s subfamily, designated as CYP74A (71-72). In contrast to P450-dependent monooxygenase, AOS is able to convert fatty acid hydroperoxide to allene oxide, a kind of unstable epoxide, without the requirement of dioxygen, NAD(P)H and NAD(P)H-dependent reductase (71). The C-O bonds formation in the epoxide ring is facilitated by substrate, fatty acid hydroperoxide, which is both substrate and oxygen atom source of the epoxide ring (71). Structurally, AOS, like catalase, is a heme protein with a tyrosine axial ligand (72).

As depicted in Figure 1-13, the reaction is believed to be initiated by the homolytic cleavage of substrate hydroperoxyl O-O bond, and subsequently the epoxide ring is closed and an epoxyallylic radical intermediate is formed (69). The epoxyallylic radical intermediate has two possible outcomes. One is that the radical species is directly oxidized to allene oxide by one electron transfer from substrate radical to enzyme and a proton abstraction. Another one is that the substrate radical is further oxidized to a carbocation, which triggers the final product allene oxide formation. The proposed carbocation intermediate was favored by a study on the AOS from Acaryochloris marina (69).
Figure 1-13. The proposed catalytic mechanism of Allene Oxide Synthase (AOS) of cyanobacteria *Acaryochloris marina*. Adapted and modified from ref. (69).
non-heme-Fe(II)/α-ketoglutarate dependent epoxidase

Although the oxygen atoms of epoxide rings installed by most epoxidases is from dioxygen, one non-heme-iron epoxidase, HppE, is different, because the oxygen atom of the epoxide ring installed by HppE is from substrate, a secondary alcohol S-HPP. Furthermore, HppE reaction is independent of the commonly used co-substrate used by non-heme-iron epoxidases, α-KG. Taken together, HppE is not only different from the heme epoxidases by substrate, but different from other non-heme-iron epoxidases by the requirement of co-substrate.

The HppE catalyzed reactions, X-ray crystal structures and catalytic mechanism have been discussed in detail before. In the following section, detailed discussion will be focused on α-KG-dependent epoxidase subfamily.

As discussed before, the Fe(II)/α-ketoglutarate dependent enzymes could generate high valent Fe(IV)-oxo intermediate, which has versatile reactivities, including hydroxylation, desaturation, halogenation and epoxidation etc. and imparts astounding array of biological functions to these enzymes. The Fe(II)/α-ketoglutarate dependent epoxidase could catalyze the dehydrogenation of alcohol substrate or oxygenation of olefin substrate to generate the epoxide products.

DdaC, PenD and PntD catalyzed epoxidation reactions with olefin substrates

In the biosynthesis of dapdiamide type antibiotic Nβ-epoxysuccinamolyl-DAP-Val in Pantoea agglomerans, an Fe(II)/α-ketoglutarate dependent enzyme DdaC was found to catalyze the key epoxidation step of Nβ-fumaramoyl-L-2,3-diaminopropionyl-S-DdaD to Nβ-epoxysuccinamolyl -L-2,3-diaminopropionyl-S-DdaD (Figure 1-14) (52). The oxygen atom of epoxide ring was proved to be derived from dioxygen, in that DdaC incubation under 18O2 resulted in the 18O incorporation into the product, as suggested by LC-MS analysis of the product
(52). Similarly, PenD and PntD, two Fe(II)/α-ketoglutarate dependent enzymes involved in pentalenolactone biosynthesis in Streptomyces exfoliates and Streptomyces arenae, respectively, were found to be responsible for the epoxidation of olefin substrates after the desaturation steps (73).
Figure 1-14. DdaC catalyzed epoxidation reaction in the biosynthesis of dapdiamide-type antibiotic.
Hyoscyamine 6β-hydroxylase (H6H) catalyzed epoxidation with alcohol substrate

Hyoscyamine and scopolamine belong to the biologically active tropane alkaloid family, which is produced by a few genera of the plant family Solanaceae (74). Tropane alkaloids like hyoscyamine and scopolamine are widely used as anticholinergic agents, which are able to block the neurotransmitter acetylcholine in the central and peripheral nervous system (74). Scopolamine has been extensively used for the treatment of Parkinson’s disease and motion sickness (75). In the biosynthesis of scopolamine (Sco), a bifunctional Fe(II)/α-ketoglutarate dependent enzyme, hyoscyamine-6β-hydroxylase (H6H) catalyzes the conversion of hyoscyamine to scopolamine in two discrete steps, as shown in Figure 1-15A (76-77). The first step entails the conversion of hyoscyamine to 6β-hydroxy-hyoscyamine (6β-OH-hyo), which involves a typical Fe(II)/α-ketoglutarate dependent enzyme catalyzed hydroxylation of an aliphatic C-H bond. In the second step, H6H catalyzes the dehydrogenation of 6β-OH-hyo to generate epoxide product, scopolamine, which is suggested by 18O-labeling studies (53, 78). This distinguishes H6H from most of known Fe(II)/α-ketoglutarate dependent epoxidases, because they all catalyze the oxygen atom insertion to olefin substrates.

Until now, there is no clear demonstration of the H6H catalytic mechanism of the epoxidation step. Recently, Bollinger, J. M., Jr and Krebs, C. have proposed a new mechanism on the basis of their continuous endeavor to investigate the catalytic mechanism of non-heme-iron enzymes. As shown in the pathway ii (green, Figure 1-15B), the ferryl intermediate could abstract a hydrogen atom from hydroxyl group on C6, and O-H bond cleavage activity of ferryl has been observed in the studies of another non-heme-iron enzyme, 2-hydroxyethylphosphonate dioxygenase (HEPD), which will be discussed in detail in Chapter 2. Following the cleavage of O-H bond are C6-C7 scission, transfer of the 7β-H atom to the aza group, and a [3+2]-cyclization. Alternatively, in pathway i (purple, Figure 1-15B), the ferryl intermediate may directly abstract a
hydrogen atom from C7, and then the resulting C7-centered radical species may be oxidized to a carbocation, which subsequently is attacked by the 6β-OH group to close the epoxide ring. Another possibility is that the C7 centered radical could directly trigger the epoxide formation without undergoing a carbocation intermediate (pathway i, purple, Figure 1-15B). Although, until now the mechanism of H6H catalyzed reaction is not clear, the studies on H6H X-ray crystal structure, transient state kinetics and spectroscopy, and the study of H6H-catalyzed reaction with specific isotope-labeled substrate will be helpful for understanding the mystery of H6H catalysis.
Figure 1-15. (A) H6H catalyzed reactions; (B) Proposed catalytic mechanism of H6H for the epoxidation step.
Non-heme-diiron-dependent epoxidase

Besides mono-nuclear-non-heme epoxidase, some non-heme-diiron monooxygenases, like toluene monooxygenase (TMO) and xylene monooxygenase (XO), display the epoxidation activity (68, 79-82). Both TMO and XO are able to effect oxidation of the side chains of aromatic hydrocarbons to the corresponding alcohols, and this activity depends on NAD(P)H and O₂. In 2000, McClay et al. identified that a strain of *E. coli* expressing TOM gene from *Pseudomonas mendocina* KR1 was able to oxidize butene, butadiene, pentene and hexene to corresponding epoxides (79). The *E. coli* strain carrying XO gene from *Pseudomonas putida* mt-2 has been proven to be capable of oxidizing the ethylene side group of styrene to styrene epoxide (80). With respect to the catalytic mechanism of XO, Groves and coworkers suggested a hydroxylated hydro-carbon via a hydrogen abstraction-carbon radical recombination mechanism by using a radical clock bicyclo[4.1.0]heptane (norcarane) in a whole-cell assay (82).

Other epoxidases

Besides the heme and non-heme-iron cofactors, some epoxidases employ organic cofactor like flavin and substrate itself to activate dioxygen and subsequently insert the oxygen atom to C=C bond to generate epoxide. Further, some epoxidase even doesn’t take olefin as substrate, but catalyze the dehalogenation of haloalcohol to form epoxide ring with intramolecular nucleophilic substitution (69).
Flavin-dependent epoxidase

Like P450-dependent epoxidase and some non-heme-iron dependent epoxidase, the flavin-dependent epoxidases could insert one oxygen atom from dioxygen to an alkene moiety. The catalytic mechanism of flavin-dependent epoxidases is believed to be similar to that of other flavin-dependent monooxygenases. As shown in Figure 1-16, firstly, the anionic reduced form flavin transfers one electron to O₂ and generates the caged semiquinone-superoxide radical pair, which then employs radical recombination and protonation to produce a flavin C₄₅-hydroperoxide intermediate (69). The distal oxygen of flavin hydroperoxide, an electrophilic intermediate, is capable to insert an oxygen atom to the olefin, which leads to the formation of epoxide ring and a flavin-C₄₅-hydroxy species (69). Subsequently, the flavin hydroxyl speice proceeds a spontaneous decay to generate the oxidized form flavin and simultaneously loses one molecular water (69). Finally, a NAD(P)H-dependent reductase transfers two electrons to the oxidized form flavin, which results in the regeneration of active form flavin-dependent epoxidase.
Figure 1-16. General catalytic mechanism of the flavin-dependent epoxidase. Adapted and modified from ref. (69).
Cofactor-independent epoxidases

Some dioxygenase is able to convert the phenolic compound substrate to epoxide by activating dioxygen without the assistance of either transition metal ions or organic cofactors. By acquiring one electron from substrate, these kind of special enzymes activate dioxygen and simultaneously produce a relative stable substrate radical species (69). The typical examples of this kind of cofactor-independent epoxidase are dihydroxyacetanilide epoxidase I and II (DHAЕ I and DHAЕ II), both of which involve in the biosynthesis of epoxyquinone compounds (83). The epoxyquinone compounds possess antitumor and anticancer activities (83). As indicated in Figure 1-17A, DHAЕ I effects the epoxidation of dihydroxyacetanilide and generates (5R,6S)-epoxyquinone by incorporating an oxygen atom into the epoxide ring, which is indicated by the $^{18}$O$_2$ incorporation experiment (83).

Epoxide Formation via Intramolecular Nucleophilic Substitution

Unlike all the above mentioned epoxidases, a unique epoxidase, haloalcohol dehydrogenase (HheC), catalyzes a dehalogenation reaction of haloalcohol substrate, rather than dehydrogenation or oxygenation, without the requirement of O$_2$ and any reductant, as shown in Figure 1-17B. HheC employs acid/base chemistry to catalyze a reversible $S_N$2-like epoxidation reaction. As indicated by crystal structure, during the catalysis, the general base Tyr145, deprotonates the OH group, which initiates the intramolecular $S_N$2 transformation. The pKa of the general base Tyr 145 is probably lowered by another conserve residue Ser132 (69).

To summarize, all the above-discussed epoxidases are listed in table 1-2, and are compared in terms of their cofactors, co-substrates/reductants, substrates, reaction type and key intermediates.
Figure 1-17. (A) Dihydroxyacetanilide epoxidase I (DHAЕ I) catalyzed epoxidation; (B) Haloalcohol dehydrogenase (HheC) catalyzed the epoxidation of 1,2-halo alcohol with an $S_N$-2 reaction. Adapted and modified from ref. (69).
<table>
<thead>
<tr>
<th>Epoxidase type</th>
<th>Enzyme cofactor</th>
<th>Co-substrate or electron donor</th>
<th>Substrate</th>
<th>Reaction type</th>
<th>Redox mode</th>
<th>Key intermediate</th>
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<td>NAD(P)H</td>
<td>Olefin/O₂</td>
<td>monoxygenation</td>
<td>4e</td>
<td>Fe(IV)-oxo prophyrin cation radical species (Cpd I)</td>
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<td>dehydrogenation</td>
<td>2e</td>
<td>Cpd I</td>
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<td>α-KG</td>
<td>olefin or alcohol/O₂</td>
<td>dioxygenation or dehydrogenation</td>
<td>4e</td>
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<td></td>
</tr>
<tr>
<td></td>
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<td>Olefin/O₂</td>
<td>monoxygenation</td>
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</tbody>
</table>

Table 1-2. The summary of epoxide-forming enzymes.
1.2.2 HEPD catalyzes an unprecedented C-C cleaving reaction

1.2.2.1 Previous studies established that HEPD was a non-heme-iron dioxygenase

In 2009, van der Donk and coworkers found that during the PTT biosynthesis, 2-HEP is converted to HMP and formate, which is catalyzed by a non-heme-iron (II) enzyme, HEPD (31). This reaction involves the cleavage of an unactivated $\text{C}(sp^3)$–$\text{C}(sp^3)$ bond in the presence of Fe(II) and $O_2$, but does not require the input of any exogenous electrons from reductants or additional cofactors/cosubstrates (31). The isotopic labeling studies suggested that one each oxygen atom from $O_2$ was inserted into both HMP and formate, and C1 and C2 of 2-HEP were retained in HMP and formate, respectively (31). Further labeling studies using either $^{18}O_2$ and $H_2^{18}O$, respectively, indicated that the hydroxyl oxygen in HMP was partially derived from $O_2$ and partially from solvent (4). Such a finding implicates that HEPD-catalyzed reaction involves an intermediate in which $O_2$-derived oxygen atom can exchange with solvent (31). Meanwhile, van der Donk and coworkers reported the X-ray crystal structures of HEPD, which suggest that HEPD employs the canonical “2H1C” facial triad (two histidines and one glutamate) to chelate the active site Fe(II) (31).

The overall structure of HEPD-Cd(II) contains imperfect tandem repeats of a two-domain architecture (Figure 1-18A). Each of the repeats is composed of an $\alpha$-helical domain linked to a $\beta$-barrel fold, which is typical character of the cupin superfamily (4). Despite the low sequence identity of HEPD and HppE, each repeat of the overall structure of HEPD is homologous to the monomer of HppE and the tandem arrangement of HEPD is analogous to half of the HppE tetramer (4). The $\beta$-barrel fold of the first repeat of HEPD possesses all of the canonical residues
responsible for Fe(II) coordinating of non-heme iron enzymes, namely “2H1C” facial triad (4). In the structure of HEPD-Cd(II)-2-HEP, the Cd(II) ion is position at the active site and is chelated by His 129, Glu 176 and His 182 (Figure 1-18B) (4). Although the spatial positioning of metal-chelating ligands is similar to that of HppE, but the position of the three ligands within the β-barrel is different from that of HppE. Glu 176 is housed in a different β-strand to its counterpart, Glu 142 of HppE (4, 43). Furthermore, the spacing between the first two metal ligands in HEPD (HX_{46}E, where X denotes any residue) is larger than most enzymes possessing facial triad (HX_{1,4}E/D) (4). The second repeat in HEPD houses an arrangement of 2-His-1-Asn, instead of 2-His-1-caboxylate (4). Based on the fact that only one equivalent of Fe(II) is required for the full activity of HEPD, the second repeat in HEPD may be vestigial and not essential for catalysis (4). The analysis of the crystal structure of SeMet-labelled Cd(II)-HEPD-2-HEP complex reveals that the electron density agree well with bidentate coordination of 2-HEP to Cd(II) (Figure 1-18B), which is similar to the binding mode of S-HPP to HppE-Fe(II) complex (4).

Based on the biochemical and crystallographic studies, van der donk and coworkers unequivocally established HEPD to be a non-heme-iron(II) dioxygenase. Their study expands the repertoire of reactions catalyzed by the mononuclear non-heme-iron enzymes containing “2H1C” facial triad.
Figure 1-18. Structures of HEPD-Cd(II) and HEPD-Cd(II)-2-HEP. (A) Orthogonal view of the overall structure of HEPD-Cd(II) showing the tandem repeats of bi-domain architecture. Each repeat contains one cupin domain (blue and purple) and one α-helix (red and cyan). The cadmium ion is shown with a white sphere; (B) Stereoview of electron density maps ($F_o - F_c$) calculated with HEPD-2-HEP model phases. 2-HEP carbons are shown in green. Adapted and modified from ref. (4).
1.2.2.2 The proposed catalytic mechanisms of HEPD

To probe the catalytic mechanism of HEPD, van der Donk group carried out extensive studies on the structural analogues and isotopologues of 2-HEP (31, 84-85). The product of HEPD-catalyzed reaction, HMP, can be taken as a substrate and be converted into formate and phosphate (Figure 1-19A) (31). The substrate analogue, (S)-1-hydroxyethylphosphonate (S-1-HEP), was converted to acetylphosphate, an inhibitor of HEPD (Figure 1-19B) (31). When R-HPP was presented to HEPD, R-HPP partitioned between the conversion to 2-OPP (2-oxopropylphosphonate) and HMP, and the subsequent oxidation of HMP generated phosphate and formate (Figure 1-19C), though HEPD is inactivated before the complete consumption of R-HPP (31). The stereochemical investigations have shown that the HEPD-catalyzed transformation of 2-HEP into HMP involves stereospecific removal of C2-pro-S-H, which is consistent with the crystal structure of HEPD-Cd(II)-2-HEP complex, and the loss of stereochemistry at C1 (Figure 1-19D) (4, 84).
Figure 1-19. HEPD catalyzed reactions of substrate analogues and isotopologues (31, 84-85).
Furthermore, the site-directed mutagenesis study was conducted to detect the binding of 2-HEP and the correlation between substrate binding and O₂ activation. The mutation of Lys 16 to Ala led to the inactivation of HEPD, and the mutations of Arg 90 to Ala and Tyr 98 to Phe resulted in a great decreasing value of \( k_{cat}/K_{m,2-HEP} \) (85). The variant Y98F cannot be saturated with O₂. Thus, the site-directed mutagenesis studies provide evidence for the roles of these residues in substrate binding (85). These findings also suggest that the proper binding of 2-HEP is essential for O₂ activation and that the binding of 2-HEP occurs before the binding of O₂ (85). The Y98F variant produces MP in a low level as a side product, which provides indirect evidence supporting the hypothesis that the last step of HEPD catalysis involves a rebound with ferric hydroxide to a methylphosphonate radical (85).

Additionally, the DFT calculation performed by Hirao et al. provides insight to the mechanism of HEPD catalysis (86). The DFT study indicates that the HEPD-catalyzed reaction starts with hydrogen abstraction from the C2 of 2-HEP by an Fe(III)-OO⁻ intermediate (86). The similar manner of hydrogen abstraction is employed in the reactions of a non-heme-diiron enzyme, myo-inositol oxygenase (MIOX) (87), and a non-heme-iron enzyme, Isopenicillin N synthase (IPNS) (88). This DFT study indicates that the initial H-abstraction step is a rate-limiting step, which implies the possibility of trapping and characterizing the postulated H-abstraction intermediate, Fe(III)-OO⁻ species (86). According to these DFT calculations carried by Haiko et al., an Fe(III)-OH/methylphosphonate radical should be generated before the formation of product and such a methylphosphonate radical can readily rotate around the C-P bond (86). Thus, the attack of methylphosphonate radical on Fe(III)-OH can occur from either side of the plane defined by methylphosphonate radical, which is able to account for the loss of stereochemistry of HMP at C1 (86, 89).
Collectively, on the basis of these biochemical studies, as well as substrate analogue studies, site-directed mutagenesis, and DFT calculations, two plausible mechanisms were formulated, as shown in Scheme 2-2 (31, 84-86). The detailed description of these two proposed mechanisms has been given in chapter 2. In both mechanisms, an Fe(III)-OO$^*$ species is proposed to be the C2-H bond cleaving intermediate, however, there is no direct spectroscopic or kinetic evidence provided. According to extensive studies on accumulating and characterizing intermediate species in Bollinger-Krebs group, the hydrogen abstracting intermediate might be trapped and identified using rapid-kinetic and spectroscopic methods (90). In consequence, the accumulation of H-abstraction intermediate is critical for direct intermediate characterization. In this study, to accumulate the postulated C-H-cleaving intermediate, Fe(III)-OO$^*$ species, a HEPD variant, E176A, which lacks the carboxylate ligand of the “facial triad”, is used in rapid-kinetic and spectroscopic experiments. Strikingly, in this study a solvent-exchangeable H-abstraction high spin Fe(IV)-oxo intermediate ($S=2$) was trapped instead of Fe(III)-OO$^*$ species, leading to a complete reconsideration of the previously proposed mechanism. The study described in chapter 2 will focus on the trap and characterization of an intermediate involving in HEDP catalysis, which contributes to a better understanding of the mechanism of HEPD catalysis.
1.2.3 MPnS catalyzes a novel oxidative C-C cleaving reaction of 2-HEP

Recently, during the investigation of methylphosphonate (MP) biosynthesis, van der Donk et al. discovered a novel enzyme, methylphosphonate synthase (MPnS), catalyzing the last step in the biosynthetic pathway of MP (5). MPnS requires only Fe(II) and O$_2$ to catalyze the conversion of 2-HEP to MP and CO$_2$ (5) without the requirement of exogenous electrons from either cosubstrates or reductants. This fact suggests that MPnS might also employ an Fe(III)-OO$^\bullet$ intermediate to finish the initial C-H bond cleaving step, as HEPD does.

The isotopic labeling studies indicated that the C1 and C2 of 2-HEP were retained in MP and CO$_2$, respectively, which suggests that MPnS catalyzes an oxidative C-C cleaving reaction (5). When (S)-[2-$^2$H$_1$]-2-HEP or (R)-[2-$^2$H$_1$]-2-HEP was presented to MPnS as a substrate, the C2-pro-S hydrogen was abstracted and C2-pro-R hydrogen was transferred to the methyl group of the final product MP (91). Thus, MPnS may use the proposed Fe(III)-OO$^\bullet$ intermediate to remove a hydrogen from C2-pro-S position in 2-HEP, and the same C-H cleaving step has been proposed in the HEPD-catalyzed reaction with 2-HEP. Such a hypothesis agrees with the X-ray structure of HEPD•Cd(II)-2-HEP (4) and the sequence homology between HEPD and MPnS (91). The studies of a substrate analogue, S-HPP, suggested that S-HPP was not a substrate for MPnS in that no O$_2$ consumption was detected (91). However, when R-HPP was presented to MPnS, nearly all the R-HPP was converted to 2-OPP and a small amount of MP and phosphate, as indicated by the analysis of $^{31}$P-NMP spectra (91). MPnS, HEPD and HppE catalyze the same transformation from R-HPP to 2-OPP with O$_2$ and H$_2$O$_2$ as an oxidant (31, 55, 92). Furthermore, HEPD and MPnS effect the oxidative C-C cleaving reactions of the same substrate, 2-HEP, with O$_2$ as a cosubstrate and share the same Fe(II)-coordinating ligands, two histidine residues (4, 91). Additionally, the HEPD variant, E176A, lacking the carboxylate ligand of “2H1C” facial triad, is structurally similar to MPnS and retains the activity of wild-type HEPD, as suggested by the
study in chapter 2. All these similarities between HEPD and MPnS suggest that there might be some correlation between the activities and catalytic mechanisms of HEPD and MPnS.

What's more, MPnS also has some similar features with HppE in structure and reaction. As above mentioned, both of them is able to convert $R$-HPP to 2-OPP with different oxidants ($H_2O_2$ used by HppE and $O_2$ used by MPnS). The natural substrates of MPnS and HppE, namely 2-HEP and S-HPP, both possess the phosphonate group and hydroxyl group and 2-HEP is just one carbon unit less than S-HPP. Structurally, both HppE and MPnS employ two histidine residues to chelate the active site Fe(II), as the members of non-heme-iron(II) enzyme superfamily. All these similarities suggest that there also might be some correlation between the reactivities and catalytic mechanisms of HppE and MPnS. Therefore, in chapter 3, the correlations among three enzymes in reactivities and mechanisms will be discussed.
1.3 The stereospecific C-H activation in HppE, HEPD and MPnS catalysis

Although, the three non-heme-iron(II) enzymes, HppE, HEPD and MPnS, catalyze completely different reactions, these reactions all involve the stereospecific C-H bond cleavage. In the biological system, high-valent and mid-valent Fe-oxo intermediates are ordinarily used to activate the high-energy C-H bond (BDE = 98.7 kcal/mol in methane). O₂-activating non-heme-iron enzymes are known to catalyze remarkably diverse oxidation reactions using an equally remarkably breadth of reaction mechanisms (93). Several well-described mechanistic intermediates include Fe^{III}-superoxo, Fe^{III}-hydroperoxo and Fe^{IV}-oxo species. In the following sections, how these Fe-oxo species activate C-H bond will be discussed in detail.
1.3.1 The C-H bond activation by Fe$^{III}$-superoxo intermediate

Enzymatic C-H bond activation by Fe$^{III}$-superoxo complex has emerged in recent several years and is summarized in the following paragraphs (94). Both HEPD and MPnS may employ this Fe-oxo species to abstract a hydrogen atom from the C2-pro-S position of 2-HEP.

**MIOX, PhnZ and HD-domain family**

Myo-inositol oxygenase (MIOX) was the first non-heme-iron enzyme, reported to employ an Fe$^{III}$-superoxo complex for cleaving aliphatic hydrocarbon bond. MIOX, a member of HD-domain protein family, is a non-heme-diiron monooxygenase, effecting a four-electron oxidation of myo-inositol (cyclohexan-1,2,3,4,5,6-hexa-ol, MI) to D-glucuronate (DG) (Figure 1-20A) (95). HD-domain family possesses a conservative α-helix containing the HX$_n$HDX$_n$D motif, which coordinates the divalent metal cofactor (96). MIOX catalyzed reaction initiates the only pathway in humans for catabolism of MI, the sugar backbone of cell-signaling phosphoinositides (97). Additionally, there is considerable evidence that both type-1 and type-2 diabetes are associated with altered inositol metabolism, especially MI (98). MIOX activates MI and O$_2$ via a mixed-valent (Fe$^{II}$/Fe$^{III}$) diiron cofactor probably at electron rich Fe$^{II}$ site, which distinguishes MIOX from other previously well-studied non-heme-diiron enzymes (94). These previously well-studied non-heme-diiron enzymes ordinarily employ Fe$^{II}$/Fe$^{II}$ diiron cofactor in the active form (Figure 1-20B) (93). Various considerations imply that Fe1 in the HD domain of MIOX is likely to be the Fe$^{II}$ site and Fe2 is the Fe$^{III}$ site (94). The crystal structure of MI-bound MIOX suggested that MI coordinates to Fe2 site (Fe$^{III}$ site) via O1 and O6, though the crystal structure almost certainly represents the inactive almost certainly represents inactive Fe$_2$$^{III/III}$ MIOX (98). Thus, the crystal structure suggested a division of labor between the two irons of MIOX: substrate activation at Fe2 site (Fe$^{III}$) and O$_2$ activation at Fe1 site (Fe$^{II}$). The C1-H bond is expected to be activated by the ionization of a heteroatom, O1 (99). A intermediate G had been trapped and assigned as an Fe$_2$$^{III/III}$-superoxo complex based on EPR spectroscopic evidence and the significant deuterium
kinetic isotope effect ($^2$H-KIE $\geq 5$) on its decay established that Fe$_2^{III/III}$-superoxo complex is a C1-H bond cleaving species (Figure 1-20B) (93, 95). The C1-H scission step results in an Fe$_2^{III/III}$-OOH complex and a C1-centered MI radical, and since then the reaction mechanisms bifurcate. The Fe$_2^{III/III}$-OOH complex undergoes either a homolytic O-O bond cleavage, which leads to the hydroxylation of the C1-radical, or a Fe2-O bond cleavage, which contributes to the hydroperoxylation of the C1-radical (Figure 1-20B). Then C1-C6 bond cleavage results in the formation of DG (Figure 1-20B).
Figure 1-20. The reactions catalyzed by enzymes belonging to the HD-domain family and the proposed mechanisms. (A) Reaction catalyzed by MIOX; (B) proposed mechanisms of MIOX catalyzed reaction; (C) PhnZ-catalyzed reaction. Adapted and modified from Ref. (94-96).
Another member of HD-domain family, PhnZ, catalyzes the conversion of 2-amino-1-hydroxyethylphosphonate to glycine and phosphate, culminating the 2-aminoethylphosphonate metabolism path in bacteria (Figure 1-20C) (96). Like MIOX, PhnZ wields a mixed-valent diiron cofactor to activate substrate and dioxygen, and employs the Fe$_{II/III}$-superoxo complex to activate C-H bond (96). The fact that Fe$_{II/III}$-superoxo complex mediates C-H bond cleavage in MIOX and PhnZ reactions, suggested that more members of HD-domain family may employ the same strategy to activate C-H bond.

Fe$_{III}$-superoxo complex of IPNS initiates β-lactam ring closure

Isopenicillin N synthase (IPNS) is a mononuclear non-heme-iron oxidase, catalyzing the formation of β-lactam and thiazolidine rings in the penicillin nucleus from a tripeptide L-δ-amino adipoyl-L-cysteinyl-D-valine (ACV) (Figure 1-21A) (93). The two ring-closure steps involve cleaving two heteroatom-activated C-H bonds along with the formation of C-S and C-N bonds, as depicted in Figure 1-21B. Two reaction intermediates are detected by combined studies using UV-vis stopped flow and rapid freeze quench Mössbauer spectroscopy studies (100). The results indicated that an Fe$_{III}$-superoxo species is likely the first intermediate that initiates the $\text{C}_\beta$-$\text{C}_{\text{Cys}}$-H bond cleavage (Figure 1-21A, green) and the β-lactam ring formation (100). This Fe$_{III}$-superoxo complex proceeds the second C-H activation intermediate, Fe$_{IV}$-oxo complex, which effects the d-valine C-H bond cleavage. Both Fe$_{III}$-superoxo and Fe$_{IV}$-oxo complexes had been trapped and fully characterized using Mössbauer spectroscopy (100); furthermore, the X-ray structural, computational and isotopologue studies all suggested that Fe$_{III}$-superoxo and Fe$_{IV}$-oxo complexes were C-H activation intermediates, and they facilitated the ring closure steps in the order as indicated in Figure 1-21B (100).

This enzymatic C-H bond activation by Fe$_{III}$-superoxo complex is characteristic of cleaving C-H bond activated by adjacent heteroatoms or π system coupled to a four-electron reduction of one molecular O$_2$ without the requirement of reducing co-substrates (93-94, 101). A
bond dissociation energy (BDE) of 91 kcal/mol has been cited for a C–H bond, which is chemically equivalent to the C1-H bond of MI in the MIOX reaction, is (102). The BDE of the β-C-H bond of L-Cys was calculated to be 93 kcal/mol (103). These bonds are relative weaker than the unactivated C-H bond cleaved by the Fe^{IV}-oxo species, like the methyl C-H bond (BDE ~98 kcal/mol) targeted by the Fe^{IV}–oxo complex in the α-ketoglutarate-dependent halogenase, CytC3, and the methane C-H bond (BDE = 98.7 kcal/mol) cleaved by the Fe_{2}^{IV/IV} complex in sMMO (94). Such analysis suggested that Fe^{III}–superoxo complexes are less potent than Fe^{IV}–oxo complexes and thus ordinarily cleave C-H bond activated by adjacent heteroatoms.
Figure 1-21. (A) Reaction catalyzed by IPNS. In this process, two hydrogens (Red and Blue) were removed from C-H bonds along with the cleavage of one N-H and one S-H bond (Green). (B) The proposed mechanism of IPNS reaction. AA denotes L-δ-amino adipoyl moiety. Adapted from ref. (93).
1.3.2 The C-H bond activation by Fe$^{III}$-hydroperoxo intermediate

Cleavage of the O-O bond of ferric-hydroperoxo complex has been considered as a key step leading to the formation of high-valent iron-oxo species, which effect the oxidation of organic substrates, in the O$_2$-activation mechanisms of cytochrome P450, Rieske dioxygenases, and even methane monooxygenase (MMO) (104-109). However, in the catalysis of these enzyme, Fe$^{III}$-hydroperoxo has never been proved to be a C-H bond cleaving species. The evidence of enzymatic C-H bond activation by Fe$^{III}$-hydroperoxo complex is in scarcity, and most studies of C-H bond activation by Fe$^{III}$-hydroperoxo complex focused on model complexes and a glycopeptide antibiotic chemotherapy agent, bleomycin (BLM) (figure 1-22A). In the following section, the C-H bond activation with the Fe$^{III}$-hydroperoxo complex in BLM catalysis will be discussed in detail.

**Bleomycin cleaving C-H bond with an Fe$^{III}$-hydroperoxo intermediate**

BLM, a kind of glycopeptides antibiotic produced by *Streptomyces verticillus*, has been used as anticancer reagent, and its therapeutic activity depends on Fe$^{II}$ and O$_2$. BLM is able to effect the single- and double-strand DNA oxidative cleavage by selectively cleaving at some GT and GC sites (110-111). BLM is suggested to provide a pentadentate ligand to the metal ion with five nitrogen atoms from histidine, a deprotonated peptide function, pyrimidine, and primary and secondary amine groups to forms a square pyramidal N5 coordination, as indicated in bold in Figure 1-22 (110-111). The six coordination site is left for the binding of exogenous ligands, like O$_2$ (110). It has been suggested that DNA degradation was carried out by activated BLM (ABLM) by cleaving C4'-H bond of deoxyribose, which results in the generation of a base propenal (112). ABLM is generated by adding one electron to dioxygen adduct of BLM-Fe(II) or by adding H$_2$O$_2$ to BLM-Fe(II), which is an analogy to the peroxide shunt pathway of cytochrome P450 (110). ABLM exhibits S=1/2 EPR signals with g value of 2.26, 2.17 and 1.94, which is
consistent with a low spin Fe(III) species, and such assignment was further corroborated by Mossbauer spectroscopy (113). EXAFS (Extended X-Ray Absorption Fine Structure) studies indicated the presence of a shell of 2.5 O/N scatters at 1.89 Å, together with a shell of 2 O/N scatters at 2.03 Å, which excludes the possibility that ABLM is an Fe(IV)-oxo complex with a short Fe-O bond (111). Solomon, E.I. and coworkers detected the kinetics of ABLM reaction and the resulting deuterium isotope effect ($k_H/k_D \approx 3$) for ABLM decay by using circular dichroism spectroscopy, which indicated the involvement of ABLM in hydrogen abstraction (114). Further evidence from density functional calculations (DFT) confirmed that ABLM is thermodynamically and kinetically competent for hydrogen abstraction (114). As BLM-Fe(II) reacting with O$_2$ or BLM-Fe(III) reacting with H$_2$O$_2$, an intermediate exhibiting similar kinetics of formation and decay to those of ABLM, and a mass to charge ration ($m/z$) agreeing with $m/z$ of BLM-Fe(III)-OOH were detected (115). The direct hydrogen abstraction by ABLM could result in a reactive Fe$^{IV}$=O species, which is able to initiate the cleavage of a second DNA strand (114). These studies of BLM provide the most well established experimental and theoretical evidence for C-H bond activation by the Fe$^{III}$-hydroperoxo complex.
Figure 1-22. Structure of bleomycin (probable metal ligating atoms indicated in bold blue).

(Inset) The crystal structure of CuII-BLM. Adapted and modified from ref. (110, 114).
1.3.3 The C-H bond activation by Fe$^{IV}$-oxo intermediate

**Groundwork of non-heme iron(IV)-oxo intermediates**

In non-heme-iron enzyme family, the high valent Fe$^{IV}$-oxo complex has been demonstrated versatile reactivities, which range from hydroxylation to halogenations, desaturation, epimerization, epoxidation, cyclization and decarboxylation (101). This Fe$^{IV}$-oxo complex is characteristic of cleaving unactivated C-H bond with a 2e¯ or 4e¯ oxidation of substrate and reduction of one equivalent dioxygen. In the 2e¯ electron oxidation effected by Fe$^{IV}$-oxo complex, two reducing equivalents are required to balance the four-electron reduction of dioxygen, which is the same mode employed by HppE to effect C-H cleavage with Fe$^{IV}$-oxo complex, as claimed by previous studies (39-40, 42). The donor of the two reducing equivalents utilized by various non-heme-iron oxidases or oxygenases includes α-KG, tetrahydrobiopterin, reduced nicotinamide and L-ascorbic acid (101).

The large diverse reactivities of Fe$^{IV}$-oxo complex could be attributed to the tuning of conserved non-heme-iron cofactor (101). Usually, three amino acid ligands of a (His)$_2$-(Asp/Glu) motif, known as “facial triad”, occupy one face of the octahedron and leave the remaining opposite face for substrate coordination (101, 116-117). HppE also utilizes the same facial triad protein ligands coordination mode (43).

**The non-heme iron(IV)-oxo intermediate**

The first Fe$^{IV}$-oxo intermediate was directly detected in the reaction of non-heme-iron enzyme, (4-hydroxyphenyl)pyruvate dioxygenase (HPPD), with 4-hydroxyphenylpyruvate and dioxygen. A transient feature at 490 nm, which formed with a second-order rate constant of $1.4 \times 10^5$ M$^{-1}$s$^{-1}$ and decayed with a catalytic relevant first-order rate constant of 7.8 s$^{-1}$, was detected by Moran and coworkers (118). However, the detected transient species has not been further characterized with spectroscopic and kinetic methods. In 2003, Carsten, Bollinger and coworkers
firstly detected and characterized a transient species in the reaction of taurine: \(\alpha\)-KG dioxygenase (TauD) (Figure 1-23), by Stopped-flow Absorption (SF) and freeze-quench (FQ) Mössbauer spectroscopy (50). This intermediate forms with a second-rate constant of \(1.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}\) and decays with a large substrate deuterium kinetic isotope effect \((k_{HD}/k_{D} \approx 50)\) (50, 116). Such a large substrate KIE indicated that this intermediate is a hydrogen-abstraction species. This species displays a maximal absorption at \(\sim 318\) nm and a new Mössbauer quadrupole doublet feature with isomer shift of \(0.31\) mm/s and quadrupole splitting of \(-0.88\) mm/s (50) (Figure 1-24). Such an intermediate has a nearly axial \(S = 2\) ground state and exhibits a positive zero-field splitting parameter of \(10.5\) cm\(^{-1}\)(50). Furthermore, its cryoreduction generates a high-spin Fe(III) species, which indicates this intermediate is a high-spin Fe(IV) species (50). The fact that the TauD intermediate has \(S = 2\) ground state and a low isomer shift (0.31 mm/s), together with the fact that cryoreduction of this intermediate generates a high-spin Fe(III) species, is strongly suggestive of a high spin Fe(IV)-oxo species. The identity of this ferryl intermediate in TauD reaction was further confirmed by resonance Raman spectroscopy, which displayed a band at \(812\) cm\(^{-1}\) that shifted to \(787\) cm\(^{-1}\) upon use of \(^{18}\)O\(_2\) (119), and X-ray absorption spectroscopy, which exhibited a short interaction of \(1.62\) Å between Fe and one of its ligands (120). Therefore, the identity of this transient species in the reaction of TauD is unequivocally established as an high-spin Fe(IV)-oxo species.

As indicated in Figure 1-24, in the reaction of another two \(\alpha\)-KG dependent enzymes, P4H and Cytc3, intermediates with the striking similar absorption features (maximum absorption at \(\sim 320\) nm) and Mössbauer features \((\delta = 0.30\) mm/s & \(\Delta E_Q = -0.82\) mm/s, \(\delta = 0.30\) mm/s & \(\Delta E_Q = -1.09\) mm/s, respectively) to those of TauD (49, 121), were detected. These studies imply that Fe\(^{IV}\)-oxo complex is a common C-H activation species shared among non-heme-iron \(\alpha\)-KG dependent enzymes. Therefore, a consensus mechanism for the non-heme-iron \(\alpha\)-KG dependent hydroxylase family is proposed, as indicated in Figure 1-25(101).
Figure 1-23. TauD catalyzed α-KG dependent hydroxylation reaction.
Figure 1-24. Spectroscopic features comparison of Fe^{IV}-oxo complex from TauD (top), P4H (middle), and CytC3 (bottom). (A) Comparison of the kinetics of Fe^{IV}-oxo complex detected by SF absorption spectra by using unlabeled (red) and selectively deuterium labeled substrates (blue); (B) Mössbauer spectra of the Fe^{IV}-oxo complex at 4.2K/53mT (left) and 4.2K/8T (right). Adapted and modified from ref. (101).
Figure 1-25. The general mechanism of α-KG-dependent dioxygenases. Adapted from ref. (101).
1.4 Outlook

1.4.1 The accumulation and characterization of the postulated substrate-derived carbocation intermediate in the HppE catalyzed reaction with R-1-HPP

In 2013, Chang et al. discovered that HppE can take R-1-HPP as a substrate and catalyze a biological unprecedented 1,2-phosphono migration reaction (Figure 1-10A) (65). The studies with substrate analogues and model reactions suggested that this unusual migration reaction probably involved a substrate-derived carbocation intermediate (Figure 1-10 and 1-11) (65). The substitution of fluoromethyl group or trifluoromethyl group for the distal methyl group of R-1-HPP, respectively, dramatically slows down the reaction rate of the 1,2-phosphono migration reaction relative to the normal reaction rate observed with R-1-HPP (Figure 1-26) (122). The similar reduction of reaction rate was also detected in the prenyltransferase-catalyzed reactions with (E)-and (Z)-3-trifluoromethyl-2-buten-1-yl pyrophosphates (Figure 1-27) (123). This prenyltransferase-effected reaction is known to undergo a carbocation intermediate (123-124).

Taken together, the reduction of reaction rate observed with fluoro-substituted analogues of R-1-HPP not only provided additional evidence for the formation of a carbocation intermediate in HppE-catalyzed phosphono-migration reaction, but also suggested the possibility of accumulating this carbocation intermediate. Therefore, using the 3-fluoro-R-1-HPP or 3,3,3-trifluoro-R-1-HPP as a substrate or inhibitor, the postulated carbocation intermediate might be accumulated and even be characterized.
Figure 1-26. HppE-catalyzed reactions with substrate analogues possessing electron-withdrawing substitutes at C3. Adapted and modified from ref. (122).
Figure 1-27. The prenyltransferase catalyzed reaction. Adapted and modified from ref. (123-124).
1.4.2 The amino acid(s) involving in the deprotonation of H$_2$O$_2$ in the HppE catalyzed peroxidation reaction

In the HppE-catalyzed peroxidation reaction, the deprotonation of H$_2$O$_2$ is required for the catalysis of HppE. In the second coordination sphere of HppE, there are two residues, which might be able to deprotonate H$_2$O$_2$ with their side chains. These two amino acids are Lys 23 and Arg 97. However, according to the X-ray structure of HppE-Fe(II)-S-HPP-NO complex, only the side chain of Lys 23 is positioned towards NO, the mimic of H$_2$O$_2$, and the distance between $\varepsilon$-NH$_3$ group of Lys 23 and the oxygen atom of NO is feasible for proton transfer (about 4.94 Å) (Figure 1-28) (43). Thus, to probe the probable function of Lys 23 as a general base, a series of HppE variants including K23A, K23M, K23C and K23R were prepared and the reactivities of each variant and wild-type HppE with S-HPP were evaluated (Figure 1-29). Intriguingly, the number of the turnover ([Fos]/[Enz]) increased, as the basicity of the side chain of each amino acid in the variants and wild-type HppE increased (Figure 1-29). Such a finding provides evidence supporting that Lys 23 is likely to act as a general base to deprotonate H$_2$O$_2$ in the HppE-catalyzed peroxidation reaction with S-HPP. Further investigations on the X-ray crystal structure of HppE-M(II)-S-HPP-H$_2$O$_2$, in which M stands for the ordinarily used metal ions in the crystallographic study, will provide a better understanding of the function of Lys 23.
Figure 1-28. The possible amino acid residues that can act as general bases in the active site of HppE
Figure 1-29. The assessment of the number of turnovers of reactions catalyzed by HppE variants. The reaction conditions are 0.10 mM each variant enzyme or wild-type HppE, 0.080 mM Fe(II), 4.0 mM S-HPP, 10 mM l-Asc, and 5.0 mM H$_2$O$_2$. This experiment was carried out as described in the ref. (55). WT denotes wild-type HppE. Fos denotes fosfomycin. Enz denotes enzyme.
1.4.3 The investigation of the iron-coordinating ligands of MPnS

MPnS is a novel type of non-heme iron(II)-dependent enzyme, catalyzing a unique oxidative C-C bond cleavage of 2-HEP to form MP and CO₂, and MP has been thought as the precursor of methane in the biosynthesis of methane in the aerobic ocean (5). Sequence alignment of the MPnS sequence with HppE and HEPD sequences led to the speculation that the conserved residues His 148 and His 190 are the Fe(II)-binding ligands of MPnS (91). According the sequence alignment, the third iron-coordinating residue might be Gln 152 (91). Substitution of these three residues with alanine, respectively, may result in the reduction of iron-binding affinity, which can be probed by analyzing the EPR spectra of the corresponding variant-Fe(II)-2-HEP-nitrosyl complex and the wild-type MPnS-Fe(II)-2-HEP-nitrosyl complex. Furthermore, the reduction or complete loss of the enzymatic activity for H148A, H190A and Q152A will provide evidence for their postulated roles as the iron-binding residues. Similar mutagenesis and spectroscopic study has been used to study the iron-binding ligands of HppE prior to the X-ray crystallographic study of HppE (125). In addition, the most direct evidence for iron-binding roles of the three residues is from the crystallographic study of MPnS, though it might not be easy to perform this study. The investigation of the iron-binding residues of MPnS will significantly contribute to the understanding of its catalytic mechanism and the promiscuity of its catalytic activities (described in chapter 3).
Reference


122. Chang, W.-c. (2011) Mechanistic studies of two iron-containing enzymes that catalyze unusual chemical transformations, In *Department of Medicinal Chemistry*, University of Texas at Austin, Austin, TX, USA.


Chapter 2

Characterization of an Unexpected O-H Bond Cleaving Intermediate during Hydroxyethylphosphonate Dioxygenase Catalysis

2.1 Introduction

Bioactive natural product phosphonates and phosphinates are widely used in agriculture and medicine. One member of the phosphinate family, phosphinothricin (PT), is the active component in commercial herbicides (Liberty, Basta, and Ignite) and widely used in conjunction with transgenic crop, like corn, soybean, cotton and canola (1). Investigation into PT biosynthesis revealed an unusual carbon-carbon bond cleaving transformation in the conversion of 2-hydroxyethylphosphonate (2-HEP) to hydroxymethylphosphonate (HMP) and formate. This transformation is catalyzed by a previously uncharacterized enzyme, 2-hydroxyethylphosphonate dioxygenase (HEPD) (Scheme 2-1) (1).
Scheme 2-1. The reaction catalyzed by HEPD in the biosynthesis of PT.
The unprecedented reaction catalyzed by HEPD involves the cleavage of an unactivated $sp^3–sp^3$ carbon-carbon bond of 2-HEP and requires only the enzyme, Fe(II) and O$_2$ to reconstitute enzyme activity (I). X-ray crystal structures of this enzyme revealed that HEPD employs two histidines and one glutamate, the typical “2-his-1-carboxylate” facial triad utilized by non-heme-iron(II) enzymes, to chelate the active site Fe(II) (I). The HEPD-catalyzed oxidative C-C cleavage of 2-HEP does not require the input of any exogenous electrons from reductants or additional cofactors or co-substrates (I), distinguishing HEPD from most members of the non-heme-iron(II) families. Previous isotopic labeling studies suggested that the oxygen atoms from O$_2$ are inserted into both HMP and formate, and C1 and C2 of 2-HEP are retained in HMP and formate, respectively (Scheme 2-1) (I). Further studies of the isotopologues of 2-HEP demonstrated the removal of C2-pro-$S$ hydrogen and the loss of stereochemistry of HMP at C1 during the catalysis (2). Also solvent-derived oxygen is partially incorporated into the hydroxyl group of HMP (I). On the basis of these experimental observations, together with substrate analogue studies, site-directed mutagenesis, and computational studies, two plausible mechanisms were formulated, as shown in Scheme 2-2 (2-5).
Scheme 2.2. Two previously proposed mechanisms that can account for the observed experimental outcomes for the transformation of 2-HEP into formate and HMP.
In both mechanisms A and B, subsequent to O₂ binding, an Fe^{III}-superoxo initiates the hydrogen atom transfer (HAT) from the C2-pro-S-H position to yield an Fe(III)-O-OH/C2• radical species (Scheme 2-2). The resulting radical intermediate is then hydroxylated (pathway A, Scheme 2-2) or undergoes a one-electron transfer to the active site iron to generate an Fe(II)-O-OH/aldehyde intermediate V (pathway B, Scheme 2-2). In pathway A, the gem-diol in intermediate II initiates the C1-C2 bond scission to generate formate and a carbanion in intermediate III. This carbonion intermediate is then oxidized to a C2-radical in intermediate IV and subsequent rebound with Fe(III)-OH affords the second product, HMP. In pathway B, a bridged peroxo intermediate VI is generated, followed by homolytic O-O bond cleavage to generate an Fe(III)-OH and a radical species VII. The radical in intermediate VII undergoes C-C bond scission to form the same methylphosphonate radical IV as in pathway A, followed by rebound with the ferric hydroxide to generate HMP.

In mechanisms A and B, an Fe^{III}-superoxo species is postulated to be the C-H bond cleaving intermediate, however, there is no direct spectroscopic or kinetic evidence provided for this intermediate. Preceding studies suggest that the hydrogen abstracting intermediate might be trapped and identified by employing rapid-kinetic and spectroscopic methods (6). Thus, the accumulation of the hydrogen abstracting intermediate is essential for direct intermediate characterization. Previous studies in Bollinger-Krebs group suggests that the observation of a steady-state kinetic isotope effect (KIE) on kcat is a positive sign of the accumulation of an C-H cleaving intermediate, which effects the chemical transformation step (7). Based on this criteria established by Bollinger-Krebs group for assessing, by steady-state kinetics, whether a C-H-cleaving intermediate might accumulate, the HEPD variant, E176A, is found to be a better candidate than wild-type HEPD. In the variant E176A, the carboxylate-ligand of the “facial triad”, Glu 176, is substituted with Ala. To further investigate the mechanism of catalysis of
HEPD, in this study, rapid-kinetic and spectroscopic methods and HEPD-E176A variant were employed to trap and characterize the putative C-H cleaving intermediate, Fe$^{III}$-superoxo species. However, instead an Fe(IV)-oxo intermediate was trapped and characterized instead, necessitating a complete reconsideration of the previously proposed mechanism.

2.2 Results

2.2.1 A steady-state kinetic isotope effect (KIE) on $k_{cat}$ is observed.

Based on the extensive studies carried out in Bollinger-Krebs group on accumulating /trapping reactive C-H cleaving intermediates, observation of a steady-state KIE on $k_{cat}$ is considered a positive sign of accumulating C-H cleaving intermediates (6). In order to assess whether the wild-type HEPD was a suitable candidate for the accumulation of C-H cleaving intermediate in rapid-kinetic and spectroscopic studies, the kinetics of wild-type HEPD-effected reactions under steady state conditions with 2-HEP and 2-[2-$^2$H$_2$]-HEP, respectively, were determined using an O$_2$ electrode. Previous investigations suggested that wild-type HEPD oxidizes 2-HEP with a catalytic efficiency typical of an enzyme involved in secondary metabolism (Table 2-1) (4). Wild-type HEPD reactions with each 2-HEP and 2-[2,2-$^2$H$_2$]-HEP exhibited a minimal KIE on $k_{cat}$ (Table 2-1). This observation contradicts the DFT calculations which predicted the rate-limiting step as the initial hydrogen atom abstraction from C2 of 2-HEP (2, 5, 8-9). It is possible that the intrinsic KIE of the C-H cleavage step is masked under steady state conditions, which might account for the observed minimal KIE of wild-type HEPD reaction. As a result, the reaction of wild-type HEPD with 2-[2,2-$^2$H$_2$]-HEP did not look promising in regards to trapping a reaction intermediate. As an alternative, a series of variants were prepared and their steady-state kinetics evaluated. The variant E176A, where the iron-coordinating
glutamate was mutated to alanine, was over-expressed in *E. coli* and the variant protein was purified. HEPD-E176A produces the same products, HMP and formic acid with 2-HEP and O₂, as wild-type HEPD (Figure 2-1). However, E176A displayed diminished affinity for Fe(II) compared to that of wild-type HEPD, which gains full activity upon reconstitution with one equivalent Fe(II) (Data was not shown). After determining the Fe(II) concentration that resulted in maximum activity of E176A, the Michaelis-Menten kinetics of 2-HEP and 2-[2,2-H₂]-HEP oxidation were determined in a manner similar to WT-HEPD (Table 2-1). Interestingly, for HEPD E176A, a small but distinct KIE on $k_{cat}$ was observed under steady state conditions (Table 2-1). The substitution of glutamate to alanine might afford such an even larger KIE. Furthermore, E176A was able to be saturated by both substrates (2-HEP and O₂), exhibited KIEs associated with a hydrogen abstraction event, and produced the wild-type products. Thus we determined E176A to be suitable for spectroscopic studies.
<table>
<thead>
<tr>
<th>System</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( K_{\text{m,2-HEP}} ) (μM)</th>
<th>( k_{\text{cat}}/K_{\text{m,2-HEP}} ) (M(^{+}) s(^{-1}))</th>
<th>( K_{\text{m,02}} ) (μM)</th>
<th>( k_{\text{cat}}/K_{\text{m,02}} ) (M(^{+}) s(^{-1}))</th>
<th>KIE (( k_{\text{cat}} ))</th>
<th>KIE (( k_{\text{cat}}/K_{\text{m,2-HEP}} ))</th>
<th>KIE (( k_{\text{cat}}/K_{\text{m,02}} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-HEPD, 2-HEP(^a)</td>
<td>0.35 ± 0.04</td>
<td>10 ± 1</td>
<td>(3.5 ± 0.7) x 10(^{4})</td>
<td>15 ± 4</td>
<td>(2.2 ± 0.6) x 10(^{3})</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>4.5 ± 1.4</td>
</tr>
<tr>
<td>WT-HEPD, 2-[2,2-(\text{H}_{2})]-HEP</td>
<td>0.35 ± 0.06</td>
<td>14 ± 2</td>
<td>(3.5 ± 0.9) x 10(^{4})</td>
<td>63 ± 9</td>
<td>(4.8 ± 0.8) x 10(^{3})</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>HEPD-E176A, 2-HEP</td>
<td>0.75 ± 0.06</td>
<td>17 ± 2</td>
<td>(4.5 ± 0.7) x 10(^{4})</td>
<td>7.1 ± 1.4</td>
<td>(9.9 ± 1.9) x 10(^{3})</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>HEPD-E176A, 2-[2,2-(\text{H}_{2})]-HEP</td>
<td>0.65 ± 0.02</td>
<td>21 ± 3</td>
<td>(3.1 ± 0.4) x 10(^{4})</td>
<td>28 ± 3</td>
<td>(2.3 ± 0.3) x 10(^{3})</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>4.3 ± 1.0</td>
</tr>
</tbody>
</table>

**Table 2-1.** Michaelis-Menten parameters for wild-type HEPD and variant E176A under steady-state.  
\(^a\) adapted from reference (4). WT-HEPD denotes wild-type HEPD.
Figure 2-1. Identification of the products generated in HEPD-E176A reaction. a, $^{31}$P NMR was used to distinguish HEPD-E176A produced HMP (17.5 ppm) from 2-HEP (20 ppm). b, Formate was determined by monitoring the formyl-nitrophenylhydrazide adduct in mass spectrometry as previously described (10) (black, wild-type HEPD; blue, HEPD-E176A; red, unreacted HEPD-E176A control).
2.2.2 Stopped-flow (SF) absorption evidence for accumulation of a long stretch intermediate in HEPD-E176A reaction

To assess the possibility of build-up of a ferric-superoxide intermediate in the HEPD-E176A-effected reaction, SF experiments initiated by mixing HEPD-E176A•Fe(II)•substrate complex with O$_2$-saturated buffer at 5°C or mixing HEPD-E176A•Fe(II) with O$_2$-saturated solution containing substrate at 5°C were carried out, as shown in Figure 2-2. Intriguingly, the time dependent SF absorption spectra acquired in the reaction of HEPD-E176A•Fe(II)•2-[2,2-$^2$H$_2$]-HEP with excess O$_2$ provides clear evidence for the accumulation of an intermediate state with absorption feature centered at ~320 nm. It develops to its maximum extent within the first 30 to 40 ms and decays within ~1 s (Figure 2-2, blue). Similar intermediate species absorbing at 320 nm have been detected in a group of reactions catalyzed by non-heme-iron dependent enzymes, including TauD, SyrB2, and CarC (11-13). These intermediates have been assigned to an Fe(IV)-oxo species based on the Mössbauer spectroscopy and Resonance Raman spectroscopy (14), as discussed in Chapter 1. Although in the reaction of HEPD-E176A•Fe(II)•2-HEP with O$_2$ the formation of an intermediate state exhibiting an identical absorption feature at 320 nm was not detected, its decay was still observable (Figure 2-2, black). The absorption of this intermediate decays within an earlier time range (~ 300 ms) (Figure 2-2, black). Comparison of the SF absorption of reaction with 2-[2,2-$^2$H$_2$]-HEP (Figure 2-2, blue) to that of reaction with unlabelled 2-HEP (Figure 2-2, black trace) is suggestive of a large kinetic isotopic effect on the decay of this intermediate.

Furthermore, when HEPD-E176A reconstituted with Fe(II) was mixed against O$_2$-saturated buffer containing 2-[2,2-$^2$H$_2$]-HEP, the formation of the intermediate absorbing at 320 nm was delayed and displayed a substantially diminished intensity (Figure 2-2, red) compared to
that of the reaction of E176A•Fe(II) •2-[2,2-2H2]-HEP complex mixed with excess O2. Such a delayed formation of the intermediate state might indicate that the substrate binding step may be partially rate limiting in HEPD-E176A catalysis and could even mask the chemical transformation step in the wild-type HEPD catalyzed reaction.
Figure 2-2. SF experiments with HEPD-E176A. **Blue,** 1.0 mM HEPD-E176A was reconstituted with 0.80 mM Fe(II) and 6.0 mM 2-[2,2-\(^{2}\)H\(_{2}\)]-HEP and then reacted with O\(_{2}\)-saturated buffer (~1.9 mM at 5\(^\circ\)C) in a 1:1 mix. **Red,** HEPD-E176A was reconstituted with Fe(II) and then reacted with 2-[2,2-\(^{2}\)H\(_{2}\)]-HEP in oxygenated buffer under same conditions as above. **Black,** HEPD-E176A was reconstituted with Fe(II) and then reacted with 2-HEP in oxygenated buffer under same conditions as above.
2.2.3 An Fe(IV)=O species is trapped and characterized by Mössbauer spectroscopy

The nature of the intermediate detected in SF experiments was further probed by rapid freeze-quench Mössbauer spectroscopy experiments. Samples were prepared by mixing E176A•Fe(II)•2-[2,2-2H₂]-HEP complex at 5 °C with O₂ saturated buffer and freeze-quenching after reaction times of 10 ms, 25 ms, 50 ms, 125 ms, and 500 ms. The sample prepared by reconstituting HEPD-E176A anaerobically with ⁵⁷Fe and 2-[2,2-²H₂]-HEP is denoted as 0-ms sample. The 4.2-K/53-mT Mössbauer spectrum of 0-ms sample (Figure 2-3A, vertical bars) can be simulated as a quadrupole doublet with an isomer shift (δ) of 1.28 mm/s and a quadrupole splitting of (ΔE_Q) of 3.18 mm/s (Figure 2-3A, blue solid line), which is likely to be attributed to the E176A•Fe(II)•2-[2,2-²H₂]-HEP complex. The analysis of the Mössbauer spectrum of 500-ms sample collected at the same conditions reveals the presence of a new quadrupole doublet associated with a δ of 1.25 mm/s and a ΔE_Q of 2.85 mm/s (Figure 2-3A, green solid line), which can be considered as the contribution of the E176A•Fe(II)•product complex. 25 ms is near the time of maximum absorbance at 320 nm of the intermediate detected in SF experiments. The 4.2-K/53-mT Mössbauer spectrum of 25-ms sample exhibits a contribution from the Fe(II) site of the reactant complex (δ = 1.28 mm/s and ΔE_Q = 3.18 mm/s) and an additional prominent peak at ~0.75 mm/s (Figure 2-3A). This peak is the high energy line of a quadrupole doublet with a δ of 0.22 mm/s and a ΔE_Q of 0.69 mm/s (Figure 2-3A, red solid line), parameters very similar to those of ferryl intermediates detected in other non-heme-iron oxidases and oxygenases (11-13). Similar spectral features, the absorption line at ~ 0.75 mm/s of a quadrupole doublet with a δ of 0.22 mm/s and a ΔE_Q of 0.69 mm/s, are readily observable in spectra of samples quenched at 10 ms, 50 ms and 125 ms, but almost not observable in the spectra of samples quenched at 0 ms and 500 ms. Thus, this new spectral feature (δ = 0.22 mm/s and ΔE_Q = 0.69 mm/s) can be assigned to a transient Fe(IV)-oxo intermediate.
The different spectrum of 0-ms sample and 25-ms sample and that of 500-ms sample and 25-ms sample (Figure 2-3B) might be clearly display the conversion of E176A●Fe(II)●2-[2,2-\textsuperscript{3}H\textsubscript{2}]-HEP complex to the Fe(IV)-oxo intermediate and the subsequent conversion Fe(IV)-oxo intermediate to the E176A●Fe(II)●product complex, respectively. Based on all the above analysis, the Mössbauer spectra of the time-dependent samples prepared by quenching at 10, 25, 50, 125 and 500 ms can be best simulated as a weighted superposition of contributions from three Fe-containing species, namely the E176A●Fe(II)●2-[2,2-\textsuperscript{2}H\textsubscript{2}]-HEP reactant complex, Fe(IV)-oxo intermediate, and E176A●Fe(II)●product complex, which are shown as solid black lines overlaying corresponding experimental spectra in Figure 2-3A. The deconvolution of these time-dependent Mössbauer spectra enables the quantification of each individual component presented at available time point independently from those obtained from SF absorption and the kinetics of HMP formation determined in the following chemical quench experiments. Furthermore, this deconvolution of these spectra allows for the determination of an extinction coefficient (Ɛ = 1500 M\textsuperscript{-1}cm\textsuperscript{-1}) of the Fe(IV)-oxo intermediate detected in the SF experiments (12, 15). This value is consistent with the extinction coefficient of Fe(IV)-oxo species reported in previous investigations. Thus, the Mössbauer spectra demonstrate the accumulation of an Fe(IV)-oxo intermediate in the reaction of HEPD-E176A●Fe(II)●2-[2,2-\textsuperscript{2}H\textsubscript{2}]-HEP complex with O\textsubscript{2}.

Additionally, this ferryl intermediate was not detected in the Mössbauer spectra of samples prepared by mixing HEPD-E176A●Fe(II)●2-HEP complex at 5 °C with O\textsubscript{2} and quenching at the earliest possible time point (≤ 10 ms). However, when wild-type HEPD reacted with 2-[2,2-\textsuperscript{2}H\textsubscript{2}]-HEP anaerobically in the presence of chlorite dismutase/chlorite, an in situ rapid O\textsubscript{2}-generating system producing up to 10 mM O\textsubscript{2}, for 10 ms, 25 ms, 80 ms and 2 s, the same Fe(IV)-oxo intermediate with a δ of 0.22 mm/s and a ΔE\textsubscript{Q} of 0.69 mm/s was observed (Figure 2-4).
Figure 2-3. (A) Mössbauer spectra (4.2 K/53 mT) illustrating the time-dependent formation and decay of a Fe(IV)-oxo intermediate. Solid blue line in the top spectrum is a simulation of the spectrum of the presumptive reactant complex, HEPD-E176A•Fe(II)•2-[2,2-2H2]-HEP. Solid red line above the spectrum of sample reacting for 25ms is a simulation of the spectrum of a high spin Fe(IV)-oxo species. Solid green line above the bottom spectrum is a simulation of the spectrum of the presumptive product complex, HEPD-E176A•Fe(II)•HMP. (B) Different spectra of samples reacting for different amounts of time, demonstrating a high spin Fe(IV)-oxo species with an isomer shift (δ) of 0.22 mm/s and a quadrupole splitting (ΔE_Q) of 0.69 mm/s.
Figure 2-4. Mössbauer spectra (4.2 K/53 mT) illustrating the time-dependent formation and decay of a Fe(IV)-oxo intermediate in the reaction of wild-type HEPD with [2,2-H]-HEP. **Top**: wild-type HEPD reacting with [2,2-H]-HEP and O_{2}, which was generated by chlorite dismutase/chlorite system. The reactions were quenched at 10, 25, 80, and 200 ms after initiation. The simulation of the spectra of a high spin ferryl species is shown in solid red line. **Bottom**: wild-type HEPD reacting with [2,2-H]-HEP and O_{2}-saturated buffer at 4°C and freeze-quenched at 20 ms after mixing.
2.2.4 The Fe(IV)=O intermediate might be kinetically competent during the catalysis of HEPD-E176A

To evaluate the kinetic competency of the Fe(IV)-oxo intermediate, the relative quantity of Fe(IV)-oxo species as determined with Mössbauer spectroscopy (Figure 2-3A) and SF absorbance at 320 nm generated in the reaction of HEPD-E176A•Fe(II)•2-[2,2-2H2]-HEP with O2 (Figure 2-2) were analyzed by curve-fit. The curve-fit of the relative quantity of Fe(IV)-oxo species with an equation describing the formation of intermediate B from reactant A and the decay of B to product C (A → B → C; A to B is controlled by \( k_{\text{form}} \) and B to C is controlled by \( k_{\text{dec}} \)) yields a ferryl formation rate of \( k_{\text{form}} = (7.4 \pm 3.9) \times 10^1 \text{ s}^{-1} \) and a decay rate of \( k_{\text{dec}} = (1.1 \pm 0.5) \times 10^1 \text{ s}^{-1} \) (Figure 2-5A, dashed red line), and values are in a good agreement with the rate constants derived from curve-fitting SF traces \([k_{\text{form}} = (6.9 \pm 0.8) \times 10^1 \text{ s}^{-1} \text{ and } k_{\text{dec}} = (1.1 \pm 0.1) \times 10^1 \text{ s}^{-1} \); Figure 2-5A, dashed blue line]. The agreement of the kinetics of formation/decay of ferryl intermediate observed in SF absorbance and Mössbauer spectra suggests its kinetic competency in HEPD catalysis.

Additional evidence that the Fe(IV)-oxo species is kinetically competent in HEPD-E176A catalysis, arises from chemical quench (CQ)/LC-MS experiments. The kinetics of HMP production were determined at 5°C with 0.40 mM E176A•Fe(II), 3.0 mM 2-[2,2-2H2]-HEP, and ~0.85 mM O2 with chemical quench (CQ)/LC-MS experiments in the similar manner to the previous study (16). The simulation of the kinetics data derived from CQ experiments requires a rate constant of 12 s\(^{-1}\) for the formation of HMP (Figure 2-5B, dashed pink line). Such a rate constant of HMP formation is in good agreement with the decay rate of ferryl species derived from the SF absorption \([k_{\text{form}} = (6.9 \pm 0.8) \times 10^1 \text{ s}^{-1} \text{ and Mössbauer spectra } [k_{\text{form}} = (7.4 \pm 3.9) \times 10^1 \text{ s}^{-1} \text{]} \) (Figure 2-5A). Furthermore, the rate constant of HMP production determined in CQ
experiment is also consistent with the rate constant of E176A•Fe(II)•HMP complex formation derived from the time-dependent Mössbauer spectra (14 s\(^{-1}\)) (Figure 2-5B, solid green line). The agreement of the decay rate constants of Fe(IV)-oxo species derived from SF absorption and from Mössbauer spectra with the rate constant of HMP formation suggests that the detected Fe(IV)-oxo species is a kinetically relevant intermediate in HEPD-E176A catalysis, rather than an intermediate in an uncoupled pathway.
Figure 2-5. (A) Kinetics of the trapped ferryl intermediate. Overlay of the relative quantity of Fe(IV)-oxo species as determined with Mössbauer spectroscopy (red dot) and the SF absorbance at 320 nm generated as HEPD-E176A•Fe(II)•2-[2,2-²H₂]-HEP reacting with O₂ (blue curve). The curve-fit of the Mössbauer data was illustrated with dashed red line. The curve-fit of the SF data was illustrated with dashed blue line. (B) Kinetics of HMP formation. Overlay of the relative quantity of E176A•HMP-complex species as determined with Mössbauer spectroscopy (green dot) and the production of HMP determined with CQ experiment (pink dot). The CQ samples were prepared by mixing a solution containing 1.0 mM HppE, 0.80 mM Fe²⁺, and 6.0 mM 2-[2,2-²H₂]-HEP with an equal volume of O₂-saturated buffer at 5°C and allowing the reaction to proceed at 5 °C for the indicated time before quenching by mixing reaction solution with 5-fold volume of the "quench" solution (80% isopropanol/20% acetic acid, v/v). The CQ data are from three independent trials, with duplicate points in each trial. The simulation of the Mössbauer data was illustrated with dashed green curve. The simulation of the CQ data was illustrated with dashed pink curve.
2.2.5 Solvent isotope effect (SIE) suggests that Fe(IV)=O complex may abstract a solvent-exchangeable hydrogen atom

Although an Fe(III)-superoxo complex was expected to be responsible for the initial hydrogen-abstraction from C2-pro-S of 2-HEP, in reality, a ferryl complex which is revealed by its SF absorption and Mössbauer quadrupole doublet only in the reaction with the deuterated substrate, is observed. Usually, the formation of the Fe(IV)-oxo intermediate requires 2e\(^-\) from either primary substrates or cosubstrates. In the HEPD-catalyzed reaction, 2e\(^-\) can only be obtained from the substrate, since this reaction does not require the injection of exogenous electrons. Thus, as indicated in Scheme 2-2B, the formation of Fe(IV)-oxo complex occurs after the hydrogen abstraction from C2 of 2-HEP by the Fe(III)-superoxo species, in which 2e\(^-\) can be derived from the substrate. However, the deuterium kinetic isotope effect (D-KIE) on C2-D cleavage would be anticipated to slow the ferryl intermediate formation and thereby disfavor its accumulation. A quantum mechanical/molecular mechanical (QM/MM) analysis of the HEPD reaction could help explain the conundrum of the accumulation of ferryl intermediate. This QM/MM study suggested that a ferryl intermediate might initiate C1-C2 radical fragmentation by abstracting H• from a hydroxyl group installed on C2 after initiation of the reaction by a superoxide intermediate (8, 17). In the proposed mechanism of HEPD catalysis (pathway A, scheme 2-2), the gem-diol species of Fe(IV)-oxo/gem-diol intermediate \(\text{II}\) would readily exchange with solvent. Thereby, a SIE is expected to be observed with respect to the accumulation of the Fe(IV)-oxo intermediate in the HEPD reaction pathway.

To assess the SIE of HEPD-E176A catalysis, all of the reagents were prepared in H\(_2\)O and 90% D\(_2\)O, respectively, and the pH of buffer in D\(_2\)O was adjusted. The SF absorption at 320 nm of a sample prepared by mixing E176A•Fe(II)•2-[2,2,2\(^-\)H\(_2\)]-HEP complex with O\(_2\) in 90%
D$_2$O exhibits a larger intensity than the absorption of the sample prepared in H$_2$O (Figure 2-6A). This observation was further confirmed by the increased intensity of the Mössbauer quadrupole doublet upon reaction in 90% D$_2$O with 2-[2-$^2$H$_2$]-HEP substrate (Figure 2-6B). Two freeze-quench Mössbauer samples were prepared by mixing E176A•Fe(II)•2-[2,2-$^2$H$_2$]-HEP complex with O$_2$ in 90% D$_2$O and H$_2$O, respectively, and terminating the reactions after 25 ms. The simulation of the spectrum of the sample prepared in 90% D$_2$O suggests the same Fe(IV)-oxo intermediate with $\delta = 0.22$ mm/s and $\Delta E_Q = 0.7$ mm/s (Figure 2-6B, bottom red solid line). The deconvolution of the Mössbauer spectra reveals that the sample in 90% D$_2$O is composed of ~27% ferryl intermediate (Figure 2-6B, bottom spectrum) and the sample in H$_2$O is composed of far less ferryl species (~18% ferryl species) (Figure 2-6B, top spectrum). The sample in H$_2$O contains less ferryl species than the 25-ms Mössbauer sample (~30%), as shown in Figure 2-3A and Figure 2-5A. This might result from the loss of E176A activity during the repeated buffer-exchange, when preparing the E176A in 90% D$_2$O and H$_2$O, respectively. Thus, the greater accumulation of Fe(IV)-oxo intermediate in 90% D$_2$O than H$_2$O indicated by both SF absorbance and Mössbauer spectra establishes the existence of a SIE in the E176A-effected reaction. This finding suggests that the Fe(IV)-oxo intermediate abstracts a solvent-exchangeable H•/D• originating on C2 of 2-HEP.
Figure 2-6. Increased accumulation of Fe(IV)-oxo intermediate in D₂O-buffer. (A) SF reactions of HEPD-E176A reacted with 2-[2,2-²H₂]-HEP and O₂ in buffer made in H₂O (purple line) or 90% D₂O (orange line); (B) Mössbauer spectra of HEPD-E176A reacted with 2-[2,2-²H₂]-HEP and O₂ in protiated buffer (top) or deuterated buffer (bottom). Each reaction was quenched after reacting for the same period of time (25 ms).
2.3 Discussion

Based on previous studies, mechanisms involving the abstraction of C2-pro-S-H of 2-HEP by an Fe(III)-superoxo species were proposed (Scheme 2-2) (2-3, 5). However, in this study, a ferryl complex, which is revealed by its SF absorption and Mössbauer quadrupole doublet in the HEPD-E176A-effected reaction with C2-dideuterated substrate, was observed. The kinetic competence of this ferryl intermediate has been demonstrated by both chemical quench experiments and kinetic analysis of SF absorption and Mössbauer spectra. Furthermore, a solvent isotope effect, which is suggested by the increased accumulation of ferryl in D2O solvent, clearly indicates that such a ferryl species is involved in abstracting a solvent-exchangeable hydrogen. The same Fe(IV)-oxo intermediate has been trapped in the reactions of several other non-heme-iron enzymes, including TauD, CarC, SyrB2 and tyrosine hydroxylase (11-13, 18). Each of these enzymes requires two electrons from the primary substrates and another two electrons from co-substrates, either α-ketoglutarate or tetrahydropterin, to activate dioxygen and generate Fe(IV)-oxo intermediates (11-13, 18). The HEPD catalyzed C-C cleavage reaction does not require the input of any exogenous electrons. Thus, in HEPD catalysis, the formation of the Fe(IV)-oxo complex can only occur after 2e\(^{-}\) have been extracted from the substrate. However, the deuterium kinetic isotope effect (D-KIE) on C2-H cleavage would be anticipated to slow the ferryl intermediate formation and thereby disfavor its accumulation. A QM/MM study of HEPD catalysis suggested that a ferryl specie might initiate C1-C2 radical cleavage by abstracting a hydrogen from a hydroxyl group installed on C2 after C2-pro-S-hydrogen abstraction by the ferric superoxide intermediate (8). Therefore, it is possible that the deuterium abstracted from C2 remains bound to the oxygen installed on C2 during the formation of the ferryl intermediate, and that the D-KIE on abstraction of the D\(^{•}\) from OD group then slows the decay of ferryl species and favors its accumulation.
A reformulated mechanism, which is able to account for experimental results obtained in this study, as well as the findings of previous studies, has been proposed (Scheme 2-3). In this reformulated mechanism, a ferric superoxo species I initiates the hydrogen atom transfer (HAT) from C2-pro-S-H position, resulting in an Fe(III)-O-OH/C2• species II, which undergoes a one electron transfer (ET) from the ketyl radical in II to the active site ferric ion through the coordinating hydroxyl oxygen and generates an Fe(II)-O-OH/aldehyde intermediate III. The Fe(II)-O-OH species in intermediate III could proceed through a heterolytic O-O cleavage, in which the distal oxygen employs a nucleophilic attack on the aldehyde carbon atom, leading to the formation of an Fe(IV)=O/gem-diolate complex IV. This ferryl intermediate IV performs the second hydrogen abstraction from the OH group bonded to C2 of 2-HEP to produce an Fe(III)-OH/gem-diol radical complex V. This complex initiates the C-C-bond cleavage via the well-documented β-scission pathway to yield a Fe(III)-OH/methylphosphonate radical complex VI and the product, formic acid. Subsequently, the attack of CH₂• of methylphosphonate radical on the hydroxyl group of Fe(III)-OH complex generates another product, HMP.
Scheme 2-3. A reformulated mechanism for HEPD catalysis.
HAT from C2 of 2-HEP by an Fe(III)-OO$^*$ intermediate. HEPD effects the C-C-cleaving 4e$^-$ oxidation of 2-HEP to formic acid and HMP without the assistance of any co-substrate or reductant, which requires that O$_2$ is activated to an Fe(III)-superoxo species to cleave the C2-pro-S-H bond. Such Fe(III)-superoxo and Fe(III)$_2$-superoxo intermediates have been demonstrated to cleave the adjacent heteroatom activated C-H bonds in two other cosubstrate-independent oxidases/oxygenase, isopenicillin N synthase (IPNS) and myo-inositol oxygenase (MIOX), respectively (19-20). Further, both DFT calculations and QM/MM studies suggest that the HEPD reaction starts with HAT from the C2 position of 2-HEP by an Fe(III)-superoxo species, which results in the formation of Fe(III)-O-OH/C2• species II (5, 9, 17).

The formation of Fe(IV)=O/gem-diolate intermediate IV through an Fe(II)-O-OH/aldehyde intermediate III. Upon the formation of the ketyl radical species in intermediate II, the electron on the ketyl radical is transferred to the active site ferric ion through the coordinating hydroxyl oxygen, yielding an Fe(II)-O-OH/aldehyde complex III. DFT calculations as well as studies of hydroxypropylphosphonate epoxidase (HppE) reaction with (R)-2-hydroxypropylphosphonate (R-HPP) and other analogues suggested the possibility of a rapid electron transfer from the ketyl radical to the Fe(II) ion (5, 21-22). The subsequent heterolytic O-O bond scission in Fe(II)-O-OH species results in a nucleophilic attack of the distal oxygen on the aldehyde carbon, affording a ferryl species in IV, the key intermediate discussed in this study. The accumulation of the ferryl species was strongly supported by the evidence of SF absorption and Mössbauer spectra of samples of HEPD-E176A reacting with 2-[2,2-²H$_2$]-HEP.

The O-H bond cleavage carried out by Fe(IV)≡O species in intermediate IV. In this reformulated mechanism, an Fe(IV)≡O species was proposed to be responsible for hydrogen abstraction from the OH group installed on C2 of 2-HEP in the formation of the ferryl species, resulting in an Fe(III)-OH/gem-diolyl radical complex V. The proposed ferryl-catalyzed O-H
bond cleavage step agrees well with experimental observations acquired in this study and previous studies. Firstly, the accumulation of ferryl intermediate was observed in the reaction of E176A with 2-[2,2-\(^2\)H\(_2\)]-HEP rather than with 2-HEP. Such fact suggested that the ferryl was involved in a hydrogen atom abstraction and the deuterium labeling on C2 of HEP slows down the hydrogen-abtracting step from IV to V (Scheme 2-3), which allows the build-up of ferryl.

Secondly, a significantly greater accumulation of Fe(IV)=O intermediate was detected in the reaction of HEPD-E176A with deuterated substrate in D\(_2\)O than in H\(_2\)O. This observation provides the most direct evidence that the Fe(IV)=O specie abstracts a solvent-exchangeable hydrogen atom from OH group. In the proposed mechanism, intermediate states, including Fe(III)-O-O-D/C2*, the Fe(II)-O-OD/aldehyde or the Fe(IV)=O/CH(O\(_D\))O\(^-\) (gem-diolate), all have the potential for partial exchange of the D with the solvent D\(_2\)O or H\(_2\)O (scheme 2-3). Since the deuterium labeling on C2 of substrate will slow down the conversion from IV to V, the lifetime of the Fe(IV)=O/CH(O\(_D\))O\(^-\) (gem-diolate) complex IV, which would ordinarily exchange the D with solvent very rapidly, will be elongated. The prolonged lifetime of intermediate IV would allow the exchange of D with protiated solvent, which, in turn, results in less build-up of ferryl intermediate in the reaction of HEPD-E176A with 2-[2,2-\(^2\)H\(_2\)]-HEP in H\(_2\)O than that in D\(_2\)O. Similarly, in the catalysis of HEPD-E176A with 2-HEP in D\(_2\)O, the conversion of IV to V is fast, which does not allow for solvent-exchange and results in the minimal ferryl accumulation in the SF absorbance.

The postulated O-H bond cleaving ferryl intermediate could satisfactorily account for the partial incorporation of oxygen atom of solvent H\(_2\)O into the hydroxyl group of HMP (I). Nam and coworkers have demonstrated that the oxygen atom(s) of non-heme-iron model compound could be derived from the solvent (23-24). The primary alcohol of HMP is not able to exchange oxygen atoms with solvent under the reaction conditions. Thus, it is very likely that an
intermediate species exchanges its oxygen atom with water. The QM/MM study of HEPD suggest an oxo-hydroxo tautomerism mechanism proposed by Du et al. that could explain the oxygen atom exchange, as shown in scheme 2-4 (8). In this mechanism, after H$_2^{18}$O binding to the active site iron, H$_2^{18}$O is able to exchange with the $^{16}$O atom in the Fe(IV)=$^{16}$O intermediate by tautomerization, resulting in the formation of some Fe(IV)=$^{18}$O species. The ferryl-derived $^{18}$O atom would be finally incorporated into the hydroxyl group of HMP.

One barrier for the O-H bond activation by ferryl species is its relative high bond dissociation energy (BDE). Typically, the BDE of an O-H bond is at least 7-10 kcal/mol higher than that of a C-H bond (25), a typical target for Fe(IV)-oxo species. A (μ-oxo)diiron(IV) complex has been reported to activate O-H bonds in aliphatic alcohols with a small but distinguishable KIE (26). This study suggested the feasibility of O-H bond cleavage by the ferryl species. Another barrier for the O-H bond cleavage by the Fe(IV)=O species might be the non-collinear alignment of the targeted H atom of the OH group on C2 of 2-HEP with the Fe(IV)=O intermediate. However, recent studies suggest that the Fe(IV)=O intermediate can activate its substrate with the π character of the Fe(IV)=O species, when the collinear arrangement is not available (27-29). Furthermore, two separate theoretical studies of HEPD catalysis postulate that such a π attack by an Fe(IV)=O species sufficient to cleave the high energy O-H bond (5, 8). Thereby, such a π attack by the Fe(IV)=O species is likely operative in the case of HEPD catalysis.

The C-C bond cleavage of 2-HEP and new C-O bond formation of HMP The gem-diol radical in complex V initiates C-C-bond cleavage via β-scission to yield intermediate VI, Fe(III)-OH/methylphosphonate radical, and the first product, formic acid. Such C-C cleavage with β-scission has been suggested by the DFT study of HEPD catalysis (5) and the study of (μ-oxo)diiron(IV) complex that catalyzed an O-H cleaving reaction with aliphatic alcohols (26).
Following C-C bond cleavage, the CH$_2\cdot$ of the methylphosphonate radical attacks the hydroxyl group of the Fe(III)-OH complex and generates the second product, HMP. The methylphosphonate radical has been suggested to readily rotate around the C-P bond (5, 8-9) and thus the attack of the CH$_2\cdot$ moiety on the Fe(III)-OH can occur from either side, resulting in the observed loss of stereochemistry of C1 in HMP (2). The hydroxide radical rebound is typically thought to be a rapid process, as the rebound occurs with the atom from which the hydrogen atom is abstracted (5, 30-31). However, in some P450 enzymes, this rebound is not sufficiently rapid to completely suppress racemization at the targeted carbon atom (32-33). As such, the active site of HEPD may employ a rearrangement that significantly impedes very rapid rebound, which leads to the racemization of HMP.
Scheme 2-4. Oxo-Hydroxo tautomerism mechanism for $^{18}\text{O}$ exchange.
2.4 Conclusion

Non-heme Fe(IV)=O intermediates have been demonstrated or been proposed to exhibit diverse activities, including hydroxylation, halogenation, desaturation, electrophilic aromatic substitution and epimerization (11-13, 34). Although diiron(IV) model complexes have been reported to activate O-H bonds (26) and a ferryl intermediate has been hypothesized to cleave an N-H bond in the non-heme iron enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (35), to our knowledge this study provides the first experimental evidence for a non-heme iron(IV)-oxo species that cleaves an O-H bond in an enzyme effected reaction. On the basis of this study and previous theoretical study, a new mechanism of HEPD catalysis has been formulated and this postulated mechanism is able to satisfactorily account for the experimental results in this study and preceding studies (1-4). Collectively, this study on the non-heme-iron(II) dioxygenase, HEPD, will add a surprising new transformation to the known repertoire of the ferryl unit.
2.5 Experimental procedures

Materials. Wild type-HEPD and HEPD-E176A were over-expressed and purified as previously described (1). Fe(NH₄)₂(SO₄)₂ was purchased from J. T. Baker (Philipsburg, NJ). ⁵⁷Fe was purchased from Advanced Materials and Technology, Inc. (New York, NY). All reagents were used directly as obtained from the commercial sources.³¹P-NMR spectra were recorded on a Varian Unity 500 or Varian Unity Inova 600 spectrometer at the Nuclear Magnetic Resonance Facility of the Department of Chemistry, University of Illinois at Urbana-Champaign. 2-HEP, [2,2-²H₂]-HEP, 2-[2-¹³C]-HEP and [1,1-²H₂]-HMP were synthesized as previously reported (1-3).

Construction and characterization of HEPD-E176A. HEPD-E176A was constructed with the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) and the following primers (mutated codons are in blue):

Forward: 5' ggcgaactctaagtggcgcctcctactgcce 3'
Reverse: 5' gaccgtggatcaagggactctacggtgcg 3'

The identity of the products of HEPD-E176A reaction with 2-HEP were confirmed to be HMP by ³¹P NMR spectroscopy and formic acid by LC-MS after derivatization as described in previous study (3). To confirm HMP formation, HEPD-E176A activity was determined as before (3, 10) except for using 3.0 equivalent of (NH₄)₂Fe(SO₄)₂ (hereafter referred to as Fe(II)). After reacting for 2 hours, EDTA (25 mM final concentration), dithionite (10 mM final concentration), and D₂O (20% final v/v) were added, and ³¹P NMR spectrum was recorded as described previously (3). The identity of HMP in NMR spectrum was confirmed by spiking in the authentic chemical. To confirm the identity of formate, reconstituted 0.0020 mM HEPD-E176A was incubated with 2.0 mM 2-[2-¹³C]-HEP in O₂-saturated buffer at room temperature. After reacting for 5 min, reaction mix was quenched, derivatized, analyzed as previously described (2).
Stopped-flow absorption and Freeze-quench Mössbauer experiments. General procedures for stopped-flow (SF) absorption and freeze quench (FQ) Mössbauer experiments were described in references (36) and (12). In the SF experiment, an oxygen free solution of 1.0 mM HEPD-E176A, 0.80 mM FeII and 6.0 mM 2-HEP or \([2,2\text{-}^2\text{H}_2]\text{-HEP}\) was mixed at 4 °C with an equal volume of \(\text{O}_2\)-saturated solution (~1.9 mM \(\text{O}_2\)). In the FQ Mössbauer experiment, samples were prepared by mixing the \(\text{O}_2\)-free enzyme reactant solution, which contains HEPD-E176A, \(^{57}\text{Fe}^\text{II}\) and \([2,2\text{-}^2\text{H}_2]\text{-HEP}\) or 2-HEP, with equal volume of \(\text{O}_2\)-saturated solution at 4 °C and freeze-quenching after indicated reaction time, as shown in figure 2-2 and figure 2-9.

Kinetics of HMP production determined with chemical quench/LC-MS experiments. For the determination of the kinetics of HMP production, a chemical quenched (CQ) experiment was carried out using an Update Instruments (Madison, WI) model 1000 freeze-quench/chemical-quench apparatus. CQF samples were prepared by mixing a solution containing 1.0 mM HEPD-E176A, 0.80 mM FeII, and 6.0 mM \([2,2\text{-}^2\text{H}_2]\text{-HEP}\) with an equal volume of \(\text{O}_2\)-saturated buffer (~1.9 mM \(\text{O}_2\) at 4 °C) and allowing the reaction to proceed at 4 °C for the indicated time before terminating by mixing the reaction solution with "quench solution" (80% isopropanol/20% acetic acid, v/v). Samples were dried completely by using a SpeedVac concentrator (Thermo Scientific, Bellefont, PA), dissolved in water and processed through Nanosep centrifugal filters (Pall Corporation, Port Washington, NY) to remove protein debris. Samples were analyzed by LC-MS (Agilent 1200 series LC system coupled to Agilent 6410 QQQ mass spectrometer, Agilent Technologies, Santa Clara, CA) equipped with a Waters C-18 column. The substrate \([2,2\text{-}^2\text{H}_2]\text{-HEP}\) \((m/z = 127)\), product HMP \((m/z = 111)\) and internal standard \([1,1\text{-}^2\text{H}_2]\text{-HMP}\) \((m/z = 113)\) were analyzed using isocratic elution at 15% methanol/ 85% 0.1 % formic acid (v/v) in negative mode by mass spectrometry.


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Chapter 3
The Cross-Reactivities between Three Organophosphonate-Processing Enzymes, HppE, HEPD and MPnS

3.1 Introduction

Phosphonates and phosphinates are an underexploited group of bioactive compounds possessing one or two carbon-phosphorous bonds, which are more resistant to chemical hydrolysis, thermal decomposition, enzymatic degradation, and photolysis than congeneric compounds containing an O-P linkage (1). Thus, phosphonates and phosphinates display diverse applications in medicine and agriculture as antibiotics, insecticides, herbicides and fungicides by mimicking phosphate esters and carboxylic acids (1). Despite the diversity of natural phosphonates and phosphinates, their biosynthetic pathways are well conserved and the investigation of these pathways has revealed a number of biochemically unusual steps, including the epoxidation of (2S)-hydroxypropylphosphonate (S-HPP) to yield fosfomycin [(1R, 2S)-epoxypropylphosphonate] catalyzed by HppE, the carbon-carbon bond cleavage of 2-hydroxyethylphosphonate (2-HEP) to produce hydroxymethylphosphonate (HMP) and formate catalyzed by 2-hydroxyethylphosphonate dioxygenase (HEPD), and the carbon-carbon bond cleavage of the same phosphonate, 2-HEP, to generate methylphosphonate (MP) and CO₂ effected by methylphosphonate synthase (MPnS) (2-4) (Scheme 3-1).
Scheme 3-1. Reactions catalyzed by HEPD, MPnS and HppE
Three structurally related non-heme-iron (II) enzymes, HppE, HEPD and MPnS, employ the same conserved two histidine residues to coordinate the active site Fe(II), despite low overall sequence identity (5-6). HEPD is structurally most similar to HppE, in that their substrates employ the same binding mode to chelate the active site Fe(II) and they highly resemble each other in the tertiary structure (Figure 3-1) (2). 2-HEP, the shared substrate of both HEPD and MPnS is simply one carbon unit shorter than S-HPP, the natural substrate of HppE. By utilizing O2 as an oxidant, both HEPD and MPnS effect 4e\textsuperscript{-} oxidative $Csp^3$-$Csp^3$ bond cleavage of 2-HEP without the input of exogenous electrons from reductants or additional cofactors or co-substrates. However, different products are generated in each case, either HMP and formate or MP and CO\textsubscript{2}, respectively (2-3). Isotopic labeling studies suggested that both HEPD and MPnS abstract the C2-pro-S hydrogen from 2-HEP to initiate C-C cleavage reactions (5, 7). However, HppE catalyzes a 2e\textsuperscript{-} epoxidation reaction of S-HPP, rather than a 4e\textsuperscript{-} C-C cleavage reaction, and converts S-HPP to fosfomycin in stoichiometry (4, 8). To effect this 2e\textsuperscript{-} oxidation reaction, HppE utilizes H2O2 instead of O2 as a oxidant, and thus does not require exogenous electrons from either reductants or additional cofactors or cosubstrate (8). Further, HppE cleaves the C2-pro-R-H bond of S-HPP, which also distinguishes itself from MPnS and HEPD (4). Interestingly, all three enzymes are able to catalyze the 2e\textsuperscript{-} oxidation of (2R)-hydroxypropylphosphonate (R-HPP), the enantiomer of S-HPP, to 2-oxopropylphosphonate (2-OPP), but using different co-substrates (H2O2 for HppE and O2 for MPnS and HEPD, respectively) (8-10). Furthermore, the microorganisms, which are able to produce HEPD, MPnS and HppE enzymes, harbor the canonical pathway for the formation of 2-HEP (Scheme 3-2) (3).
**Scheme 3-2.** Shared biosynthetic pathway of fosfomycin, PTT and methane. PEP denotes phosphoenolpyruvate. PnPy denotes phosphonopyruvate. PnAA denotes phosphonoacetaldehyde. PTT denotes phosphinothricin tripeptide. MP denotes methylphosphonate.
Figure 3-1. (A) Superpositions of the active sites of the HppE•Fe(II)•R-HPP complex (pdb accession 3SCG) and the HEPD•Cd(II)•2-HEP complex (pdb accession 3GBF); (B) the superimposed one monomer of HEPD (cyan) and Chain A of HppE (light brown). Adapted from ref. (8).
Taken together, previous studies demonstrate that HppE, HEPD and MPnS employ the non-heme-iron as a cofactor, similar residues to chelate the active site Fe(II) and natural substrates with identical or similar chemical structures, and even catalyze the same 2e⁻ dehydrogenation reaction of R-HPP. However, in the presence of different oxidants, O₂ or H₂O₂, they catalyze different natural reactions with identical substrate or similar substrates. In the presence of O₂, HEPD and MPnS catalyze the 4e⁻-oxidative C-C cleaving reactions of 2-HEP, but in the presence of H₂O₂, HppE effects a 2e⁻-dehydrogenation reaction of S-HPP. Such a fact leads to a hypothesis that there may be a correlation between the reactivities these enzymes display and the oxidant they employed in each reaction. Explicitly, in the presence H₂O₂, HEPD and MPnS might also be able to catalyze 2e⁻-dehydrogenation reactions, and HppE might effect a 4e⁻-oxidative C-C cleaving reaction with O₂ as the oxidant. Furthermore, this postulated correlation between enzyme activity and oxidant employed may shed light on the catalytic mechanisms of three organophosphonate-processing enzymes. Therefore, the possible cross-reactivities of HppE, HEPD and MPnS with S-HPP and 2-HEP in the presence of either H₂O₂ or O₂, were probed in this study. All the chemicals used in this study and the possible products of enzymatic reactions are listed in table 3-1, and the m/z value of each compound is also included in table 3-1. The unusual cross-reactivities detected in this study suggest that these enzymes adopt different mechanisms utilizing different oxidants and imply that substrate positioning and subtle structural tuning may dictate reaction outcomes.
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Table 3-1. The structure and corresponding $m/z$ values of the chemicals involved in this study.
3.2 Results

3.2.1 HEPD catalyzed the dehydrogenation of 2-HEP by using H$_2$O$_2$ as co-substrate

As a peroxidase, HppE catalyzes the dehydrogenation reactions of $S$-HPP and $R$-HPP, using H$_2$O$_2$ as a co-substrate generating an epoxide product, fosfomycin, and a ketone product, respectively (Scheme 3-1). Likewise, HEPD might catalyze a similar dehydrogenation reaction with H$_2$O$_2$ as a co-substrate, rather than O$_2$. To test this hypothesis, I added H$_2$O$_2$ (5.0 mM) slowly to the solution of HEPD•Fe(II) (0.80 mM), l-ascorbate (10 mM), and deuterium-labeled 2-HEP at C1 or C2 (2-[1,1-$^2$H$_2$]-HEP or 2-[2,2-$^2$H$_2$]-HEP, 5.0 mM) anaerobically. Reaction mixtures were analyzed by liquid chromatography-mass spectrometry (LC-MS). All the chemical structure of each chemical involved in this study and their $m/z$ values are listed in table 3-1. In the HEPD reaction mixture with 2-[1,1-$^2$H$_2$]-HEP and H$_2$O$_2$, three new co-eluting peaks (~2 min) at mass/charge ratio ($m/z$) of 125, 124 and 123 were detected (Figure 3-2A) and substrate was slightly consumed compared with the control reaction without H$_2$O$_2$ added (Figure 3-2C). If HEPD catalyzed a dehydrogenation reaction with co-substrate H$_2$O$_2$, the peaks at $m/z$ of 125 and 124 (Figure 3-2A) might be assigned to a phosphonate aldehyde product, 2-[1,1-$^2$H$_2$]-oxoethylphosphonic acid (2-[1,1-$^2$H$_2$]-OEP), and a phosphonate epoxide product, (1,2)-[1-$^2$H$_1$]-epoxyethylphosphonate ([1-$^2$H$_1$]-EEP), respectively. The postulated product 2-[1,1-$^2$H$_2$]-OEP would ordinarily undergo exchange with solvent rapidly, and thus 2-[1-$^2$H$_1$]-OEP and 2-OEP could account for the peaks at the $m/z$ of 124 and 123. Both [1-$^2$H$_1$]-EEP and 2-[1-$^2$H$_1$]-OEP are able to contribute to the peak at $m/z$ of 124. To further evaluate the postulated dehydrogenation reaction catalyzed by HEPD, the HEPD reaction with 2-[2,2-$^2$H$_2$]-HEP and H$_2$O$_2$ was analyzed by LC-MS. Similarly, besides the peak arising from substrate, 2-[2,2-$^2$H$_2$]-HEP, at a $m/z$ of 127, only two new peaks at $m/z$ of 124 and $m/z$ of 125 with nearly the same elution time of ~2 min were
observed (Figure 3-2B). The two new peaks at \( m/z \) of 124 and \( m/z \) of 125 could be assigned to a phosphonate aldehyde, \( 2-[2-{^2}H_1]-OEP \) and a phosphonate epoxide product, \( 2-[2,2-{^2}H_2]-EEP \), respectively, both of which are not able to exchange the C2-deuterium with solvent and are in a good agreement with the postulated phosphonate aldehyde product and phosphonate epoxide product of HEPD catalyzed dehydrogenation reaction.

Thus, according to LC-MS analysis, in the presence of \( H_2O_2 \), HEPD could catalyze a dehydrogenation reaction of \( 2-HEP \), though the substrate is not fully converted. Further evidence, indicating the structure of the products of the HEPD reaction, is required to confirm the dehydrogenation reaction catalyzed by HEPD.
Figure 3-2. LC-MS analysis of HEPD effected reactions with deuterium-labeled 2-HEP and H₂O₂, anaerobically. Brown trace, m/z 127; black trace, m/z 125; red trace, m/z 124; blue trace, m/z 123. (A) HEPD•Fe(II) (0.080 mM) reacted with 2-[1,1²H₂]-HEP (5.0 mM), H₂O₂ (5.0 mM) and l-ascorbate (10 mM), anaerobically; (B) HEPD•Fe(II) (0.080 mM) reacted with 2-[2,2²H₂]-HEP (5.0 mM), H₂O₂ (5.0 mM) and l-ascorbate (10 mM), anaerobically; (C) The control reaction is without H₂O₂ added, but contained HEPD•Fe(II) (0.080 mM), 2-HEP(5.0 mM), and l-ascorbate (10 mM).
3.2.2 MPnS catalyzed the dehydrogenation of 2-HEP with H$_2$O$_2$ as oxidant

Like HppE and HEPD, MPnS might catalyze a dehydrogenation reaction using H$_2$O$_2$ as co-substrate, rather than O$_2$. To test this hypothesis, H$_2$O$_2$ (2.5 mM) was slowly added to solutions of Fe(II)-MPnS (0.080 mM), l-ascorbate(10 mM), and dideuterium-labeled 2-HEP at C1 and C2, separately, (2-[1,1-$^2$H$_2$]-HEP or 2-[2,2-$^3$H$_2$]-HEP, 2.5 mM) anaerobically. Then reaction samples were analyzed by LC-MS. All the chemical structure of each chemical involved in this study and their m/z values are listed in table 3-1.

As shown in Figure 3-3A, the intensity of the peak arising from the 2-[1,1-$^2$H$_2$]-HEP substrate at a m/z ratio of 127 with an elution time of ~3.5 min was diminished, compared to the control reaction (Figure 3-3D), in which H$_2$O$_2$ was absent. New peaks at m/z = 123, 124 and 125 with the same elution time of ~ 2 min were observed. The peaks at m/z of 125 (Figure 3-3A, black) and 124 (Figure 3-3A, red) are likely to be assigned to a phosphonate aldehyde product, 2-[1,1-$^2$H$_2$]-OEP and a phosphonate epoxide product, [1-$^2$H$_1$]-EEP, respectively, if MPnS catalyzed a dehydrogenation reaction. The proposed phosphonate aldehyde product, 2-[1,1-$^2$H$_2$]-OEP, would exchange the C1-deuterium with solvent rapidly, which is able to account for the peaks at m/z of 124 and 123 (Figure 3-3A, blue). However, both the postulated epoxide product [1-$^2$H$_1$]-EEP and 2-[1-$^2$H$_1$]-OEP, which formed due to solvent-exchange of the possible aldehyde product, are able to contribute to the peak at m/z of 124. Additionally, oxygen contamination in the MPnS reaction of 2-[1,1-$^2$H$_2$]-HEP and H$_2$O$_2$ resulted in the formation of a small amount of MP, which is consistent with a peak at m/z of 97 eluting at ~1.5 min (Figure 3-3A, Green). A similar peak at m/z of 97 eluting at ~1.5 min was also detected in the reaction solution of MPnS and 2-[1,1-$^2$H$_2$]-HEP in the presence of O$_2$ (Figure 3-3C).
Further, analysis of the MPnS reaction mixture of 2-[2,2-$^2\text{H}_2$]-HEP and $\text{H}_2\text{O}_2$ by LC-MS was conducted to support the identity of products. Besides the peak arising from the substrate 2-[2,2-$^2\text{H}_2$]-HEP at a $m/z$ of 127, only one new peak at $m/z$ of 124 with the same elution time of ~2 min was observed. This $m/z$ value agrees well with the postulated aldehyde product (2-[2-$^2\text{H}_1$]-OEP), as shown in Figure 3-3B. Such a phosphonate aldehyde product (2-[2-$^2\text{H}_1$]-OEP) is not subject to exchange with solvent. In the presence of $\text{H}_2\text{O}_2$, MPnS might catalyze a dehydrogenation reaction of 2-HEP and produce the corresponding aldehyde.
**Figure 3-3.** LC-MS analysis of MPnS reactions of deuterium-labeled 2-HEP and \( \text{H}_2\text{O}_2 \), anaerobically. Brown trace, \( m/z \) 127; green trace, \( m/z \) 97; black trace, \( m/z \) 125; red trace, \( m/z \) 124; blue trace, \( m/z \) 123. (A) MPnS•Fe(II) (0.080 mM) reacted with 2-[1,1-\(^2\text{H}\)]-HEP(2.5 mM), \( \text{H}_2\text{O}_2 \) (2.5 mM) and \( l \)-ascorbate(10 mM), anaerobically; (B) MPnS•Fe(II) (0.080 mM) reacted with 2-[2,2-\(^2\text{H}\)]-HEP (2.5 mM), \( \text{H}_2\text{O}_2 \) (2.5 mM) and \( l \)-ascorbate (10 mM), anaerobically; (C) MPnS•Fe(II) (0.080 mM) reacted with 2-[1,1-\(^2\text{H}\)]-HEP(2.5 mM) in the presence of \( \text{O}_2 \) on bench top; (D) The control reaction is without MPnS added, but contained 2-[1,1-\(^2\text{H}\)]-HEP(2.5 mM), \( \text{H}_2\text{O}_2 \) (2.5 mM) and \( l \)-ascorbate (10 mM).
The identity of the phosphonate aldehyde product was finally confirmed by $^{13}$C-NMR analysis. To investigate whether the phosphonate aldehyde is the only product of the MPnS-catalyzed reaction with 2-HEP and H$_2$O$_2$, H$_2$O$_2$ was slowly added to the solution containing $^{13}$C-enriched 2-HEP (both C1 and C2 are $^{13}$C-enriched) and MPnS•Fe(II) anaerobically at room temperature. $^1$H-NMR spectra of regular 2-HEP and $^{13}$C-enriched 2-HEP and $^{13}$C-NMR spectrum of $^{13}$C-enriched 2-HEP were shown in figure 3-4. Following removal of particulate by filtration, $^{13}$C-NMR spectroscopy was employed to identify each component in the MPnS-effected reactions. For $^{13}$C-NMR spectra, chemical shifts (δ in ppm) are provided relative to that of DMSO-$d_6$ (δ ~38 ppm). A reaction was also carried out with $^{13}$C-enriched 2-HEP and H$_2$O$_2$, but without MPnS added. Under these reaction conditions, a doublet of doublets at δ ~32 ppm and a doublet at δ ~58 ppm were observed by using $^{13}$C-NMR spectroscopy (Figure 3-5, bottom). These spectral features correspond to the substrate, $^{13}$C-enriched 2-HEP. In contrast, the $^{13}$C-NMR spectrum of reaction sample containing MPnS, H$_2$O$_2$ and $^{13}$C-enriched 2-HEP, clearly indicates the formation of a single product, 2-OEP, when the signals corresponding to substrate were nearly diminished (Figure 3-5, top). A doublet of doublets at δ ~48 ppm ($J = 38$, 103 Hz) and a doublet at δ ~204 ppm ($J = 38$ Hz) could be assigned to resonances of the C1 and C2 of 2-OEP, respectively (Figure 3-5, top). The δ of ~59-64 ppm could be assigned to the solvent or buffer salt used in this experiment. Therefore, the $^{13}$C-NMR spectra unambiguously demonstrated that MPnS could nearly fully convert 2-HEP to 2-OEP by dehydrogenation with H$_2$O$_2$ as a co-substrate.
Figure 3-4. NMR spectra of 2-HEP and $^{13}$C-enriched 2-HEP. (A) $^1$H-NMR spectra of regular 2-HEP and $^{13}$C-enriched 2-HEP; (B) $^{13}$C-NMR spectrum of $^{13}$C-enriched 2-HEP.
Figure 3-5. $^{13}$C-NMR spectroscopy analysis of MPnS reaction of $^{13}$C-enriched 2-HEP and H$_2$O$_2$ anaerobically. (Top) MPnS reaction was run to completion; (Bottom) The control reaction without MPnS added.
3.2.3 HppE is not able to convert S-HPP or R-HPP using O₂ as an oxidant

Although HppE is related to two oxygenases, MPnS and HEPD, in structure, substrates identity and biosynthetic pathway, HppE failed to show any detectable oxygenase activity by LC-MS when reacting with S-HPP or R-HPP in the presence of O₂.

3.2.4 HppE catalyzes the dehydrogenation of 2-HEP using H₂O₂

2-HEP shares the phosphonate and hydroxyl functional groups, with S-HPP, the natural substrate of HppE, but has one less carbon than S-HPP. S-HPP utilizes its C2-OH group and another phosphonate-derived OH group to chelate the Fe(II) in the active site of HppE with a bidentate mode, which leads to the postulation that 2-HEP might be able to bind to HppE and even be converted by HppE with H₂O₂ as co-substrate. To test the peroxidase activity of HppE towards 2-HEP, H₂O₂ was slowly infused to the solution of HppE•Fe(II) and 2-[1,1-²H₂]-HEP or 2-[2,2-²H₂]-HEP, and after the removal of particulate by filtration, the reaction sample was analyzed with LC-MS. All the chemical structure of each chemical involved in this study and their m/z values are listed in table 3-1.

As shown in Figure 3-6, the intensity of the peak arising from 2-[1,1-²H₂]-HEP at a m/z of 127 with an elution time of ~3.5 min was diminished (Figure 3-6A), compared to the control reaction without H₂O₂ added (Figure 3-6C), and new peaks at m/z of 123, 124 and 125 all eluting at ~ 2 min formed (Figure 3-6C). The peaks at m/z of 125 (Figure 3-6A, black) and 124 (Figure 3-6A, red) can be assigned to a phosphonate aldehyde product, 2-[1,1-²H₂]-OEP, and an epoxide product, [1-²H₁]-EEP, respectively. The phosphonate aldehyde product 2-[1,1-²H₂]-OEP would ordinarily undergo rapid exchange at the C1-deuterium with solvent, which would result in the formation of 2-[1-²H₁]-OEP and 2-OEP with m/z of 124 and 123, respectively (Figure 3-6A, red
and blue traces). However, both the postulated epoxide product, \([1-\text{H}_1]\text{-EEP}\), and aldehyde product, \(2-\text{[1-\text{H}_1]}\text{-OEP}\) contribute to the peak at \(m/z\) of 124.

Further, the HppE reaction of 2-[2,2-\text{D}_2]-HEP and \(\text{H}_2\text{O}\) was analyzed by LC-MS to confirm the identity of the products. The peak arising from the substrate 2-[2,2-\text{D}_2]-HEP at a \(m/z\) of 127 almost vanished (Figure 3-6B) compared with that of the control reaction (Figure 3-6C), and three new peaks at \(m/z\) of 125, 124 and 123 with the same elution time of \(\sim 2\) min were detected (Figure 3-6B). The peaks at \(m/z\) of 125 and 124 agree with the \(m/z\) ratio of a phosphonate epoxide product, \([2,2-\text{D}_2]\text{-EEP}\), and a phosphonate aldehyde product, \(2-[2-\text{H}_1]\text{-OEP}\), both of which are not able to exchange the C2-deuterium with solvent. Additionally, the small peak at \(m/z\) of 123 eluting at \(\sim 2\) min might result from the dehydrogenation reaction product of small amount of unlabeled 2-HEP in 2-[2,2-\text{D}_2]-HEP.
Figure 3-6. LC-MS analysis of HppE reactions of deuterium-labeled 2-HEP and H$_2$O$_2$. Brown trace, m/z 127; black trace, m/z 125, red trace; m/z 124; blue trace, m/z 123. (A) HppE•Fe(II) (0.090 mM) reacted with 2-[1,1-$^2$H$_2$]-HEP (5.0 mM) and H$_2$O$_2$ (5.0 mM); (B) HppE•Fe(II) (0.090 mM) reacted with 2-[2,2-$^2$H$_2$]-HEP(5.0 mM) and H$_2$O$_2$ (5.0 mM); (C) The control reaction is without H$_2$O$_2$ added, but contained 2-[1,1-$^2$H$_2$]-HEP (5.0 mM) and HppE•Fe(II) (0.090 mM).
The identity of the phosphonate epoxide product, EEP, and the phosphonate aldehyde product, OEP, was further probed with $^{13}$C-NMR. $\text{H}_2\text{O}_2$ was slowly added to the solution of $^{13}$C-enriched 2-HEP and HppE•Fe(II) at room temperature. After the removal of enzyme from reaction mix by filtration, $^{13}$C-NMR spectroscopy was used to identify each component in the HppE-catalyzed reaction. For $^{13}$C-NMR spectra, chemical shifts ($\delta$ in ppm) are provided relative to that of DMSO-d$_6$ ($\delta=\sim 38$ ppm). Firstly, the control sample was prepared by mixing HppE•Fe(II) and $^{13}$C-enriched 2-HEP, but without $\text{H}_2\text{O}_2$. Under such reaction conditions, a doublet of doublets at $\delta \sim 32$ ppm and a doublet at $\delta \sim 58$ ppm were observed by $^{13}$C-NMR spectroscopy (Figure 3-7, bottom), which correspond to the C1 and C2 of $^{13}$C-enriched 2-HEP, respectively. However, the $^{13}$C-NMR spectra of the reaction sample containing HppE•Fe(II), $\text{H}_2\text{O}_2$, and $^{13}$C-enriched 2-HEP, clearly revealed the presence of 2-OEP, and the signals corresponding to the substrate almost vanished (Figure 3-7, top). The doublet of doublets at $\delta \sim 48$ ppm ($J = 38, 103$ Hz) and the doublet at $\delta \sim 204$ ppm ($J = 38$ Hz) can be assigned to resonances of the C1 and C2 of 2-OEP, respectively (Figure 3-7, top). In the $^{13}$C-NMR spectra of the reaction sample, doublets arising at $\delta \sim 45$ ppm ($J = 26$ Hz) and $\delta \sim 58$ ppm ($J = 35$ Hz) can be assigned to the C1 and C2 of the epoxide product, EEP, respectively, however, the doublet arising at $\delta \sim 58$ ppm ($J = 35$ Hz) is not distinguishable with the doublet corresponding to the C2 of $^{13}$C-enriched 2-HEP. The $\delta$ of $\sim 59$ to 64 ppm stems from the solvent and buffer salt used in this study. Therefore, the $^{13}$C-NMR spectra unequivocally demonstrate that HppE could convert 2-HEP to 2-OEP by dehydrogenation with $\text{H}_2\text{O}_2$ as co-substrate, but further evidence is required to confirm the identity of the epoxide product. As a peroxidase, HppE is not only able to convert S-HPP to epoxide, fosfomycin, but catalyze the dehydrogenation reaction of 2-HEP, the natural substrate of HEPD and MPnS.
Figure 3-7. $^{13}$C-NMR spectroscopy analysis of HppE reaction of $^{13}$C-enriched 2-HEP and H$_2$O$_2$. (Top), $^{13}$C-NMR spectra of sample prepared by mixing HppE•Fe(II)•$^{13}$C-enriched 2-HEP with H$_2$O$_2$; (Bottom), $^{13}$C-NMR spectra of sample of control reaction without H$_2$O$_2$ added.
3.3 Discussion

HEPD, MPnS and HppE, three non-heme-iron (II) phosphonate-processing enzymes, have quite striking structural homology, have a canonical biosynthetic pathway, and utilize identical or similar phosphonate substrates but employ different oxidants, O₂ or H₂O₂, to effect three completely different reactions (6). In the presence of O₂, HEPD and MPnS catalyze the 4e⁻-oxidative C-C cleaving reactions of 2-HEP and insert the oxygen atoms to the final products, but in the presence of H₂O₂, HppE effects 2e⁻-dehydrogenation reactions of S-HPP and R-HPP, respectively. On the basis of this fact, the hypothesis that the reactivities of HppE, HEPD and MPnS are related to the oxidant they used in each reaction was probed in this study. As oxygenases, both HEPD and MPnS catalyze the unactivated sp⁳-sp⁳ C-C scission of 2-HEP upon O₂ used as an oxidant (2-3). However, according to the evidence provided in this study, both of them can also catalyze the dehydrogenation reaction of 2-HEP when using H₂O₂ as an oxidant in the absence of O₂. As a peroxidase, HppE is able to catalyze the 2e⁻ epoxidation reaction of S-HPP or the dehydrogenation reaction of 2-HEP with H₂O₂ as a co-substrate (8), but it fails to use O₂ to convert S-HPP and R-HPP. In this study, it was found that using H₂O₂ as oxidants, both HEPD and MPnS are able to catalyze the 2e⁻ dehydrogenation reaction of 2-HEP, but when using O₂ as oxidants, they are oxygenases, catalyzing the 4e⁻ C-C cleavage reactions. With respect to HppE, it only exhibits peroxidase activity and no oxygenase activity is detected. Therefore, this study reveals the correlation of the reactivities of three structurally related enzymes and the oxidants they used. The reactivities of HppE, HEPD and MPnS with O₂ and H₂O₂, respectively, were shown in Table 3-2. All three enzymes demonstrate the peroxidase activity, but only HEPD and MPnS exhibit the oxidative C-C-cleavage activity.
<table>
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<tr>
<th>Oxidant</th>
<th>Reaction type</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Product</th>
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<tr>
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<td>HPP</td>
<td>$\text{HO}^{\text{-}}\text{PO}_3^{2-}$</td>
<td>$\text{OH}^{\text{-}}\text{PO}_3^{2-}$ formate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MPnS</td>
<td>$\text{HO}^{\text{-}}\text{PO}_3^{2-}$</td>
<td>$\text{H}_3\text{C}^{\text{-}}\text{PO}_3^{2-}$ CO$_2$</td>
</tr>
<tr>
<td>HppE</td>
<td></td>
<td></td>
<td>$\text{OH}^{\text{-}}\text{PO}_3^{2-}$</td>
<td>No reaction</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>2e dehydrogenation</td>
<td>HPP</td>
<td>$\text{HO}^{\text{-}}\text{PO}_3^{2-}$</td>
<td>$\text{CH}_3\text{C}^{\text{-}}\text{PO}_3^{2-}$</td>
</tr>
</tbody>
</table>

**Table 3-2.** Reactions catalyzed by HppE, HEPD and MPnS with $O_2$ and $H_2O_2$ as oxidants, respectively. The structure of all the products shown in red color has been supported by the study of LC-MS and NMR spectra. The identity of all the products shown in black color was only suggested by the study of LC-MS.
HEPD, a non-heme-Fe(II) dioxygenase, catalyzes the C-C cleavage of 2-HEP and inserts one oxygen atom into each product, HMP and formate, respectively. This reaction is initiated by a stereospecific C2-pro-S hydrogen abstraction (2, 7, 10). The wild type HEPD uses the canonical “2-His-1-Carboxylate” facial triad to chelate the ferrous ion in the active site (2), but one of iron-coordinating ligands, Glu176, is not necessary for Fe(II) binding and catalysis (described in Chapter 2). Similar findings have emerged from the studies of other non-heme-iron (II) enzymes, including an asparagine hydroxylase (FIH, factor inhibiting α subunit of hypoxia-inducing factor) and halogenases, SyrB2 and CytC3 (11-12). E176A, a variant of HEPD, catalyzes the same reaction as wild-type HEPD does and proceeds through a Fe(IV)-oxo intermediate, which was trapped and characterized as a O-H cleaving species (as suggested in chapter 2). On the basis of the study, a mechanism was proposed involving the initial C2-pro-S hydrogen abstraction by an Fe(III)-superoxo species followed by an O-H cleavage by the Fe(IV)-oxo complex, a step which initiates the C-C bond cleavage of 2-HEP (Scheme 3-3, green). However, according to the isotopologue studies with LC-MS, wild-type HEPD converts 2-HEP to the corresponding phosphonate aldehyde (2-OEP) and phosphonate epoxide (EEP) as using H₂O₂ as an oxidant, rather than the products of C-C cleavage reaction with O₂ as an oxidant, although this activity is observed only in low levels. Using H₂O₂ as an oxidant in HEPD catalysis, it is not feasible to form an Fe(III)-superoxo species to mediate the initial C2-H abstraction. After deprotonating one hydrogen, H₂O₂ must bind to the Fe(II) and it is almost certain that an Fe(IV)-oxo complex is generated by the heterolytic O-O bond cleavage of H₂O₂ (Scheme 3-4). This is similar to the proposed catalytic mechanism of HppE-catalyzed epoxidation of S-HPP (8) and that of heme peroxidases (13). The finding that an Fe(IV)-oxo intermediate was involved in E176A-HEPD catalyzed C-C cleavage reaction, suggests that HEPD might be able to tune the redox potential to accommodate an Fe(IV)-oxo species in the dehydrogenation reaction. In the HppE-catalyzed reaction of S-HPP, the C1-pro-R hydrogen was positioned towards the iron-oxo complex and
removed by an Fe(IV)-oxo intermediate, followed by the formation of a new C-O bond (Scheme 3-3) (8, 14). Compared to S-HPP, the C2 of 2-HEP loses chirality, and thus both C2-H and C1-pro-R-H might be directed towards the reactive Fe(IV)-oxo species, as suggested by the structure of HEPD•Cd(II)•2-HEP (Figure 3-1 and Scheme 3-4). This may result in the formation of two dehydrogenation products, 2-OEP and EEP. Additionally, the structural similarity of the secondary coordination sphere of HEPD and HppE might account for the observed peroxidase activity of HEPD. In the active site of the peroxidase HppE, Lys23 was suggested to be necessary for catalysis based on mutational analysis (15) and crystallographic evidence which revealed that the ε amino group of Lys 23 was positioned towards NO group in the structure of the HppE-Fe(II)-NO complex (14). The site-directed mutagenesis study further established that Lys23 maybe serve as a general base to deprotonate H2O2 to facilitate its binding to Fe(II) in the active site (Figure 1-28 and Figure 1-29). Likewise, in the active site of HEPD, a lysine residue (Lys 16) was observed and envisaged to be the general base which is responsible for the deprotonation of H2O2 in the HEPD-catalyzed dehydrogenation reaction. Additionally, Tyr 105, which interacts with S-HPP in the active site of HppE, is conserved. The counterpart of Tyr 105 in HEPD is Tyr 98 (2, 15). Although, there is evidence for the peroxidase activity of HEPD, further evidence to elucidate the chemical structures of the products will contribute to a better understanding of this reaction. The biggest obstacle to characterization of reaction products is the low level of product formation. Therefore, efforts to improve the turnover number of HEPD-catalyzed dehydrogenation reaction are required.
Scheme 3-3. Proposed mechanisms of non-heme-iron enzymes, HppE, HEPD and MPnS with native substrates.
**Scheme 3-4.** Proposed mechanisms of dehydrogenation reactions catalyzed by HppE, HEPD and MPnS.
MPnS, another non-heme-Fe(II) oxygenase, effects a 4e⁻ oxidative $sp^{3}-sp^{3}$ C-C scission of 2-HEP without the input of any exogenous electron, and inserts two oxygen atoms into the product, CO₂, rather than another product, MP (2-3). The studies of isotopologue have suggested that like HEPD, MPnS also abstracts the C2-pro-S hydrogen from 2-HEP to initiate the C-C cleavage reaction (5, 7). The variant of HEPD, the substitution of Tyr 98 with Phe (Y98F), could generate MP in low levels with 2-HEP and O₂ (16), which implies that HEPD and MpnS might employ the same methylphosphonate radical intermediate during catalysis. Further, the C2-pro-R-H of 2-HEP is transferred to the product, MP, according to isotopologue studies (5). These findings have led to a proposal that MPnS and HEPD share a consensus mechanism, which differs only in the last step of catalysis, which involves a hydroxyl group rebound in HEPD-catalyzed oxygenation and a hydrogen abstraction from formate in MPnS-catalyzed reaction (Scheme 3-3). But MPnS distinguishes itself from wild-type HEPD and HppE by using only two histidine residues to chelate the ferrous iron in the active site, as suggested by the sequence alignment of HppE, HEPD and MPnS (5). Evidence from isotopologue studies with LC-MS and NMR analysis suggested that MPnS is able to catalyze the reaction with 2-HEP and H₂O₂ in the absence of O₂, with nearly full conversion of all the substrate to phosphonate aldehyde (2-OEP). This observation clearly demonstrates that MPnS could catalyze a 2e⁻ peroxidation/dehydrogenation reaction, in addition to the previously detected oxygenation reaction. The catalytic promiscuity of MPnS might be attributed to its non-canonical “2H” Fe(II)-coordinating mode, which provides vacancy in the active site of MPnS and allows this enzyme to accommodate different co-substrates and tune redox potentials in a larger range. Thus, MPnS is capable of catalyzing both a 4e⁻ oxygenation reaction and a 2e⁻ dehydrogenation reaction. Such catalytic flexibility has been observed in another non-heme-iron(II) enzyme, SyrB2, which also employs the non-canonical “2H” Fe(II)-coordinating mode (17-18). SyrB2 is able to catalyze not only halogenation of aliphatic carbons of L-2-aminobutyrate and other L-amino acids, but also
hydroxylation, nitration and azidation of aliphatic carbon of L-2-aminobutyrate \((18)\). Utilizing 
\(\text{H}_2\text{O}_2\) as the sole oxidant, HEPD is able to abstract a hydrogen atom from either C1 or C2 of 2-
HEP in order to convert it into corresponding phosphonate aldehyde (2-OEP) and phosphonate 
epoxide (EEP). However, for the reaction of MPnS with \(\text{H}_2\text{O}_2\), only one aldehyde product, 2-
OEP, was observed, which indicates that MPnS only abstracts a hydrogen atom from the C2 
position of 2-HEP, even though both C2-H and C1-pro-R-H of 2-HEP could be directed towards 
the Fe(IV)-oxo complex in the active site, as suggested by the crystal structure of 
HEPD\(\cdot\)Cd(II)\(\cdot\)2-HEP complex (Scheme 3-4) (Figure 3-1). As X-ray crystal structure of MPnS 
might be available in the future, it would explain how the orientation of C1-H and C2-H is 
controlled in the active site of MPnS.

HppE distinguishes itself from HEPD and MPnS by catalyzing a 2e\(^-\) epoxidation reaction 
of a secondary alcohol, S-HPP, as a non-heme-iron(II) peroxidase \((8)\). In this study, HppE failed 
to convert its natural substrate, S-HPP, or substrate analogue, R-HPP to product with O\(_2\), which 
agrees well with the peroxidase nature of HppE. Furthermore, HppE could catalyze the 
dehydrogenation reaction of 2-HEP with \(\text{H}_2\text{O}_2\), and one product was confirmed to be the 
corresponding aldehyde (2-OEP). More studies are required to confirm the identity of another 
product, phosphonate epoxide (EEP). Similar to the HEPD-effected peroxidation reaction of 2-
HEP, both C2-H and C1-pro-R-H might be positioned towards the reactive Fe(IV)-oxo species in 
HppE, which may result in the formation of two dehydrogenation products, 2-OEP and EEP 
(Scheme 3-4). In conclusion, HppE exhibits the peroxidase activity, not oxygenase activity, and 
does not possess the catalytic promiscuity of MPnS and HEPD.
3.4 Experimental procedures

Materials. H₂O₂ (30% w/w), L-ascorbic acid, dimethyl sulfoxide-d₆ (DMSO-d₆) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Fe(NH₄)₂(SO₄)₂ was purchased from J. T. Baker (Philipsburg, NJ). All reagents were used directly as obtained from the commercial sources. 2-[1,1-²H₂]-HEP, 2-[2,2-²H₂]-HEP and ¹³C-enriched 2-HEP were synthesized by published procedures. ¹H-NMR spectra of regular 2-HEP and ¹³C-enriched 2-HEP and ¹³C-NMR spectrum of ¹³C-enriched 2-HEP were shown in Figure 3-4.

NMR spectra were recorded on a Bruker AV-III-600 MHz spectrometer at the Nuclear Magnetic Resonance Facility of the Department of Chemistry of The Pennsylvania State University. For ¹³C-NMR spectra, chemical shifts (δ in ppm) are provided relative to that of DMSO-d₆, with the coupling constant reported in Hertz (Hz).

Cell growth and preparation of recombinant enzyme. These steps were conducted as described in previous studies (2-4).

LC-MS assay. Assays contained HEPD, HppE and MPnS (initially in their apo forms), 0.8 equivalent Fe(II) (from Fe(NH₄)₂(SO₄)₂), substrate and L-ascorbic acid. The reaction was initiated either by slow infusion of H₂O₂ into the reaction mix at room temperature in an anoxic chamber, as described in a published procedure (8). The specific reaction conditions for each experiment were given in figure legends. Reactions were allowed to proceed for ~ 10 min, and the reaction mixtures were processed through Nanosep centrifugal filters (Pall Corporation, Port Washington, NY) to remove particulate. Samples were analyzed by LC-MS (Agilent 1200 series LC system coupled to Agilent 6410 QQQ mass spectrometer, Agilent Technologies, Santa Clara, CA) equipped with a Kinetex 2.6 μm HILIC (50 × 2.1 mm) column (Phenomenex Inc., Torrance, CA). The substrates 2-[1,1-²H₂]-HEP and 2-[2,2-²H₂]-HEP (m/z = 127), products (m/z = 123, 124, 125, 97) were eluted with a gradient of 1.0 × 10² mM ammonium formate at pH 3.2 as mobile
phase A and 4.0 mM ammonium formate in 90% acetonitrile at pH 3.2 as mobile phase B for 10 min, as described in reference (8).

$^1$H-NMR-spectroscopic and $^{13}$C-NMR-spectroscopic characterization of the reaction products. Reactions were conducted as described above for the LC-MS assay. After removing debris from samples by filtration, the flow-through was dried completely and dissolved in D$_2$O for NMR-spectroscopic analysis.
Reference


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Appendix

Evidence that the Fosfomycin-Producing Epoxidase, HppE, Is a Non-Heme-Iron Peroxidase

Acknowledgements

There are a number of people contributing to the work containing in this chapter. Firstly of all, I’d like to sincerely thank my co-advisor, Prof. J. Martin Bollinger, Jr., for providing tremendous guidance for the experiment design, kinetics data analysis and writing manuscript. My co-advisor, Prof. Carsten Krebs significantly contributed to revise the manuscript and supervised all the Mössbauer experiment. I’d like to express my thankfulness to Dr. Wei-chen Chang of the Bollinger-Krebs group. He purified HppE enzyme, synthesized all the substrates and isotopologues and gave insightful discussions. I’d like to give deep gratitude to my collaborator, Prof. Hung-wen Liu of the University of Texas at Austin, for his continuous support for this work, lots of discussions and revising this manuscript. Prof. Yisong Guo of the Carnegie Mellon University collected and analyzed Mössbauer spectra. Prof. Mike Green of the Pennsylvania State University provided very helpful discussions and inspired me a lot at the early stage of this work. To everyone who has made contributions to this work, I expressed my sincere gratitude and thanks. Without the help of all of yours, this work could not be published in the high-profile journal.
Evidence that the Fosfomycin-Producing Epoxidase, HppE, Is a Non–Heme-Iron Peroxidase

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The iron-dependent epoxidase HppE converts (S)-2-hydroxypropyl-1-phosphonate (S-HPP) to the antibiotic fosfomycin (1R,2S)-epoxypropylphosphonate in an unusual 1,3-dehydrogenation of a secondary alcohol to an epoxide. HppE has been classified as an oxidase, with proposed mechanisms differing primarily in the identity of the O2-derived iron complex that abstracts hydrogen (H•) from C1 of S-HPP to initiate epoxide ring closure. We show here that the preferred cosubstrate is actually H2O2 and that HppE therefore almost certainly uses an iron(IV)-oxo complex as the H• abstractor. Reaction with H2O2 is accelerated by bound substrate and produces fosfomycin catalytically with a stoichiometry of unity. The ability of catalase to suppress the HppE activity previously attributed to its direct utilization of O2 implies that reduction of O2 and utilization of the resultant H2O2 were actually operant.

The drug fosfomycin ([1R,2S]-epoxypropylphosphonate, Fos; see Fig. 1 for structure) kills pathogenic bacteria by inactivating uridine diphosphate (UDP)-N-acetylglucosamine enolpyruvyl transferase (MurA) and thereby blocking synthesis of peptidoglycan, which constitutes the backbone of the cell wall (1, 2). The chemical “warhead” of Fos is its strained epoxide ring, which is opened upon attack by a cysteine residue in the active site of MurA, resulting in an inactivating covalent modification (1, 2). The epoxide of Fos is installed in the last step of its biosynthesis by the non–heme-iron(II) enzyme (S)-2-hydroxypropyl-1-phosphonate (S-HPP) epoxidase (HppE), which mediates an unusual 1,3-dehydrogenation of the secondary alcohol in the substrate to form the new carbon-oxygen bond of the three-membered ring (3).

HppE has been described as an oxidase, i.e., purported to use O2 as its oxidizing cosubstrate (Fig. 1A, reaction I) (3). This formulation of the epoxidation reaction would require that two electrons, in addition to the two provided by S-HPP, in forming the epoxide, be transferred to the HppE active site to achieve redox balance in the four-electron reduction of O2. The source of these electrons and the manner in which they might be delivered have remained unclear, as published structures of the enzyme do not reveal an obvious reductase domain (4–6), and gene clusters specifying the Fos-biosynthetic enzyme machinery do not encode a readily identifiable, dedicated reductase protein (7, 8). In vitro investigations of the HppE reaction have relied on either a heterologous reductase protein (called E3) from the 3,6-dideoxyhexose biosynthetic pathway of Yersinia pseudotuberculosis with its reducing cosubstrate nicotinamide adenine dinucleotide (NADH) or the combination of the biochemical reductants NADH and flavin mononucleotide (NADH/FMN) (3, 9). That these reducing systems support only very slow Fos production (~1 min−1) has reinforced the notion that additional components (e.g., the natural reducing system) might remain to be identified (9).

By contrast to simpler chemical and enzymatic epoxidation reactions involving addition of an oxygen atom (e.g., from a high-valent metal-oxo complex) to a carbon-carbon double bond (10, 11), the HppE reaction involves cleavage of a carbon-hydrogen bond of S-HPP (the pro-R C1–H bond) and formation of a new carbon-oxygen single bond to C1. In the product, the epoxide oxygen takes up the position on C1 originally occupied by the pro-S rather than the abstracted pro-R hydrogen, implying that C1 is inverted in the ring-closure step (3, 12). Although the mechanisms by which the C1–H bond is cleaved and the new C1–O bond is formed remain unknown, the observation by x-ray crystallography that S-HPP chelates the FeII cofactor via the C2 hydroxyl and the phosphate

References

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(5) and precedent from studies on other O2-activating iron enzymes (13, 14) led to the suggestion of three alternative pathways (Fig. 1B, blue arrow) (4, 15). These hypothetical O2-dependent mechanisms are distinguished by the nature of the C1–H–cleaving iron complex and the order of bond-breaking, bond-forming, and electron-injection steps.

As depicted in Fig. 1B, abstraction of the pro-R hydrogen atom (H+) from C1 could be mediated by a Fe(III)-superoxo (Fe(III)-O2–) complex formed by simple addition of O2 to the Fe(II) cofactor (pathway I, beige arrows); a Fe(II)-hydroperoxo complex, formed by addition of O2, an electron, and a proton to the cofactor (pathway II, green arrows); or a Fe(IV)-oxo (ferryl) complex, formed by addition of O2, transfer of two electrons and two protons, and cleavage of the O–O bond (pathway III, red arrows). Of these possibilities, a ferryl complex was favored in a recent computational study (16). The mechanism of epoxide-ring closure after formation of the C1 radical is equally uncertain. The most straightforward possibility, suggested by studies on other iron enzymes that mediate formation of new carbon-heteroatom bonds, would be a radical group transfer of the Fe-coordinated C2–O atom to the C1 radical (17–22). However, the inversion of C1 remains challenging to explain according to this mechanism. On the basis of a remarkable oxidative 1,2-phosphonate migration mediated by HppE upon the substrate analog, (R)-1-hydroxypropyl-1-phosphate (Fig. 1, A and C, reactions II), a recent study posited a different ring-closure mechanism, involving formation of a C1 carbocation by electron transfer from the C1 radical to the iron cofactor and subsequent nucleophilic capture of the C1 carbocation by the C2 alkoxy (Fig. 1C, reaction I) (23). In this mechanism, neighboring group participation (anachimeric assistance) by the phosphonate (with retention of the C1 stereochemistry, dashed red line) could promote formation of the formal carbocation and then also dictate the net inversion of C1 after subsequent attack by the C2-O.

Precedent suggests that the H-abstracting complex might be identified by use of rapid-kinetic and spectroscopic methods to detect it and demonstrate a kinetic isotope effect on its decay upon use of [1,1-2H2]-S-HPP as the substrate (24). However, related O2-activating iron oxidases and oxygenases have catalytic rate constants typically in the range of 1 to 100 s−1 (24, 25). Therefore, if generation of the C1–H–cleaving intermediate requires one or two electrons, a reductant considerably more efficient than the reported E3/NADH and NADH/FMN systems [which support maximum turnover rates of ~0.01 s−1 (9)] might be required to gain entry into the reaction sequence fast enough to accumulate the C1–H–cleaving complex (24). In seeking a more suitable reductant, we determined that sodium dithionite (Na2S2O4) can support multiple turnovers at rates more than a thousand times greater (10 to 100 s−1) than those supported by the NADH-based reducing systems. Dithionite is itself reactive toward O2 on this same time scale (26), creating a conundrum as to how it could deliver electrons to HppE without first being oxidized by O2.

Closer examination of the reaction led to the new hypothesis that dithionite might actually function by reducing O2 directly rather than donating electrons to the HppE active site during an O2-initiated catalytic cycle. We therefore evaluated whether the expected O2-reduction product, H2O2, can serve as the oxidant for Fos production by HppE.

To test this hypothesis, we added varying quantities of H2O2 slowly to solutions of Fe(III)-HppE, l-ascorbate, and [2,3,3,3-2H4]-2-hydroxypropyl-1-phosphonate (d4-S-HPP), remotely labeled to distinguish the enzymatic product ([2,3,3,3-2H4]-1-phosphonate; d4-Fos) from the commercially available Fos standard. Reaction samples were analyzed by liquid chromatography/mass-spectrometry (LC-MS). As shown in fig. S1, the intensity of the peak arising from the d4-S-HPP substrate at a mass/charge ratio (m/z) of 143 (negative-ion mode) with an elution time of ~3 min is diminished (A), and a new peak at m/z = 141 (consistent with the mass of the d4-Fos product) coeluting at ~2 min with the commercial Fos (m/z = 137; dotted orange trace) grows (B) as more H2O2 is added. The identity of the new product was established to be Fos by 1H-nuclear magnetic resonance (1H-NMR) analysis of a reaction sample to which H2O2 was added to a final 1:1 molar ratio with respect to the unlabeled S-HPP substrate (Fig. 2). Although line broadening, possibly arising from the presence of iron from the enzyme in the NMR sample, is evident, comparison of the spectrum of the reaction product (spectrum B) to that of com-

Fig. 1. Proposed and demonstrated reactions of HppE and their possible mechanisms. (A) Putatively O2-dependent reactions purportedly catalyzed by HppE on S-HPP (I) and its stereo- and structural isomers (II to IV). (B) Mechanisms previously proposed for the putatively O2-dependent fosfomycin-generating epoxidation reaction (blue arrow) and the mechanism indicated by the finding that H2O2 rather than O2 is the oxidizing co-substrate (magenta and red arrows). (C) Proposed mechanisms for H2O2-driven (I) epoxide ring closure to produce fosfomycin and (II) phosphonate migration with the (R)-1-hydroxypropyl-1-phosphonate analog (23).
mercial Fos (spectrum A) reveals features with the proper chemical shifts and integrated intensities at ~3.05 parts per million (ppm) (multiplet from C2-H), ~2.62 ppm (doublet of doublets from C1-H), and ~1.25 ppm (doublet from C2-Me). None of these features is present in the spectrum of the S-HPP starting material (spectrum C). Additional minor features at ~2.72, ~2.66, and ~2.43 ppm in the spectrum of the product sample (B) arise from the 2-keto oxidation product known to be generated by HppE from the (R)-2-hydroxypropyl-1-phosphonate (R-HPP) enantiomer (27), which is present as a minor (~6%) contaminant in our synthetic S-HPP substrate.

The nearly complete consumption of S-HPP by one molar equivalent of H2O2 evident in the NMR spectra implies an experimental Fos:H2O2 stoichiometry close to the predicted value of unity. The experimental stoichiometry was accurately determined by LC-MS with commercial Fos as an internal standard (Fig. 3A, gray traces) to quantify the d4-Fos product from the HppE peroxidase reaction (black traces). The stoichiometry was determined to be 1.00 ± 0.05 (Fig. 3B). Coupling between H2O2 reduction and Fos production is thus extremely tight.

The requirements for the productive HppE peroxidase reaction were defined and its catalytic nature verified by analysis of reactions carried out with high H2O2/HppE ratios from which individual reaction components were serially omitted (fig. S2 and associated text). With all components present, the enzyme could effect at least 50 turnovers. The kinetics of a single turnover of H2O2-driven Fos production were then determined at 4°C. With 0.096 mM HppE, 0.050 mM H2O2, and 0.50 mM d4-S-HPP, d4-Fos production was complete in ~0.1 s (Fig. 4). Simulation of the kinetic data requires a second-order rate constant of at least 1.2 × 10^5 M^-1 s^-1 for reaction of the HppE-Fe^3+S-HPP complex with H2O2 (- - -), and the data are simulated best with a value of 4.8 × 10^5 M^-1 s^-1 for this rate constant (——). It is possible that the first-order steps after addition of H2O2 to the enzyme could be slower than the addition step itself; in this case, the second-order rate constant for H2O2 addition could be underestimated by the analysis. Regardless, the determined rate constant is only a factor of 10 to 100 less than the value of kcat/Km reported for bovine liver catalase, an enzyme for which H2O2 is the established physiological substrate and which is often touted as having evolved almost to catalytic perfection (i.e., to operate at the diffusion limit) (28). Quantitative assessment of the ability of commercial bovine liver catalase to compete with HppE for reaction with added H2O2 yielded an estimate of 5 × 10^5 M^-1 s^-1 for the kcat/Km (H2O2) of HppE at 21°C (fig. S3 and associated text). It is likely that the experimental error in the rate constants obtained at 4°C and 21°C (we estimate a factor of 2 for each) coincidently obscures the actual temperature dependence of the reaction rate.

Both reducing systems used in previous in vitro studies on HppE (E3/NADH and NADH/FMN)
generate H$_2$O$_2$ from O$_2$ (29, 30). The demonstration that HppE is an efficient peroxidase therefore raises the possibility that its reported oxidase activity in the presence of the NADH-based reducing systems might actually have resulted from reduction of O$_2$ by the reducing system and utilization of the resultant H$_2$O$_2$ by HppE. As a test of this possibility, HppE reactions with O$_2$ and one of the two reducing systems were carried out in the presence of varying concentrations of catalase. As shown in fig. S3, increasing the concentration of catalase diminishes the Fos yield in these reactions with catalase-concentration ([catalase]) dependencies (blue and green points) essentially identical to that for the direct reaction with H$_2$O$_2$ (red). These data strongly suggest that the Fos production previously attributed to HppE oxidase activity actually reflects its peroxidase activity. Consistent with this conclusion, use of H$_2$O$_2$ as oxidant and H$_2$O$_2$-enriched solvents does not result in incorporation of a detectable quantity of $^{18}$O into the Fos product. This observation confirms that the epoxide is formed from the oxygen atom already present in the substrate, as previously demonstrated when the reaction was thought to involve O$_2$. In addition, when added directly, H$_2$O$_2$ also supports the alternative oxidations of substrate analogs that were previously attributed to O$_2$, including the aforementioned oxidative 1,2-phosphonate migration with (R)-1-hydroxypropyl-1-phosphonate (Fig. 1A, reaction II (23)) and the C2 dehydrogenation of (R)-2-hydroxypropyl-1-phosphonate to the corresponding ketone (Fig. 1A, reaction III (27)). The observations suggest that all HppE activities reported in previous studies arose from the enzyme’s reaction with H$_2$O$_2$ generated by the reducing systems rather than its reaction directly with O$_2$.

Additional evidence that HppE is not an oxidase is the failure of the Fe$^{III}$-O$_2$ complex that should result from addition of O$_2$ to its Fe$^{II}$ cofactor (17, 31–34) to accumulate in the absence of a reducing system. Freeze-quench Mössbauer spectroscopic experiments using an efficient enzymatic O$_2$-generation system that can drive formation of even dissociable O$_2$ adducts (35) revealed that O$_2$ adds either very slowly or with low affinity (or both) to the HppE cofactor, irrespective of the presence of bound substrate (fig. S4 and associated text). The apparent failure of substrate binding to promote O$_2$ addition contrasts with the behavior of most proven oxidases and oxygenases that use non–heme-iron cofactors (24, 36–40). It also contrasts with the behavior of HppE toward H$_2$O$_2$: Reaction of the substrate-free enzyme with H$_2$O$_2$ was found to be about a factor of 50 slower (fig. S5 and associated text) than the productive reaction in the presence of S-HPP, implying that substrate binding does indeed promote reaction with H$_2$O$_2$. Conversely, the inability of 2-hydroxyethyl-1-phosphonate (HEP) dioxygenase (HEPD), the non–heme-iron enzyme that is structurally most similar to HppE (fig. S6) (41), to use H$_2$O$_2$ also suggests that the peroxidase activity seen with HppE reflects its natural function (fig. S7 and associated text). HppE and HEPD share the ability to oxidize HEP (fig. S7A, blue bars and fig. S7B, green bars) [also R-HPP (42)], but HEPD unequivocally uses O$_2$ as its cosubstrate for this oxidation (41). It does not use H$_2$O$_2$ for catalytic oxidation of HEP (fig. S7A, green bars) under the conditions that support efficient consumption of S-HPP by HppE (red bars). Even in its cross-reactivity toward HEP, HppE is still an efficient peroxidase (blue bars) but has no activity with O$_2$ as the oxidant in the absence of a reducing system to first convert the O$_2$ to H$_2$O$_2$ (fig. S7B, blue bars). The enzymes’ nearly orthogonal use of the oxidants, despite their very similar structures and overlapping substrate profiles, further supports the hypothesis that HppE is an authentic peroxidase rather than an oxidase capable of an adventitious “peroxide-shunt” reaction. Unlike the previously postulated O$_2$-dependent epoxidation reaction, in which the iron complex responsible for the crucial abstraction of the pro-R H- from C1 of S-HPP could potentially have been in any of three different overall oxidation states (the three pathways in Fig. 1B), reaction of the Fe$^{II}$ cofactor with H$_2$O$_2$ is redox balanced to bypass the first two oxidation states and proceed directly to a Fe$^{IV}$-oxo (ferryl) complex have been demonstrated in the reactions of other non–heme-iron enzymes (13, 18, 38, 40, 45–47). The use of H$_2$O$_2$ as the literature precedents, and the recent computational study (16) all point to a ferryl complex as the most likely initiator of the HppE reaction. The conclusion that HppE uses a ferryl complex generated directly from H$_2$O$_2$ rather than O$_2$ to initiate its epoxidation reaction suggests that the Fos-biosynthetic machinery should not require a specific HppE reductase. Instead, the epoxidation could be supported by any endogenous or exogenous oxidase that generates H$_2$O$_2$. Conceivably, Fos-synthesizing Streptomyces might encounter H$_2$O$_2$ in their environments, turning a general toxin produced by competing organisms to their advantage by using it to produce a countertoxin of their own. Such a scenario would add to the growing list of known uses of H$_2$O$_2$, which had previously been considered primarily as a metabolic toxin, in productive physiological reactions related to intercellular interactions (48, 49).
Cancer is a disease in which cells accumulate genetic aberrations that are believed to confer a clonal advantage over cells in the surrounding tissue. However, the quantitative benefit of frequently occurring mutations during tumor development remains unknown. We quantified the competitive advantage of Apc loss, Kras activation, and P53 mutations in the mouse intestine. Our findings indicate that the fate conferred by these mutations is not deterministic, and many mutated stem cells are replaced by wild-type stem cells after biased, but still stochastic events. Furthermore, P53 mutations display a condition-dependent advantage, and especially in colitis-affected intestines, clones harboring mutations in this gene are favored. Our work confirms the previously theoretical notion that the tissue architecture of the intestine suppresses the accumulation of mutated lineages.

Defining Stem Cell Dynamics in Models of Intestinal Tumor Initiation

Louis Vermeulen,1,2,† Edward Morrissey,2,† Maartje van der Heijden,1,2 Anna M. Nicholson,1 Andrea Sottoriva,3 Simon Buczacki,1 Richard Kemp,1 Simon Tavaré,1,4 Douglas J. Winton1

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Models of Intestinal Tumor Initiation

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Refereences

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Supplementary Materials for

Evidence that the Fosfomycin-Producing Epoxidase, HppE, Is a Non-Heme-Iron Peroxidase

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Materials and Methods

Materials. H₂O₂ (30% w/w), fosfomycin disodium salt, L-ascorbic acid, bovine liver catalase (C40), sodium dithionite, and lipase CALB (Candida antarctica Lipase B) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Fe(NH₄)₂(SO₄)₂ was purchased from J. T. Baker (Philipsburg, NJ). ⁵⁷Fe was purchased from Advanced Materials and Technology, Inc. (New York, NY). All reagents were used directly as obtained from the commercial sources. NMR spectra were recorded on a Varian 400, 500 or 600 MHz spectrometer at the Nuclear Magnetic Resonance Facility of the Department of Chemistry and Biochemistry, The University of Texas at Austin. NMR spectra of the H₂O₂-generated HppE reaction product, the S-HPP substrate, and fosfomycin standard were recorded on a Bruker 400 MHz spectrometer in the Department of Chemistry of The Pennsylvania State University. Chemical shifts (δ in ppm) are given relative to that of solvent (CDCl₃ or D₂O), with the coupling constant reported in Hertz (Hz). Analytical thin layer chromatography (TLC) was carried out on pre-coated TLC aluminum plates (silica gel, grade 60, F₂₅₄, 0.25 mm layer thickness) acquired from EMD Chemicals (Gibbstown, NJ). Flash column chromatography was performed using silica gel (230-400 mesh, grade 60) obtained from Sorbent Technologies (Norcross, GA). (S)-2-hydroxypropyl-1-phosphonate (S-HPP) was synthesized by a published procedure (3). [2,3,3,3-²H₄]-(-)-2-hydroxypropyl-1-phosphonate (d₄-S-HPP) was synthesized from diethyl-(2-oxo-propyl)-1-phosphonate starting material, as shown in the scheme and described in the text below.

A few drops of Et₃N were added to a solution of diethyl-(2-oxo-propyl)-1-phosphonate (1.98 g, 10 mmol) in 10.0 mL of D₂O. The reaction was stirred at room temperature for 12 h. The reaction mixture was extracted with Et₂O (3 × 20 mL). After evaporation, this procedure was repeated twice to afford diethyl-[(1,1,3,3,3-²H₅)-2-oxo-propyl]-1-phosphonate as a colorless oil (1.96 g, 90%, >95% deuterium incorporation). ¹H NMR (CDCl₃): δ 1.336 (t, J = 7.2 Hz, 3 H), 1.337 (t, J = 7.2 Hz, 3 H), 4.11-4.18 (m, 4
H). To exchange the C1-deuteria back to protia, a few drops of H₂O were added to a solution of diethyl-[(1,1,3,3,3-²H₅)-2-oxo-propyl]-1-phosphonate (2.03 g, 10 mmol) in 10.0 mL MeOH. The reaction was stirred at room temperature for 1 h. After concentration, this procedure was repeated four times to afford diethyl-[(3,3,3-²H₃)-2-oxo-propyl]-1-phosphonate as a colorless oil (98%). ¹H NMR (CDCl₃): δ 1.336 (t, J = 7.2 Hz, 3 H), 1.337 (t, J = 7.2 Hz, 3 H), 3.08 (d, J_H-P = 23.2 Hz, 2H), 4.10-4.19 (m, 4 H). The resulting ketone was reduced via NaBD₄ to introduce deuterium at the C2 position.

NaBD₄ (670 mg, 16 mmol) was added to a solution of diethyl-[(3,3,3-²H₃)-2-oxo-propyl]-1-phosphonate (3.00 g, 15 mmol) in MeOH (80 mL) at room temperature, and the solution was stirred for 2 h. The reaction was quenched by adding acetone (5.0 mL) and partitioned between ethyl acetate (3 × 30 mL) and water (20 mL). The combined organic layer was washed with brine (40 mL), dried over MgSO₄, and concentrated under reduced pressure to afford racemic diethyl-[(2,3,3,3-²H₄)-2-hydroxypropyl]-1-phosphonate as a colorless oil (88%). ¹H NMR (CDCl₃): δ 1.287 (t, J = 7.2 Hz, 3 H), 1.291 (t, J = 7.2 Hz, 3 H), 1.88 (d, J = 18.0 Hz, 2H), 3.57 (s, 1H, exchangeable), 4.04-4.11 (m, 4 H); ³¹P NMR (CDCl₃) δ 30.0. ¹³C NMR (CDCl₃) δ 16.1 (d, J = 3.3 Hz), 16.1 (d, J = 3.3 Hz), 23.2 (m), 35.0 (d, J = 6.4 Hz), 61.5 (d, J = 6.6 Hz), 61.5 (d, J = 6.4 Hz), 62.0 (dt, J = 3.9, 21.9 Hz).

The two enantiomers were separated by adapting the published kinetic-resolution approach (50) that uses the lipase CALB for stereospecific introduction of an acetyl group on the (R)-enantiomer and subsequent separation by chromatography on silica gel. Lipase CALB (2.50 g) and vinyl acetate (6.5 mL) were added to a solution of diethyl-([2,3,3,3-²H₄]-2-hydroxypropyl)-1-phosphonate (1.30 g, 6.4 mmol) in 40 mL benzene. The reaction was stirred at room temperature and the progress of the reaction was monitored by ¹H- and ³¹P-NMR spectroscopies. Once the reaction reached ~ 50% conversion (~ 48 h), the reaction mixture was filtered through a celite pad. Concentration under reduced pressure yielded an oily residue that was purified and separated by flash chromatography on silica gel with ethyl acetate/Methanol (10:1(v/v)) as the eluting solvent to give (diethoxyphosphoryl)-(S)-2-(2H)-2-propyl acetate (45%) and diethyl-([2,3,3,3-²H₄]-((S)-2-hydroxypropyl)-1-phosphonate (40%) as a colorless oil. The enantiomeric excess (e.e.) of the diethyl-([2,3,3,3-²H₄]-((S)-2-hydroxypropyl)-1-phosphonate was determined to be 95% and the ¹H NMR and ³¹P NMR spectra were identical to those of diethyl-([2,3,3,3-²H₄]-2-hydroxypropyl)-1-phosphonate. ¹H NMR for [2,3,3,3-²H₄]-((R)-2-(diethoxyphosphoryl)propyl acetate (CDCl₃): δ 1.29 (t, J = 7.2 Hz, 6 H), 2.00 (s, 3H); 1.92-2.18 (m, 2H) overlap with previous peak, 4.05-4.08 (m, 4 H); ³¹P NMR (CDCl₃) δ 26.4. ¹³C NMR (CDCl₃) δ 16.2 (d, J = 6.2 Hz), 19.9 (m), 21.0, 32.3 (d, J = 139.2 Hz), 61.5 (d, J = 6.3 Hz), 61.6 (d, J = 6.3 Hz), 65.5 (d, J = 22.8 Hz), 169.8.

Bromotrimethyl silane was used to cleave the phosphonic ester bonds to afford the final product. TMSBr (660 µL, 5.0 mmol) was added to a solution of diethyl-([2,3,3,3-²H₄]-2-hydroxypropyl)-1-phosphonate (203 mg, 1.0 mmol) in CH₂Cl₂ (10 mL) at room temperature, and the solution was stirred at the same temperature overnight. After solvent removal under reduced pressure, the residue was dissolved in CHCl₃ (10 mL) and extracted with 5.0 mL of 0.05 M NH₄OAc. The aqueous layer was collected and lyophilized to afford d₄-S-HPP as a white solid (85%, > 95% deuterium incorporation). ¹H NMR (D₂O): δ 1.72 (dd, J = 53.4, 15.0 Hz, 2H); ³¹P NMR (D₂O) δ 21.5; ¹³C NMR (D₂O) δ 22.3 (m), 37.3 (d, J = 130.8 Hz), 63.8 (t, J = 21.0 Hz).
2-Hydroxyethyl-1-phosphonate (HEP) and [2,2-2H₂]-2-hydroxyethyl-1-phosphonate ([2,2-2H₂]-HEP) were synthesized from ethyl 2-(diethoxyphosphoryl)acetate as starting material.

Borane (1 M in THF, 30 mL, 30.0 mmol) was added at 0 °C through an addition funnel to a solution of ethyl 2-(diethoxyphosphoryl)acetate (2.24 g, 10.0 mmol) in THF (20 mL), resulting in evolution of gas. The clear solution was then warmed back to room temperature and stirred overnight. MeOH (~10 mL) was added slowly to quench the reaction. The crude reaction was concentrated under reduced pressure. The residue was re-dissolved in MeOH (10 mL) and subjected to reduced pressure to remove the borane complex. After this process was repeated three times, the concentrated crude product was used directly for the next step without further purification.

Following a procedure similar to that used in preparing d₄-S-HPP, TMSBr (6.6 mL, 50 mmol) was added to a solution of crude diethyl (2-hydroxyethyl)phosphonate obtained from the previous step in CH₂Cl₂ (100 mL) at room temperature, and the solution was stirred at the same temperature overnight. After solvent removal under reduced pressure, the residue was dissolved in CHCl₃ (30 mL) and extracted with 50 mL of 0.5 M NH₄OAc. The aqueous layer was collected and lyophilized to afford 2-hydroxyethyl-1-phosphonate as a white solid (80%). The spectroscopic data are consistent with those reported in the literature (51).

Analogously to the synthesis of 2-hydroxyethyl-1-phosphonate, [2,2-2H₂]-2-hydroxyethyl-1-phosphonate was obtained in 81% overall yield by using d₃-borane (1.0 M in THF, 98% atom % ²H, purchased from Cambridge Isotope laboratories) as the reductant. The spectroscopic data are consistent with those reported in the literature (51-52).

**Cell Growth and Preparation of Recombinant HppE.** These steps were conducted as previously described (53).

**LC-MS Assay for Fosfomycin Production.** Assays contained HppE (initially in its apo form), 0.8 equiv Fe⁴⁺ [from Fe(NH₄)₂(SO₄)₂], d₄-S-HPP and, when indicated, L-ascorbic acid in 100 mM Tris-HCl buffer (pH 7.5). The reaction was initiated either by injection of an aliquot of H₂O₂ solution into the assay solution or by slow infusion of H₂O₂ into the solution (by turning the volume dial of an adjustable pipettor while the assay solution was continuously stirred) at room temperature in an anoxic chamber. The specific reaction conditions for each experiment are given in the figure legends. Reactions were allowed to proceed to completion (for ~ 10 min), unlabeled fosfomycin was added as an internal standard, and the reaction solution was processed through a Nanosep centrifugal filter (Pall Corporation, Port Washington, NY) to remove particulate. Samples were analyzed by LC-MS (Agilent 1200 series LC system coupled to Agilent 6410 QQQ mass spectrometer, Agilent Technologies, Santa Clara, CA) equipped with a Kinetex 2.6 µ HILIC (50 × 2.1 mm) column (Phenomenex Inc., Torrance, CA). The substrate d₄-S-HPP (m/z = 143), product 2,3,3,3-[²H₄]-(1R,2S)-epoxypropyl-1-phosphonic
acid (d₄-Fos, m/z = 141) and internal standard fosfomycin (Fos, m/z = 137) were eluted with a gradient of 100 mM HCOONH₄ at pH 3.2 as mobile phase A and 4 mM HCOONH₄ in 90% acetonitrile at pH 3.2 as mobile phase B. The following gradient was used: 100% B for 2 min, from 100% B to 85% B for 3 min, and from 85% B back to 100% B for 5 min.

_H-NMR-Spectroscopic Characterization of H₂O₂-generated Products._ Reactions were carried out as described above for the LC-MS assay. After removing particulate from samples by filtration, the flow-through was dried completely and dissolved in D₂O for _¹H-NMR-spectroscopic analysis._

**Kinetics of Fos Production by Chemical-Quenched-Flow Experiments.** For the determination of the kinetics of d₄-Fos production in a single turnover with limiting H₂O₂, a chemical quenched-flow (CQF) experiment was carried out using an Update Instruments (Madison, WI) model 1000 freeze-quench/chemical-quench apparatus. CQF samples were prepared by mixing a solution containing 0.24 mM HppE, 0.19 mM Fe⁺², and 1.0 mM d₄-S-HPP with an equal volume of 0.10 mM H₂O₂ and allowing the reaction to proceed at 4 °C for the indicated time before terminating by mixing the reaction solution with an equal volume of the "quench solution" (80% isopropanol/20% acetic acid, v/v). Samples were dried completely using a SpeedVac concentrator (Thermo Scientific, Bellefonte, PA), dissolved in water, processed through Nanosep centrifugal filters (Pall Corporation, Port Washington, NY) to remove protein, and analyzed by LC-MS (see above for conditions).

**Stopped-Flow (SF) Absorption and Freeze-Quench (FQ) Mössbauer Experiments.** General procedures for stopped-flow absorption and freeze-quench Mössbauer experiments were described in references (54) and (45). In the SF experiment, an oxygen free solution of 0.48 mM HppE and 0.38 mM Fe⁺² was mixed at 4 °C with an equal volume of H₂O₂ solution delivering varying [H₂O₂]. The final concentration of H₂O₂ after mixing for each reaction is given in the legend to Figure S4. In the FQ Mössbauer experiment, the O₂-free enzyme reactant solution contained 2.5 mM HppE, 2.0 mM Fe⁺², 0.025 mM chlorite dismutase and (±) 12.5 mM S-HPP. Samples were prepared by mixing this reactant solution with 0.25 equivalent volume of an O₂-free solution of 80 mM NaClO₂ at 4 °C and freeze-quenching after a reaction time of 0.012 ms.

**Inhibition of HppE Activity by Catalase.** Reactions were carried out at 21 °C. The assay solution included HppE, Fe⁺², d₄-S-HPP and varying amounts of catalase, as indicated in the legend to Figure 4. In the direct reaction of HppE with H₂O₂, H₂O₂ was slowly infused into the reaction mix in the absence of O₂. After the standard Fos was added (to 0.50 mM) and the protein removed by filtration, samples were analyzed by LC-MS. In the reactions of HppE with either E₃/NADH or NADH/FMN in the presence of O₂, assay solutions were stirred in air to allow the continuous infusion of O₂. Sample processing was the same as described above. The final concentrations were 0.050 mM HppE, 0.040 mM Fe⁺², 2.0 mM d₄-S-HPP and either 0.50 mM H₂O₂, 0.80 mM NADH and 0.30 mM FMN, or 0.80 mM NADH and 0.15 mM E₃.

**Substrate Consumption by HppE or HEPD with H₂O₂ or O₂ as oxidant.** In reactions of either HppE or HEPD with H₂O₂, the assay solution contained 0.020 mM HppE or HEPD, 0.016 mM Fe⁺², 10 mM L-ascorbic acid and 2.0 mM d₄-S-HPP or HEP. H₂O₂ was slowly infused (in the absence of O₂) at 21 °C to a final concentration of 2.0
mM by turning the dial of an adjustable pipettor. In reactions of either HppE or HEPD with O₂, the assay solution contained 0.020 mM HppE or HEPD, 0.016 mM Fe²⁺ and 2.0 mM HEP. O₂ was infused by stirring in air at 21 °C for 30 min. With either oxidant, quantification of the remaining substrate was identical. After the standard solution of S-HPP or [2,2-²H₂]-HEP was added and the protein removed by filtration, samples were analyzed by LC-MS.

Supplementary Results

Figure S1. Consumption of S-HPP and generation of a new product with the mass of Fos in the reaction of HppE with H₂O₂. The LC-MS chromatograms show A, the m/z = 143 peak of d₄-S-HPP substrate and B, the m/z = 141 peak expected for d₄-Fos in samples prepared by addition of varying [H₂O₂] (indicated at right) to a solution containing 1.0 mM HppE, 0.80 mM Fe²⁺, and 2.0 mM d₄-S-HPP. Reactions were carried out at 21°C in the absence of O₂. Details of sample preparation and the LC-MS analysis are provided in the Material and Methods.

Requirements for and Catalytic Nature of Fos Production by HppE with H₂O₂. Omission of the enzyme (panel A, red), its Fe²⁺ cofactor (blue), or either of the substrates, S-HPP (green) or H₂O₂ (black), resulted in production of very little Fos (< 1 HppE equivalent). Including the common biochemical reductant, L-ascorbate, in the complete reaction (pink) led to more than 50 enzyme equivalents of fosfomycin being produced (Fig. S2, panel B), whereas omission of L-ascorbate diminished the yield of Fos to ~25 turnovers (purple). The effect of L-ascorbate probably arises from its ability to protect the enzyme against, or reactivate it after, adventitious cofactor oxidation resulting from reaction with the substrate-free form of enzyme (see below), as is commonly seen with the Fe²⁺- and α-ketoglutarate-dependent dioxygenases (55-57). Although further optimization of the reaction conditions might permit a greater number of turnovers to occur, the data of Figure S2 are sufficient to establish that the HppE peroxidase reaction is fully catalytic.
Figure S2. Definition of the requirements for the H₂O₂-driven production of Fos by HppE and assessment of the number of turnovers. A, LC-MS chromatograms monitoring m/z = 141 for the d₄-Fos product. B, Quantities, in HppE equivalents, of Fos produced in each reaction. The reaction conditions were: [HppE] = 0.010 mM, [FeII] = 0.080 mM, [d₄-S-HPP] = 2.0 mM, [H₂O₂] = 2.0 mM, [L-ascorbic acid (Asc)] = 10 mM. Reactions were carried out at 21°C in the absence of O₂. The error bars in panel B represent the standard deviations from the mean values of six trials for each condition.

Estimation of kcat/KM of HppE for H₂O₂ by its Competition with Catalase. In addition to the direct determination of a lower limit for kcat/KM of HppE for H₂O₂ by the single-turnover kinetic experiment of Figure 4, a second measure of the efficiency of the HppE peroxidase reaction was sought by testing for suppression of H₂O₂-driven Fos production in the presence of bovine liver catalase (Figure S3). As alluded to in the main text, the kinetic parameters for this enzyme [kcat = 9 x 10⁵ s⁻¹ and kcat/KM = 9 x 10⁶ M⁻¹s⁻¹ on a per heme basis at 21 °C (28)] reflect a very efficient catalyst. Diminution of the Fos:H₂O₂ yield in HppE reactions (with excess and saturating S-HPP) containing increasing concentrations of catalase and a fixed concentration of HppE (red points) confirms the expected competition. The data can be analyzed according to eq. 1, which gives the relative yield of Fos in terms of the concentrations of the competing enzymes ([HppE] and [catalase]) and the ratio, R, of their kcat/KM values (apparent second-order rate constants) for H₂O₂, where R = (kcat/KM)catalase/(kcat/KM)HppE. The fit (solid line) corresponds to R = 20. From this value and the published kcat/KM of catalase (28), kcat/KM of HppE for H₂O₂ is estimated to be 5 x 10⁵ M⁻¹s⁻¹ (21 °C), in acceptable agreement with the second-order rate constant for the reaction at 4 °C measured in the single-turnover experiment of Figure 4.

\[
\text{relative yield of Fos} = \frac{[\text{HppE}]}{([\text{HppE}] + R \cdot [\text{catalase}])}
\]  \hspace{1cm} (1)
**Figure S3.** Inhibition by catalase of HppE-catalyzed Fos production. The data points shown are the relative yields of Fos at the indicated concentrations of catalase in the direct reaction with H$_2$O$_2$ (red) and the reactions with either the E$_3$/NADH (blue) or the NADH/FMN (green) reducing system in the presence of O$_2$. The solid black line is the fit of Equation 1 to the data. The error bars are the standard deviations from the mean of six measurements at each catalase concentration. Reaction conditions are given in Materials and Methods.

*Addition of O$_2$ to the Fe$^{II}$ Cofactor of HppE is Slow or Unfavorable (or Both).* As indicated in Fig. 1, O$_2$-dependent Fos production would require a reducing system to provide the redox-balancing two additional electrons (3). It was previously reported and confirmed by experiments carried out during this study that Fos production does not occur (or occurs only on the $10^3$-s timescale and with an ultimate yield of less than one enzyme equivalent) in the presence of O$_2$ but absence of a reducing system (53). However, the first step in the putative oxidase reaction, addition of O$_2$ to the Fe$^{II}$ center in the HppE•S-HPP complex (Fig. 1B, blue arrow), should not require a reductant. Experimental and computational precedents establish that this step would produce a Fe$^{III}$-superoxo (Fe$^{III}$–O$_2$•–) complex, which should be distinguishable from the reactant complex on the basis of its Mössbauer spectrum (17, 31-34). We therefore used freeze-quench Mössbauer spectroscopy to test for the accumulation of a Fe$^{III}$–O$_2$•– complex during incubation of HppE with O$_2$ but no reducing system. To do so, we applied our recently developed method that employs the enzyme chlorite dismutase to generate very high concentrations of O$_2$ (~10 mM) from ClO$_2^-$ (35). The high [O$_2$] that this method affords should favor saturation of the Fe$^{II}$ cofactor in the event that O$_2$ can bind but does so only weakly, a situation that is thought to be prevalent among mononuclear iron(II)-dependent oxidases and oxygenases. The effect of the substrate was further evaluated, because the O$_2$-addition steps of many such enzymes are favored by bound substrate (24, 36-40). Spectra of HppE•Fe$^{II}$•S-HPP samples quenched after a 0.012-s exposure to the high [O$_2$] afforded by the chlorite dismutase system (Fig. S4, top, hashes) reveal that no more than 8% of the total iron is found in a potential Fe$^{III}$–O$_2$•– adduct suggests that reaction of the HppE•Fe$^{II}$•S-HPP complex with O$_2$ is either much slower than its reaction with H$_2$O$_2$, thermodynamically disfavored even at the high [O$_2$]
afforded by the chlorite dismutase system, or both. For example, if it is assumed that an effective \([O_2]\) of only 5 mM was generated, then the second-order rate constant for a slow but favorable \(O_2\)-binding step would have to be less than \(2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}\) to account for the observed conversion. This value is \(~100\)-fold less than we have typically seen for authentic oxidases and oxygenases (38-39, 58). Alternatively, for a fast \(O_2\)-addition step, a dissociation constant \((K_D)\) in excess of 40 mM would be required to account for the modest conversion. This value is \(~100\) times greater than that observed for the reversible \(O_2\)-addition step in the dinuclear non-heme-iron enzyme, \(\text{myo}-\text{inositol oxygenase}\) (59). Thus far, the only reported \(\text{Fe}^{\text{III}}-\text{O}_2^-\) adduct in a mononuclear non-heme-iron oxygenase or oxidase is formed in an active-site variant of homoprotocatechuate dioxygenase with a slow, non-native substrate (44). Although no \(K_D\) value was reported, examination of data presented in that study indicates that \(K_D\) must be significantly less than 1 mM to rationalize the high levels of \(\text{Fe}^{\text{III}}-\text{O}_2^-\) accumulation observed (60). Thus, if rapid and reversible addition of \(O_2\) to the \(\text{Fe}^{\text{II}}\) center of HppE is assumed, the lower limit that can be set for \(K_D\) is \(~100\) times greater than the corresponding parameters for these two reported reversible \(O_2\)-addition steps in authentic oxygen-activating enzymes.

**Figure S4.** Mössbauer spectra (4.2-K/zero-field) illustrating the modest conversion of HppE•\(\text{Fe}^{\text{II}}\)•S-HPP to the corresponding \(\text{Fe}^{\text{III}}-\text{O}_2^-\) complex upon exposure to high \([O_2]\). **Top:** spectrum of a sample of the HppE•\(\text{Fe}^{\text{II}}\)•S-HPP reactant complex reacted for 0.012 s with \(O_2\) as described in Experimental Procedures (black vertical bars). The red curve above the spectrum is a simulation of the spectrum of the presumptive \(\text{Fe}^{\text{III}}-\text{O}_2^-\) complex with isomer shift \(\delta = 0.50 \text{ mm/s}\) and quadrupole splitting parameter \(\Delta E_Q = 0.70 \text{ mm/s}\). It is plotted at an intensity corresponding to 8% of the total iron in the sample. **Bottom:** spectrum of a sample of the HppE•\(\text{Fe}^{\text{II}}\) complex reacted for 0.012 s with \(O_2\) as described in Experimental Procedures (black vertical bars). The solid lines in both
panels are experimental spectra of the HppE•FeII•S-HPP reactant solutions.

Substrate Binding Does Not Demonstrably Activate for Addition of O₂ to the FeII Cofactor: Figure S4 also shows that bound S-HPP does not augment conversion to the presumptive FeIII-O2•− adduct relative to the reaction of the substrate-free enzyme. The spectrum of the 0.012-s no-substrate control sample has a slightly greater intensity that is not attributable to the HppE•FeII starting material, although the new features are somewhat different from those in spectrum of the sample with S-HPP. This result indicates that substrate binding has little effect on O₂ addition to the active-site FeII center in HppE, which stands in contrast to the behavior of other known oxidases and oxygenases (18, 58).

Substrate Binding Does Activate for Reaction of the FeII Cofactor with H₂O₂. The hypothesis that HppE is actually a peroxidase suggests that bound substrate might instead facilitate addition of hydrogen peroxide to the FeII cofactor (Fig. 1B, magenta pathway). To test this possibility, we determined the second-order rate constant for addition of H₂O₂ to HppE•FeII in the absence of S-HPP for comparison to the values estimated above for productive combination of the HppE•FeII•S-HPP complex with H₂O₂. As shown in Figure S5, the substrate-free enzyme reacts with H₂O₂ to generate a stable and absorbing (presumably FeIII) complex, but the second-order rate constant for the reaction is only $(9 \pm 4) \times 10^3$ M⁻¹s⁻¹. This value is ~ 50-fold less than that determined for the productive reaction of the HppE•FeII•S-HPP complex. In other words, binding of S-HPP appears to activate the FeII cofactor for reaction with H₂O₂ but not O₂. These observations are consistent with our hypothesis that HppE is a peroxidase rather than an oxidase.

**Figure S5.** Kinetics of oxidation of the HppE•FeII cofactor upon reaction of its substrate-free form with H₂O₂. **A,** development of absorbance at 311 nm, presumably as a result of formation of a FeIII species. **B,** dependence of the observed first-order rate constant for the reaction on [H₂O₂], from which the second-order rate constant is determined. Reactions were performed by mixing equal volumes of HppE•FeII and H₂O₂ reactant solutions at 4°C. Final concentrations of HppE and FeII were 0.24 and 0.19 mM, respectively. The [H₂O₂] after mixing for each reaction is given in the legend to panel **A.**
Inability of the Structurally Similar 2-Hydroxyethyl-1-phosphonate Dioxygenase (HEPD) to Use H$_2$O$_2$. The data presented above both establish that HppE can efficiently use H$_2$O$_2$ to catalyze production of Fos and controvert all previously published evidence for its oxidase activity (3, 9, 53), but they leave open the question of whether the enzyme is, biologically, a peroxidase. The ability of some heme- and non-heme-iron oxidases and oxygenases to use H$_2$O$_2$ in place of O$_2$ is well documented. For the case of the heme-dependent cytochromes P450, the term "peroxide shunt" was coined to denote the productive reaction of the Fe$^{III}$ form of the enzyme with H$_2$O$_2$ in the absence of reductant (61). Similarly, soluble methane monooxygenase and several Rieske dioxygenases are capable of turnover upon reaction of their oxidized forms with H$_2$O$_2$ (62-65). These reactions are quite distinct from the demonstrated productive HppE peroxidase reaction, because they are mediated by the oxidized forms of the metallocofactors. Functional reaction of H$_2$O$_2$ with a reduced iron cofactor is not, however, entirely without precedent: the non-heme-Fe$^{II}$ center of the H$_2$O$_2$ sensor, PerR, from Bacillus subtilis reacts with H$_2$O$_2$ to effect the oxidation of histidine-37 to 2-oxo-histidine and initiate the associated signaling cascade (66-67).

Additional evidence that HppE is actually a peroxidase, rather than an oxidase that is capable of an adventitious "peroxide shunt" reaction, is provided by comparison to the enzyme's closest structural relative, 2-hydroxyethyl-1-phosphonate (HEP) dioxygenase (HEPD) (41). HEPD catalyzes the four-electron oxidation of HEP to hydroxymethylphosphonate and formate (Fig. 1C, reaction III). The two enzymes have overlapping substrate specificities: HEPD can oxidize (R)-2-hydroxypropyl-1-phosphonate (which, as noted above, is converted by HppE to the corresponding 2-ketone (27)) to a mixture of two-electron- and four-electron-oxidation products (42), and HppE can oxidize HEP (to products that remain to be identified; vide infra). X-ray crystallographic studies (5-6, 41) have shown that the coordination spheres of their non-heme-iron(II) cofactors are very similar, especially when the complex of HppE with their common substrate, R-HPP, is considered (Figure S6A). Their protein folds are also very similar (Figure S6B). HEPD is, unequivocally, an oxygenase (41). As such, it affords the opportunity to assess how efficient an adventitious "peroxide shunt" reaction might be in an authentically O$_2$-activating but otherwise homologous enzyme. Figure S7A shows that HEPD is vastly inferior to HppE at mediating substrate oxidation by H$_2$O$_2$. Upon addition of 125 enzyme equivalents of H$_2$O$_2$ to an O$_2$-free solution of HppE and S-HPP (also 125 equiv), approximately 63% of the S-HPP is consumed (~ 80 turnovers, red bars), consistent with other data. By contrast, HEPD does not significantly deplete HEP (< 5 turnovers) under these conditions (green bars), although ~1 equiv of product(s) could be detected at higher enzyme concentration. Remarkably, HppE is more active than HEPD under the peroxidase conditions even toward HEP (~ 70 turnovers, blue bars), whereas only HEPD can efficiently oxidize HEP with O$_2$ (Fig. S7B). The enzymes' nearly orthogonal use of the oxidants, despite their very similar structures and overlapping substrate specificities, supports the hypothesis that HppE is an authentic peroxidase.
**Figure S6.** Superpositions of the published structures of the HppE•Fe•R-HPP complex (pdb accession 3SCG) and the HEPD•Cd•HEP complex (pdb accession 3GBF) (6, 41). A, the active sites. B, the overlaid protein folds of HEPD (cyan) and Chain A of HppE (light brown). The protein sequences were aligned with the programs ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and MAFFT (http://mafft.cbrc.jp/alignment/software/). Structural alignment was accomplished by using the UCSF Chimera program (http://www.cgl.ucsf.edu/chimera/). The artwork was created with Adobe Illustrator CS4.

**Figure S7.** Demonstration that only HppE, and not the structurally similar oxygenase, HEPD, can efficiently use H\(_2\)O\(_2\) to oxidize 2-hydroxy-1-phosphonate substrates. A: Quantitites of substrate remaining before (solid bars) and after (striped bars) the slow addition (in the absence of O\(_2\)) of a total of 2.0 mM H\(_2\)O\(_2\) to a solution containing final concentrations of: 20
µM HppE, 16 µM Fe\textsuperscript{II}, 10 mM \textit{L}-ascorbic acid and 2.0 mM d\textsubscript{4}-S-HPP \textcolor{red}{(red)}; 20 µM HppE, 16 µM Fe\textsuperscript{II}, 10 mM \textit{L}-ascorbic acid and 2.0 mM HEP \textcolor{blue}{(blue)}; or 20 µM HEPD, 16 µM Fe\textsuperscript{II}, 10 mM \textit{L}-ascorbic acid and 2.0 mM HEP \textcolor{green}{(green)}. \textbf{B}: Quantities of HEP remaining before \textcolor{blue}{(solid bars)} and after \textcolor{green}{(striped bars)} solutions of 20 µM HppE \textcolor{blue}{(blue)} or HEPD \textcolor{green}{(green)}, 16 µM Fe\textsuperscript{II} and 2.0 mM HEP were stirred under air for 30 min at 21 °C. The error bars are the standard deviations from the mean values of four measurements under each condition.
References and Notes


22. H. M. Hanauske-Abel, V. Günzl er, A stereochemical concept for the catalytic mechanism of prolylhydroxylase: Applicability to classification and design of


44. H. Hirao, F. Li, L. Que Jr., K. Morokuma, Theoretical study of the mechanism of oxoiron(IV) formation from H₂O₂ and a nonheme iron(II) complex: O-O cleavage


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