

Site-directed mutagenesis and spectroscopic studies of the iron-binding site of (*S*)-2-hydroxypropylphosphonic acid epoxidase

Feng Yan^a, Tingfeng Li^c, John D. Lipscomb^b, Aimin Liu^{b,c}, Hung-wen Liu^{a,*}

^a Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas, Austin, TX 78712, USA

^b Department of Biochemistry, Molecular Biology, and Biophysics and Center for Metals in Biocatalysis, University of Minnesota, Minneapolis, MN 55455, USA

^c Department of Biochemistry, University of Mississippi Medical Center, University of Mississippi, 2500 N. State St., Jackson, MS 39216, USA

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Abstract

(*S*)-2-Hydroxypropylphosphonic acid epoxidase (HppE) is a novel type of mononuclear non-heme iron-dependent enzyme that catalyzes the O₂ coupled, oxidative epoxide ring closure of HPP to form fosfomycin, which is a clinically useful antibiotic. Sequence alignment of the only two known HppE sequences led to the speculation that the conserved residues His138, Glu142, and His180 are the metal binding ligands of the *Streptomyces wedmorensis* enzyme. Substitution of these residues with alanine resulted in significant reduction of metal binding affinity, as indicated by EPR analysis of the enzyme–Fe(II)–substrate–nitrosyl complex and the spectral properties of the Cu(II)-reconstituted mutant proteins. The catalytic activities for both epoxidation and self-hydroxylation were also either eliminated or diminished in proportion to the iron content in these mutants. The complete loss of enzymatic activity for the E142A and H180A mutants *in vivo* and *in vitro* is consistent with the postulated roles of the altered residues in metal binding. The H138A mutant is also inactive *in vivo*, but *in vitro* it retains 27% of the active site iron and nearly 20% of the wild-type activity. Thus, it cannot be unequivocally stated whether H138 is an iron ligand or simply facilitates iron binding due to proximity. The results reported herein provide initial evidence implicating an unusual histidine/carboxylate iron ligation in HppE. By analogy with other well-characterized enzymes from the 2-His-1-carboxylate family, this type of iron core is consistent with a mechanism in which both oxygen and HPP bind to the iron as a first step in the conversion of HPP to fosfomycin.

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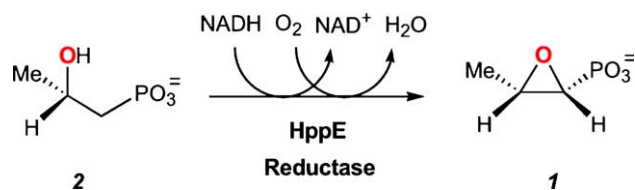
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(*S*)-2-Hydroxypropylphosphonic acid epoxidase (HppE) catalyzes the last step in the biosynthesis of the clinically useful antibiotic fosfomycin (**1**) as shown in [Scheme 1](#) [1–3]. Molecular oxygen and two reducing equivalents supplied by an NAD(P)H-coupled reductase are required for the reaction [2]. Recently, we have shown that HppE also requires a single iron atom for

catalysis [2]. Together, these observations would suggest a mechanism in which the molecular oxygen is activated at the mononuclear iron for insertion into the substrate to form fosfomycin. However, direct experiments have shown that neither of the oxygen atoms from O₂ is incorporated into the fosfomycin product [4,5]. Instead, the oxygen atom of the oxiranyl ring in fosfomycin (**1**) is derived from the secondary hydroxyl group of HPP (**2**) ([Scheme 1](#)) [2,4,5]. Hence, the unusual epoxidation catalyzed by HppE is effectively a dehydrogenation rather than an oxygenation reaction.

* Corresponding author. Fax: +1 512 471 2746.

E-mail address: h.w.liu@mail.utexas.edu (H.-w. Liu).



Despite the unusual chemistry of its native reaction, HppE can also catalyze more typical oxygenase reactions. For example, HppE is capable of self-hydroxylation of an active site tyrosine, most likely Tyr105, to 3,4-dihydroxyphenylalanine (DOPA105) [6]. Subsequent chelation of the active site ferric ion by DOPA105 forms a catecholate-to-Fe(III) charge transfer complex, giving HppE a green color. This reaction is distinct from self-hydroxylation reactions of other non-heme iron-dependent enzymes, such as ribonucleotide reductase (RNR) R2 F208Y mutant [7], taurine dioxygenase (TauD) [8], and 2,4-dichlorophenoxyacetic acid dioxygenase (TfdA) [9], because the newly introduced oxygen atom in the DOPA is derived from dioxygen and not H₂O [6]. A similar incorporation pattern has only been found for the tyrosine hydroxylase (TyrH)-catalyzed DOPA production [10]. The discovery of true oxygenase activity for HppE has significant mechanistic implications since the same reactive intermediate responsible for self-hydroxylation may also participate in the reaction cycle for the formation of fosfomycin.

The use of NAD(P)H as a source of reducing equivalents distinguishes HppE from many other non-heme iron containing oxygenases which commonly derive electrons from the substrate or a co-substrate like α -ketoglutarate, ascorbate, or tetrahydropterin [11–15]. Two exceptions to this are the large family of oxygen-bridged diiron cluster containing monooxygenases [16] and the Rieske-type mono- and dioxygenases which contain both an iron sulfur and a mononuclear iron site [17]. These oxygenase classes have two redox active metals or clusters that can accept both electrons (indirectly) from NAD(P)H at the outset of the reaction. In contrast, HppE has only one iron, so it must channel the addition of electrons during the reaction cycle in some manner.

The unique features of HppE described above suggest that it represents a distinct new member of the non-heme oxygen activating enzyme class. It is important to deter-

mine whether the unique catalytic features derive from special features of the metal ligand environment, of the active site, or of both. Thus far, only the HppEs from *Streptomyces wedmorensis* [18] and *Pseudomonas syringae* PB-5123 [19] have been isolated and biochemically characterized. Sequence analysis shows that they both belong to the cupin structural superfamily, and contain two sequence motifs that are poorly conserved among the members of this family: motif 1, **GX₅HXHX_{3,4}EX₆G**, and motif 2, **GX₃PXGX₂HX₃N** [20]. The crystal structures of several cuprin metalloproteins have been solved, including Mn(II)-bound barley germin [21], Zn(II)-bound phosphomannose isomerase [22], Fe(II)-bound α -KG-dependent dioxygenases [23], and Fe(II)-bound aromatic ring cleaving dioxygenases [24,25]. In all of these structures, the divalent metal centers have similar coordination environments consisting of two (or occasionally three) histidines and one glutamate or aspartate from these two motifs. The two histidine motif has been termed a 2-His-1-carboxylate facial triad because all of the fixed ligands occur on one face of the iron, leaving the opposite face available for substrates [26]. Interestingly, sequence alignment of the only two known HppE sequences suggested that conserved residues from both sequence motifs might serve as iron ligands in the *S. wedmorensis* enzyme [2], His138 and Glu142 from “motif 1” and His180 from “motif 2” (Fig. 1).

Here, we have directly tested the postulated ligation of the iron-binding site in HppE by site-directed mutagenesis. The effects of mutation of the putative ligand residues on iron binding, substrate binding, self-hydroxylation, and enzymatic epoxidation are investigated. These studies show that HppE is likely to be a new member of the 2-His-1-carboxylate class of enzymes which generally initiate catalysis by binding substrate and oxygen to exchangeable sites in the iron coordination. Thus, these studies support and extend our proposals for the molecular mechanism of this novel enzyme.

Materials and methods

General methods

Culture medium ingredients were products of Difco (Detroit, MI). All electrophoresis materials were purchased from Gibco-BRL (Gaithersburg, MD) or Bio-

<i>S. wedmorensis</i>	DYYVYNCLVR	TKRAPSLVPL	VVDVLTDPD	DAKFNSGHAG	NEFLFVLEGE	150
<i>P. syringae</i>	HYTYEHLVT	TNQDPLMAL	RLDLHSDDEQ	PLRLNGGHGS	REIVYVTRGA	140
Consensus	.YY.Y..LV.	T...P.L..L	..D...D...	...N.GH..	.E...V..G.	150
<i>S. wedmorensis</i>	IHMKW-GDKE	NPKEALLPTG	ASMFVEEHVP	HAFTAAGTGT	SAKLIIVNPF-	198
<i>P. syringae</i>	VRVRWVGDN	ELKEDVLNEG	DSIFILPNVP	HSFTNHVGG	KSEIIAINYG	190
Consensus	...W.GD..	..KE..L..G	.S.F....VP	H.FT...G..	...IA.N..	200

Fig. 1. Protein sequence alignment of HppE from *Streptomyces wedmorensis* and *P. syringae* (amino acid mutated in this study are shaded).

Table 1
Oligonucleotides used for site-directed mutagenesis

Mutant	Oligonucleotide sequence ^a
H138A	5'-GAAGTTCAACTCGGGCGCCGCCGGCA ACGAG-3'
E142A	5'-GCCACGCCGGCAACGCGTTCCTCTTC GTGCTCG-3'
H180A	5'-GGAGGAGCACGTGCCGGCCGCCTTCA CGGCGGC-3'

^a The sites of mutation are underlined.

Rad (Hercules, CA). All chemicals were analytical grade or the highest quality commercially available. Biochemicals including fosfomycin disodium salt standard were purchased from Sigma–Aldrich Chemical (St. Louis, MO), unless noted otherwise. The construction of plasmid pPL1001 that contains the gene coding for HppE, *fom4*, was previously reported [2]. Protein concentrations were determined by the procedure of Bradford method [27] using bovine serum albumin as the standard. The substrate, HPP (2), was chemically synthesized according to a literature procedure [28].

Site-directed mutagenesis

Mutagenesis of the HppE gene (*fom4*) was carried out using QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The oligonucleotides used for mutagenesis were customarily synthesized by Integrated DNA Technology (Coralville, IA) (Table 1). The constructed mutant plasmids were amplified in *E. coli* strain DH5 α and purified with Qiaprep spin miniprep kit (Qiagen, Valencia, CA). Once the mutation was verified by DNA sequencing performed by the core facility in the Institute for Cellular and Molecular Biology of the University of Texas at Austin, the mutant plasmids were used to transform *Escherichia coli* cell BL21(DE3) (Novagen, Madison, WI) for gene expression.

Expression and purification

The wild-type HppE and its mutants were produced by growing the corresponding recombinant strains in LB medium and then the proteins were purified according to previously reported procedures [2]. SDS–PAGE was used to monitor the elution processes during purification. The purified enzymes were desalted, concentrated, and stored at -80°C .

Nitroblue tetrazolium (NBT) quinone staining

Proteins were first electrophoresized on a SDS–PAGE gel with pre-stained broad-range protein marker (New England Biolabs, Beverly, MA) and then transferred onto a nitro-cellulose membrane using the Mini Trans-Blot Cell Assembly (Bio-Rad). The transblotting

was conducted in the transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) at 100 V for 1 h. The DOPA-containing proteins were visualized with a temporary staining solution (0.1% (w/v) ponceau S, 5% (v/v) acetic acid) [29]. After the temporary stains were removed by washing with H₂O, the protein-containing nitro-cellulose membrane was immersed in a solution of 0.24 mM NBT and 2 M potassium glycinate, pH 10, in dark for 45 min to visualize the DOPA-containing protein band [30].

Preparation of ferric samples

The apo-proteins of the wild-type and mutant enzymes were made anaerobic by repeated cycles of evacuation and flushing with argon. A molar equivalent of Fe(NH₄)₂(SO₄)₂ from an anaerobic stock solution was added to the apo-proteins under anaerobic conditions. The Fe(II)-loaded proteins were subsequently exposed to air until all of the iron was oxidized to the ferric state.

Preparation of cupric samples

One molar equivalent of cupric ion from a CuSO₄ stock solution (20 mM) was slowly added to the apo-proteins of the wild-type and mutant enzymes. Rapid addition of the buffered cupric solution often led to protein precipitation. To prepare substrate-containing samples, the Cu(II)-reconstituted proteins were further incubated with ten molar equivalents of HPP.

Preparation of ferrous nitrosyl samples

The Fe(II)-loaded proteins were prepared as described above. Subsequent incubation of each sample with ten molar equivalents of HPP (2) was carried out anaerobically in the EPR tubes. Nitric oxide gas was passed over NaOH pellets to remove any acid impurities and then introduced into the substrate-bound enzyme samples through a gas-tight Hamilton syringe under argon. The samples were frozen by slow immersion in liquid nitrogen for later EPR analysis.

Epoxidase activity assay

The assay conditions varied slightly from those of previously reported [2] for better comparison between mutant proteins. The major change is that, instead of using Fe(III)-reconstituted HppE, the apo-enzyme was reconstituted in situ aerobically with Fe(NH₄)₂(SO₄)₂. A typical assay solution (200 μL) contains 70 μM enzyme, 70 μM Fe(NH₄)₂(SO₄)₂, 15 mM HPP (2), 60 μM FMN, and 22.5 mM NADH in 20 mM Tris–HCl buffer, pH 7.5. The reaction was carried out at room temperature with vigorous shaking and then quenched by the addition of 40 μL EDTA (0.5 M). The reaction mixture was kept on

ice for immediate NMR analysis or frozen at -80°C and thawed shortly before analysis. The enzyme activity was calculated based on integration of the ^{31}P NMR peaks assigned to product (δ 10.9) and substrate (δ 19.9). A sample of wild-type HppE was run in parallel as a standard in order to compare the enzyme activity of different mutants.

In vitro self-hydroxylation

As purified apo-proteins were diluted with H_2O to 1 mg/mL and were aerobically incubated with one equivalent of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and 10 equivalents of ascorbate at 4°C for 1 h. Such reaction conditions have been shown to be most effective for *in vivo* self-hydroxylation of HppE [6]. The mixtures were concentrated by Amicon concentrator with a membrane pore size of 10 kDa (Millipore, Bedford, MA). The final protein concentrations were between 10 and 20 mg/mL. The extent of self-hydroxylation of each HppE mutant was analyzed by NBT quinone staining and UV-vis absorption spectroscopy.

EPR spectroscopy

EPR first derivative spectra of HppE were collected at X-band microwave frequency with 100 kHz field modulation using either a Bruker Elexsys E500 or an EMX spectrometer, both of which were equipped with an Oxford Instruments ESR910 cryostat. A calibrated frequency meter and a Bruker ER035M NMR Gauss meter were used for the g value determination. Spin quantitation on the EPR spectra of the mutant enzymes was performed by comparing the double integration using the wild-type enzyme as the standard.

Results

Preparation of HppE mutants

Using plasmid pPL1001, which contains HppE-encoding gene (*fom4*), as the template, the H138A, E142A, and H180A mutant plasmids were constructed. The mutations were confirmed by DNA sequencing, and the mutant genes were expressed and the resulting proteins purified by protocols previously developed for the wild-type HppE [2]. The Stokes radius and spectroscopic features of each mutant protein as purified were identical to those of wild-type protein (all apo, see below) indicating that no significant conformational change due to mutation occurs. These purified mutant proteins, along with the wild-type HppE, were subjected to SDS-PAGE followed by transblotting onto a nitro-cellulose membrane. The protein bands on the membrane were visualized by a reversible red dye, ponceau S [29], and all proteins were shown to have been purified to near

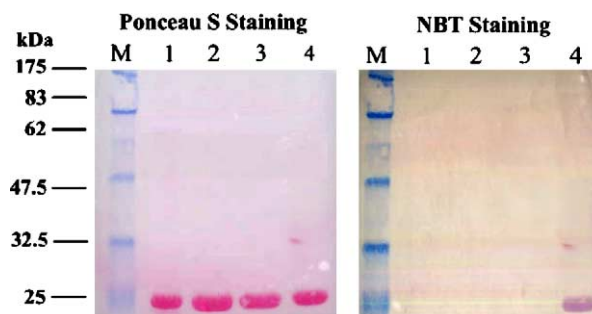


Fig. 2. Ponceau S (left) and NBT (right) staining of purified HppE and its mutants. (M) protein marker; (1) H138A; (2) E142A; (3) H180A; and (4) wild-type (Note: Ponceau S staining shows all proteins while NBT staining only shows modified proteins).

homogeneity (Fig. 2). After a thorough rinse to remove the red dye, the membrane was subjected to the NBT quinone staining [30]. This quinone staining method was designed to identify quinonoid compounds, such as 1,2,4-trihydroxybenzene, menadione, and DOPA. It is also a convenient assay for quinonoid proteins, and has been applied to detect DOPA residue formation in HppE as a result of self-hydroxylation [6]. Here, the H138A, E142A, and H180A mutants were found to be insensitive to the NBT staining (Fig. 2). Evidently, mutation at H138, E142, or H180 significantly impairs the *in vivo* oxygenase activity of HppE, resulting in no discernible self-hydroxylation of these three mutants.

Electronic absorption of Fe(III)-reconstituted HppE and its mutants

Since EDTA and DTT were used throughout purification, the isolated proteins were essentially iron-free. To study the iron binding properties of HppE and its mutants, the isolated enzymes were subjected to metal reconstitution. The low aqueous solubility of ferric ion at neutral pH precludes the reconstitution of apo-HppE by mixing the protein with ferric ion directly to generate the Fe(III)-HppE resting state. Instead, the fully reconstituted Fe(III)-HppE was prepared by aerobic incubation of apo-enzyme with a stoichiometric amount of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ [2] which, after air oxidation, was converted to its ferric form in the enzyme active site. As shown in Fig. 3, the optical spectrum of Fe(III)-reconstituted wild-type HppE displays a broad peak centered around 680 nm ($\epsilon \approx 450 \text{ M}^{-1} \text{ cm}^{-1}$) that has been assigned to the ligand-to-metal charge transfer (LMCT) band of the Fe(III)-catecholate complex [6]. When the H138A, E142A, and H180A mutants were aerobically reconstituted with an equivalent of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, a bright yellow color characteristic of Fe(III) slowly appeared in all protein solutions. After exposing the reconstituted mutants to air for 1 h to give the maximum intensity of the yellow color, the electronic absorption spectra were recorded. As shown in Fig. 3,

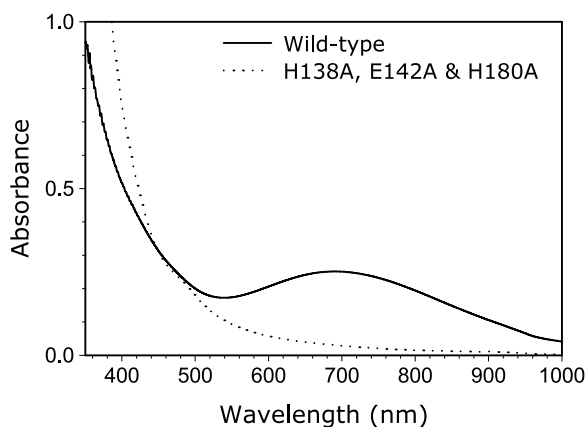


Fig. 3. Electronic absorption spectra of Fe(III)-HppE (solid line) and its mutants (dotted line) (protein concentrations, 0.5 mM).

the spectra of these mutants are superimposable, and are clearly different from that of the wild-type HppE, lacking the characteristic absorption band of the Fe(III)-catecholate complex at 680 nm. These data are in accord with the results obtained from the NBT staining experiments, reflecting the absence of the iron-dependent *in vivo* self-hydroxylation in these mutant enzymes, the failure to bind Fe(III) in these mutants, or both.

Spectral properties of Cu(II)-substituted HppE

The lack of an EPR signal and the facile oxidation of the enzyme bound Fe(II) center make direct spectroscopic studies of Fe(II)-dependent enzymes challenging. To circumvent these problems, Fe(II) in enzymes is often replaced by other divalent metal ions that are more aerobically stable and spectroscopically accessible. The most commonly used metal ion substitute is Cu(II) because of its well characterized spectral properties. Accordingly, Cu(II) was chosen as EPR spectroscopic probe and was reconstituted with the wild-type apo-HppE at 1:1 molar ratio. The electronic and EPR spectra of the resulting Cu(II)-HppE exhibit typical spectral features of a type II copper center. These include an absorption maximum at 690 nm ($\epsilon = 84 \text{ M}^{-1} \text{ cm}^{-1}$) which is characteristic for the low-intensity $d \rightarrow d$ transitions in type II Cu(II) (Fig. 4), and the EPR spectrum with a g_{\parallel} value of 2.276 and an A_{\parallel} value of $172 \times 10^{-4} \text{ cm}^{-1}$, which are indicative of a type II Cu center with a tetragonal coordination geometry (Fig. 5). In the presence of HPP (2), the absorption maximum shifts to 860 nm ($\epsilon = 92 \text{ M}^{-1} \text{ cm}^{-1}$) (Fig. 4). The Cu EPR signal also changes, exhibiting a smaller A_{\parallel} value of $135 \times 10^{-4} \text{ cm}^{-1}$ and an increased g_{\parallel} value of 2.366 (Fig. 5), both of which are consistent with a more oxanion-rich environment [31]. Such a large increase in g_{\parallel} value and the shift of the absorption maximum accompanying the binding of the substrate HPP suggest

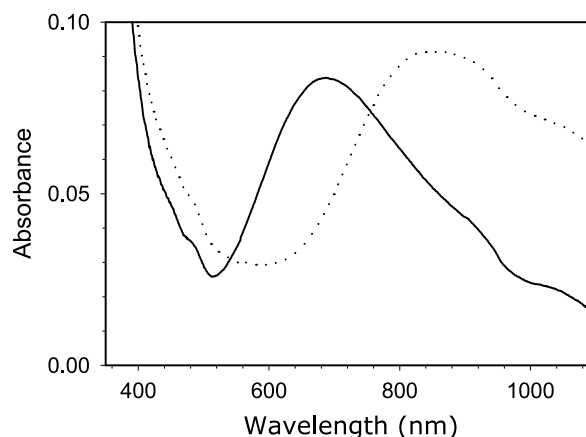


Fig. 4. Electronic absorption spectra of Cu(II)-substituted HppE (solid line) and bound with substrate (dotted line) (protein concentration, 0.8 mM).

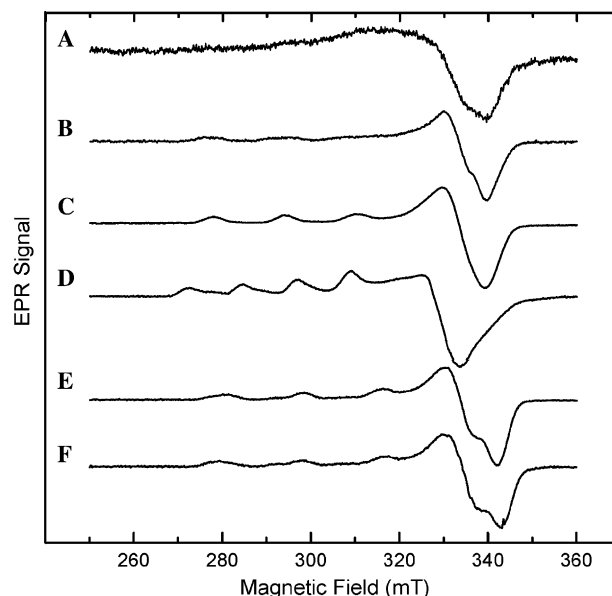


Fig. 5. EPR spectra of the Cu(II)-substituted HppE. (A) CuSO_4 in Tris-HCl buffer; (B) complex of substrate HPP and CuSO_4 ; (C) Cu(II)-substituted HppE; (D) Cu(II)-substituted HppE in the presence of excess amount of substrate HPP; (E) Cu(II)-substituted H142A HppE; and (F) Cu(II)-substituted H142A HppE in the presence of excess amount of substrate HPP. Samples contained $625 \mu\text{M}$ Cu(II). HppE concentration was $625 \mu\text{M}$ (monomer). EPR conditions: temperature, 20 K; microwave power, 0.6 mW; and modulation amplitude, 0.3 mT.

that Cu(II) is bound to the active site, and may be directly coordinated with substrate in the active site. In addition, Cu(II) (1 M equivalent) was found to be inhibitory toward HppE when added together with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ during reconstitution. Inhibition is likely due to the competition of Cu(II) with Fe(II) for the same metal-binding site. This observation is also consistent with the above conclusion, indicating that, while the Cu(II) reconstituted HppE is catalytically inactive, Cu(II) is a suitable substitute of Fe(II) for probing metal binding in HppE.

Table 2
Spectral parameters of copper-substituted HppE mutants

Cu ^{II} -substituted protein	HPP	Optical features		EPR parameters		
		λ_{\max} (nm)	ϵ (M ⁻¹ cm ⁻¹ /Cu)	g_{\parallel}	A_{\parallel}^a	g
Wild-type	–	690	84	2.276	172	2.063
	+	860	92	2.366	135	2.099
H138A	–	611	107	2.233	187	2.054
	+	611	111	2.234	195	2.054
E142A	–	615	75	2.234	185	2.055
	+	618	76	2.238	187	2.054
H180A	–	610	142	2.233	183	2.053
	+	608	130	2.230	180	2.052
Cu(II) (Tris–HCl buffer)	–	648	50	NR ^b	NR	2.076
	+	640	55	2.279	172	2.061

^a EPR hyperfine coupling parameter A is given in 10⁻⁴ cm⁻¹.

^b NR, the spectrum has no resolved features to record the corresponding parameters.

Spectral properties of Cu(II)-substituted HppE mutants

The HppE mutants were also reconstituted with Cu(II) and characterized by electronic absorption and EPR spectroscopies. The observed spectral parameters are listed in Table 2. Typical EPR spectra are shown in Figs. 5E–F. Overall, the spectra of the H138A, E142A, and H180A mutants are similar to each other but significantly different from those of the wild-type enzyme. In particular, they do not respond to the addition of substrate HPP in the same way as the Cu(II)-substituted wild-type enzyme. For example, the spectra of the Cu(II)-HppE mutants, without bound substrate, differ from the wild-type enzyme by a blue-shift of the weak cupric d → d transition from 690 to 610–615 nm and the less rhombic EPR signals with smaller g_{\parallel} and larger A_{\parallel} values. When HPP is added, dramatic changes are observed for the spectra of Cu(II)-HppE, while those of Cu(II)-reconstituted H138A, E142A, and H180A mutants are essentially unchanged (Table 2). These results show that the copper added to the mutants is neither free in solution, bound exclusively to HPP, nor in the same protein environment as observed for wild-type enzyme. It may be that the copper is bound adventitiously to the mutant enzyme in a site where it cannot interact with HPP. In any event, mutation of any of the three candidate ligand residues results in a very significant change in the Cu binding behavior of HppE.

Fe(II)-HPP nitrosyl complexes of HppE H138A, E142A, and H180A mutants

An Fe(II)-HppE-HPP nitrosyl ternary intermediate, which presents a nearly homogeneous EPR signal with principal g values of 1.97, 3.63, and 4.42 ($S = 3/2$) and E/D of 0.066 (Fig. 6A), was characterized in our previous studies [2]. The EPR spectrum of this intermediate resembles that of the Fe(II)-enzyme-substrate nitrosyl complexes observed for many non-heme Fe(II) containing enzymes such as isopenicillin N synthase (IPNS) [32] and

1-aminocyclopropane-1-carboxylate oxidase (ACCO)¹ [14]. This ternary complex is considered to be representative of the catalytically active form of enzyme-substrate-O₂ intermediate. Mutation of the iron ligands is expected to decrease the intensity of the signal due to lower iron affinity and also change the spectroscopic fingerprint of the ternary complex because the EPR lineshape has been shown to be very sensitive to changes in the ligation and environment of iron for many other enzymes. To assess the roles of H138, E142, and H180 on the iron coordination in HppE, the H138A, E142A, and H180A mutants were anaerobically reconstituted with ferrous ion, and (*S*)-HPP was added. Then the sample was exposed to NO and analyzed by EPR spectroscopy.

As shown in Figs. 6B–D, in the presence of substrate and NO under anaerobic conditions, these mutants exhibit a major EPR spectrum with g values of 2.013 and 2.039, in addition to an anomalous signal from unbound NO at about $g = 1.96$. The signals slightly above $g = 2$ are absent in the EPR spectrum of the wild-type enzyme (Fig. 6A). We assign these signals to nonspecifically bound Fe(II) nitrosyl species that have previously been

¹ Abbreviations used: ACCO, 1-aminocyclopropane-1-carboxylate oxidase; ACC, 1-aminocyclopropane-1-carboxylic acid; ACV, δ -(L- α -amino acidipoyl)-L-cysteinyl-D-valine; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; CAS, clavaminic acid synthase; DAOCS, deacetoxycephalosporin C synthase; DEAE, diethylaminoethyl; DOPA, L-3,4-dioxyphenylalanine; DTT, dithiothreitol; E₃, CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase reductase; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HPP, (*S*)-2-hydroxypropylphosphonic acid; HppE, (*S*)-2-hydroxypropylphosphonic acid epoxidase; IPNS, isopenicillin N synthase; IPTG, isopropyl- β -D-thiogalactopyranoside; α -KG, α -ketoglutaric acid; LB medium, Luria–Bertani medium; LMCT, ligand-to-metal charge transfer; NBT, nitroblue tetrazolium; NDO, naphthalene 1,2-dioxygenase; PCR, polymerase chain reaction; PheH, phenylalanine hydroxylase; NO, nitric oxide; PMI, phosphomannose isomerase; RNR, ribonucleotide reductase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TauD, taurine dioxygenase; TfdA, 2,4-dichlorophenoxyacetic acid dioxygenase; TrpH, tryptophan hydroxylase; TyrH, tyrosine hydroxylase; Tris, Tris(hydroxymethyl)aminomethane.

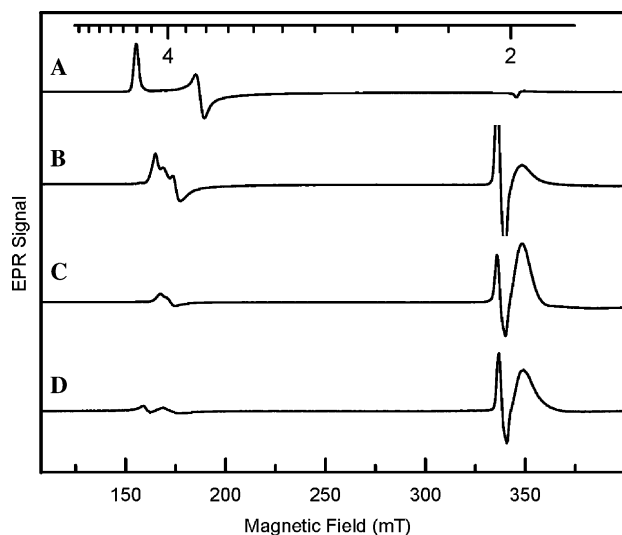


Fig. 6. EPR spectra of the Fe(II)-substrate-NO complexes obtained from (A) wild-type enzyme, (B) H138A, (C) E142A, and (D) H180A. See Table 3 for quantitations. EPR parameters: temperature, 2 K; microwave frequency, 9.6 GHz; modulation frequency, 100 KHz; and power, 0.6 mW; modulation amplitude, 0.3 mT. Samples C and D possess large excess NO (as shown at $g = 1.96$) to detect the full amount of Fe(II) at the active site.

noted with many non-heme iron-nitrosyl complexes in proteins and model systems [33–38]. In the $g = 4$ region, signals from an $S = 3/2$ species with different E/D values and much lower intensities than that exhibited by the wild-type enzyme were observed for the mutant enzymes. The $S = 3/2$ species in each EPR spectrum were quantified by double integration and compared with that of the wild-type enzyme at the same concentration (Table 3). It becomes immediately clear that the individual mutations of His138, Glu142, and His180 significantly reduce the iron binding affinity in the site where it can interact with both substrate and a small molecule O_2 surrogate. Moreover, the change in E/D value shows that each mutation alters the environment of the small amount of iron that does bind in the active site. The data is fully consistent with Glu142 and His180 serving as iron ligands. It is likely that H138 is also a ligand based on the significant changes observed (Table 3), but the fact that some iron binds and a proportionate amount of activity is retained after reconstitution of the H138A mutant opens the possibility that H138 is simply located proximal to the iron binding site.

Table 3
Properties of iron-reconstituted HppE mutants

Enzymes (%)	H138A	E142A	H180A	Wild-type
Active site iron ^a	26.6	2.1	5.8	100
Epoxidase activity ^b	19.9	0	0	100

^a Calculated from the EPR spectra of Fe(II)-enzyme-HPP nitrosyl complexes.

^b Obtained under new assay conditions as reported in the text.

Epoxidase activity of mutant enzymes

In our previous studies, holo-HppE was generated by reconstitution of the apoenzyme with Fe(II) under aerobic conditions [1,2]. The resulting Fe(III)-enzyme was passed through a gel-filtration column to remove the unbound iron prior to activity assay. However, we encountered problems when applying these same procedures to the HppE mutants. Due to their reduced iron binding affinities, a larger amount of iron was depleted from the HppE mutants during the gel-filtration step, making a comparison of enzyme activity based on protein concentration between the wild-type and mutant enzymes invalid. To ensure comparable conditions for all the enzymes, a stoichiometric amount of $Fe(NH_4)_2(SO_4)_2$ was added directly to the apo-enzyme (1:1 ratio) in the assay solution to reconstitute the enzyme in situ. The epoxidase activities determined under the new assay conditions are given in Table 3. It should be noted that the enzyme activity of the wild-type HppE determined by this method is nearly identical to that determined with Fe(III)-reconstituted enzyme [2]. However, the new assay method proved to be more effective for the mutant proteins. In general, there is a good correlation between the concentration of iron that gives an $S = 3/2$ type EPR signal in the enzyme-substrate-nitrosyl complex and the epoxidase activity despite the changes in iron environment revealed by the shifts in E/D values.

Self-hydroxylation activity of mutant enzymes

The data presented in Figs. 2 and 3 shows that in vivo self-hydroxylation was eliminated by mutations of His138, Glu142, and His180. This loss of activity is correlated with the loss of iron in the purified mutant enzymes (Table 3), in accord with the previous observation that this activity is iron-dependent [6]. However, some residual self-hydroxylation activity would have been expected for the H138A mutant because the mutant retains a small amount of iron and epoxidase activity. Hence, the purified mutants were examined more carefully under optimal conditions for in situ iron reconstitution and self-hydroxylation (1 equiv. of $Fe(NH_4)_2(SO_4)_2$ and 10 equiv. of ascorbate). The results showed that no self-hydroxylation occurs for the E142A and H180A mutants, but limited self-hydroxylation is observed for H138A mutant based on both the optical spectrum and NBT staining after gel electrophoresis (data not shown).

Discussion

In this study, site-directed mutagenesis and spectral analyses were used to assess the possible roles of His138,

Glu142, and His180, as active site ligands for metal binding in HppE. It was shown that mutating these residues individually is sufficient to either eliminate or greatly inhibit both the native epoxidase activity and the self-hydroxylation of the enzyme. As compared to the wild-type enzyme, the small amount of iron bound to the purified mutant enzymes under optimal reconstitution conditions is in a different electronic environment which remains unchanged upon substrate binding (Table 3). Similarly, substitution of Cu for iron in the reconstitution process showed that mutation of the putative metal ligands results in the alteration of the metal environment and the loss of response to added substrate (Fig. 5). These results suggest that HppE is a new member of the 2-His-1-carboxylate family of enzymes. This has implications for the mechanism of the enzyme which are discussed here.

His138 as an iron ligand

The results presented here uniformly support the proposal that Glu142 and His180 are two of the iron binding ligands. In contrast, mutation of His138 did not result in the complete loss of iron binding following reconstitution, and the reconstituted enzyme retained some epoxidation and self-hydroxylation activities. In comparison to other known 2-His-1-carboxylate enzymes, retention of 20% of the wild-type activity of the HppE H138A mutant is unusual. In most cases, including phenylalanine hydroxylase (PheH) [39], TyrH [40], IPNS [41], toluene dioxygenase [42], ACCO [43], TfdA [44], and clavaminic acid synthase (CAS) [45], the catalytic activities are completely abolished when one of the metal binding ligands is mutated to alanine. A few exceptions are known, such as the CAS H145Q and H280Q mutants [46] and the TyrH H336E and H336Q mutants [47], where residual catalytic activities ranging from <3% for the CAS mutants [48] to about 4–12% for the TyrH mutants were reported [49]. The residual activities found in these H → Q mutants may be attributed to the partial competence of glutamine to replace histidine in binding of iron. However, the same reasoning is not applicable to the case of HppE since the histidine at position 138 is replaced by an alanine, which cannot serve as a metal ligand. Thus, the role of His138 as an iron ligand cannot be unequivocally confirmed based on these results.

It is possible that H138 is indeed the iron ligand but that another amino acid residue near the active site may serve the role of His138 after its mutation, thereby attenuating the effects of mutation on iron binding and catalytic activity of the H138A mutant. Alternatively, the small size of the side chain of Ala versus His at position 138 may allow iron to bind with a vacant ligand site or with solvent in place of an amino acid in one position. This might be expected to reduce the affinity of the

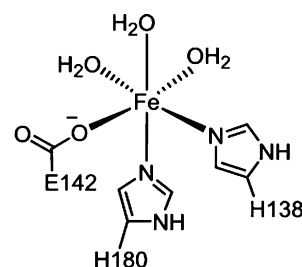
enzyme for the iron (as observed), but it may not eliminate iron binding if the relative orientations of the other iron ligands are not perturbed. This has precedent in the case of protocatechuate 3,4-dioxygenase where either of the two tyrosine ligands can be replaced without complete loss of the active site iron [48].

It is worth mentioning that a DOPA residue has been identified in HppE as a product of self-hydroxylation on Tyr105 [6]. However, the self-modification is far below completion. Hence, the as isolated HppE exists as a mixture of modified and unmodified forms, and the iron coordination environment must be very different in these two forms of HppE. Interestingly, the ternary complex of Fe(II)-HppE, substrate, and nitric oxide demonstrated a strong homogeneous EPR signal accountable for >95% iron bound in the active site [2]. The uniformity of substrate-bound iron active site observed in this ternary complex is inconsistent with the possibility of DOPA ligating to the ferrous center in the HppE-substrate complex.

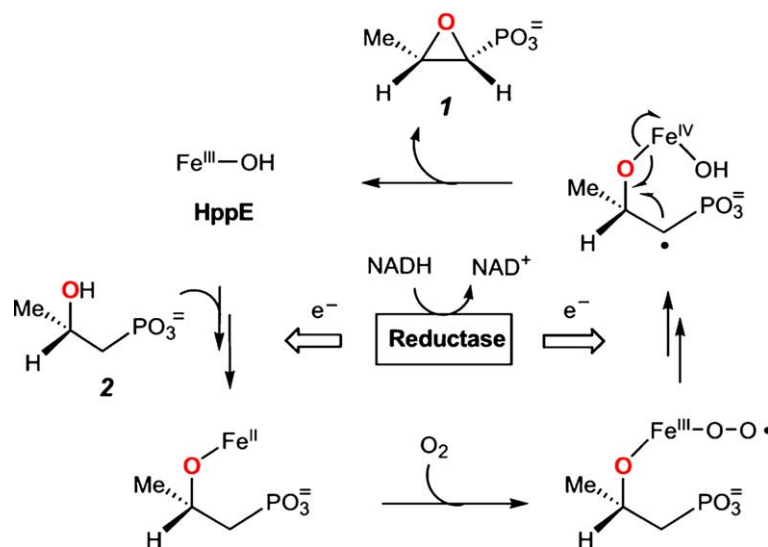
Significance of the iron ligation

Although some doubt remains as to whether His138 is an iron ligand, the significant changes caused by its mutation support such a role. At least some of the remaining three iron ligand sites are occupied by displaceable ligands such as solvents to allow NO and probably substrate to bind. Overall, this would yield the correct stoichiometry for a 2-His-1-carboxylate facial triad ligation with the other ligand sites occupied by 2 or 3 solvents. By analogy with other enzymes that utilize this binding, a model of the HppE active site can now be proposed as depicted in Scheme 2.

The 2-His-1-carboxylate facial triad metal center has been found in many mononuclear non-heme iron-dependent enzymes [49], including 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) [24,25], IPNS [50], the Rieske-type naphthalene 1,2-dioxygenase (NDO) [51], the pterin-dependent PheH [52], TyrH [53], and tryptophan hydroxylase (TrpH) [54], and the α -KG-dependent deacetoxycephalosporin C synthase (DAOCS) [23], CAS [55], and TauD [56]. Clearly, enzymes with this motif catalyze a great variety of reactions. Despite this diversity,



Scheme 2.



Scheme 3.

there is a common mechanistic theme shared among them. The 2-His-1-carboxylate motif provides an iron-coordination platform in these enzymes, on which Fe(II)-dependent dioxygen activation takes place in the presence of substrate or cofactor [57]. The apparent presence of a similar iron-binding fold in HppE strongly suggests that an analogous oxygen activation mechanism is also operative in HppE catalysis. Our proposed enzyme mechanism for HppE [2,6] fits well with this general mechanistic model (Scheme 3).

As illustrated in Scheme 3, the initial steps likely involve substrate binding and a single electron reduction of the metal center. Although it remains unclear whether HPP (2) binds occurs prior to or subsequent to the reduction of the iron center, its direct binding to the metal center in the active site is strongly suggested by the EPR data presented here and in our previous study [2]. The activation of dioxygen by the Fe(II) center, in the presence of exogenous electron(s) from NAD(P)H, can then produce any of various oxidative iron–oxygen intermediates, such as Fe(III)-superoxo, Fe(III)-hydroperoxo, or Fe(IV)-oxo. One of the intermediates is expected to be responsible for the abstraction of a C-1 hydrogen atom from enzyme-bound HPP to generate a substrate radical intermediate. Radical induced homolytic cleavage of the Fe–O bond in this intermediate will produce 1 and also regenerate the iron core.

Conclusion

The 2-His-1-carboxylate facial triad motif has proven to be a remarkably versatile and widely employed metal site architecture. The current results suggest that it is used in yet another role, internal carbon-oxygen bond formation. The presence of a 2-His-

1-carboxylate facial triad could also account for the observation of true oxygenase chemistry in the self-hydroxylation reaction of HppE, since this is a commonly observed reaction for this family of enzymes. Another common theme for reactions catalyzed by members of this family is the initial step of reducing bound oxygen at the expense of substrate or cofactors. The diverse reaction mechanisms of the family diverge at this point. In the case of HppE, the resulting reduced oxygen species may act as both a reagent to promote radical chemistry and a terminal electron acceptor to complete the reaction. Identification of the metal ligands allows the mechanism of HppE to be associated with mechanistic studies of other members of this family and helps to define the active site region. The latter will allow mutagenesis of potential second sphere residues to permit a detailed characterization of their roles in the mechanism of this unusual catalyst.

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