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

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Abstract

(*S*)-2-Hydroxylpropanylphosphonic acid epoxidase (HppE) is a novel type of mononuclear non-heme iron-dependent enzyme that catalyzes the O_2 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 in the conversion of HPP to fosfomycin.

Keywords: Fosfomycin biosynthesis; Non-heme iron; EPR; 2-H-1-D/E motif

(*S*)-2-Hydroxylpropanylphosphonic 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 O_2 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.

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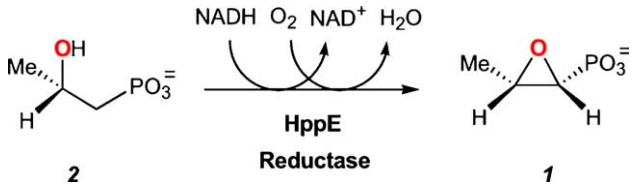

Scheme 1.

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 H_2O [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 determine 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, $\mathbf{G}X_5\mathbf{HXH}X_{3,4}\mathbf{E}X_6\mathbf{G}$, and motif 2, $\mathbf{G}X_5\mathbf{PXG}X_2\mathbf{H}X_3\mathbf{N}$ [20]. The crystal structures of several cupin 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 (Gaitherburg, MD) or Bio-

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S. wendmorensis   DYYVYNCLVR TKRAPSLVPL VVDVLTDNPD DAKFNSGHAG NEFLFVLEGE  150
P. syringae       HYYTYEHLVT TNQDPGLMAL RLDLHSDDEQ PLRLNGGHGS REIVYVTRGA  140
Consensus         .YY.Y..LV. T...P.L..L ..D...D... ....N.GH.. .E...V..G.  150

S. wendmorensis   IHMKW-GDKE NPKEALLPTG ASMFVEEHVP HAFTAAKGTG SAKLIAVNF-  198
P. syringae       VRVRWVGDND ELKEDVLNEG DSIFILPNVP HSFTNHVGGA KSEIIAINYG  190
Consensus         ....W.GD.. ..KE..L..G .S.F....VP H.FT...G.. ....IA.N..  200
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Fig. 1. Protein sequence alignment of HppE from *Streptomyces wendmorensis* and *P. syringae* (amino acid mutated in this study are shaded).