Biosynthesis of Fosfomycin, Re-Examination and Re-Confirmation of a Unique Fe(II)- and NAD(P)H-Dependent Epoxidation Reaction†

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ABSTRACT: (*S*)-2-Hydroxypropylphosphonic acid epoxidase (HppE) catalyzes the epoxide ring closure of (*S*)-HPP to form fosfomycin, a clinically useful antibiotic. Early investigation showed that its activity can be reconstituted with Fe(II), FMN, NADH, and O_2 and identified HppE as a new type of mononuclear non-heme iron-dependent oxygenase involving high-valent iron−oxo species in the catalysis. However, a recent study showed that the Zn(II)-reconstituted HppE is active, and HppE exhibits modest affinity for FMN. Thus, a new mechanism is proposed in which the active site-bound Fe^{2+} or Zn^{2+} serves as a Lewis acid to activate the 2-OH group of (*S*)-HPP and the epoxide ring is formed by the attack of the 2-OH group at C-1 coupled with the transfer of the C-1 hydrogen as a hydride ion to the bound FMN. To distinguish between these mechanistic discrepancies, we re-examined the bioautography assay, the basis for the alternative mechanism, and showed that Zn(II) cannot replace Fe(II) in the HppE reaction and NADH is indispensable. Moreover, we demonstrated that the proposed role for FMN as a hydride acceptor is inconsistent with the finding that FMN cannot bind to HppE in the presence of substrate. In addition, using a newly developed HPLC assay, we showed that several non-flavin electron mediators could replace FMN in the HppE-catalyzed epoxidation. Taken together, these results do not support the newly proposed "nucleophilic displacement−hydride transfer" mechanism but are fully consistent with the previously proposed iron-redox mechanism for HppE catalysis, which is unique within the mononuclear non-heme iron enzyme superfamily.

Fosfomycin (**1**) is a clinically useful antibiotic (*1*) for the treatment of lower urinary tract infections (*2*) and limb-threatening diabetic foot infections (*3*). It is also effective against methicillin-resistant (*4*) and vancomycin-resistant (*5*) strains of *Staphylococcus aureus*. The antimicrobial activity of fosfomycin has been attributed to the inactivation of UDP-Glc*N*Ac-3-*O*-enolpyruvyltransferase (MurA), which catalyzes the first committed step in the biosynthesis of peptidoglycan, the main component of the cell wall (*6*, *7*).

Fosfomycin is biosynthetically derived from (*S*)-2-hydroxypropylphosphonic acid [**2**, (*S*)-HPP][1] (*8*, *9*). The conversion of (*S*)-HPP to fosfomycin (**1**) is catalyzed by HPP epoxidase (HppE) (*10*, *11*). A mononuclear non-heme iron in the HppE active site is essential for enzyme activity (*12*). Coordination of the iron by His138, Glu142, and His180, the 2-H-1-D/E facial triad, was first implied by sequence alignment (*13*) and site-directed mutagenesis studies (*14*) and was later confirmed by an X-ray crystal structure (*15*). Earlier research also showed that molecular oxygen is essential for the reaction (*11*, *12*). However, no oxygen atoms from O_2 are incorporated into the fosfomycin product (*9*, *11*, *12*). Instead, the oxygen atom of the epoxy ring in **1** is derived from the secondary hydroxyl group of (*S*)-HPP (**2**) (Scheme 1). Thus, the conversion of **2** to **1** by HppE is effectively a dehydrogenation reaction, not an oxygenation reaction.

This unusual epoxidation reaction is NAD(P)H-dependent and involves four-electron redox chemistry with full reduction of dioxygen to water (*11*, *12*). In this process, two electrons are generated through epoxide ring formation, and the other two electrons are supplied by NAD(P)H. The use of NAD(P)H as a source of reducing equivalents distinguishes HppE from most other mononuclear non-heme iron-dependent oxygenases, where the source of electrons is either the substrate or a cosubstrate such as α-ketoglutarate, ascorbate, or tetrahydropterin (*16*−*19*).

Despite the unusual chemistry of its physiological reaction, HppE can also catalyze more typical oxygenase reactions. In particular, HppE can self-hydroxylate an active site tyrosine to form 3,4-dihydroxyphenylalanine (DOPA) (*20*). The modified residue has been identified as Tyr105, on the basis of its proximity to the iron center (8.7 Å) (*15*) and the results of site-directed mutagenesis (*20*). The chelation of DOPA105 with the active site ferric ion gives HppE a green

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[1] Abbreviations: DOPA, L-3,4-dioxyphenylalanine; DTT, dithiothreitol; E_3, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HPP, 2-hydroxypropylphosphonic acid; HppE, (*S*)-2-hydroxypropylphosphonic acid epoxidase; α-KG, α-ketoglutaric acid; LB, Luria-Bertani; LMCT, ligand-to-metal charge transfer; NO, nitric oxide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RNR, ribonucleotide reductase; TauD, taurine dioxygenase; TfdA, 2,4-dichlorophenoxyacetic acid dioxygenase; Tris, tris(hydroxymethyl)aminomethane.

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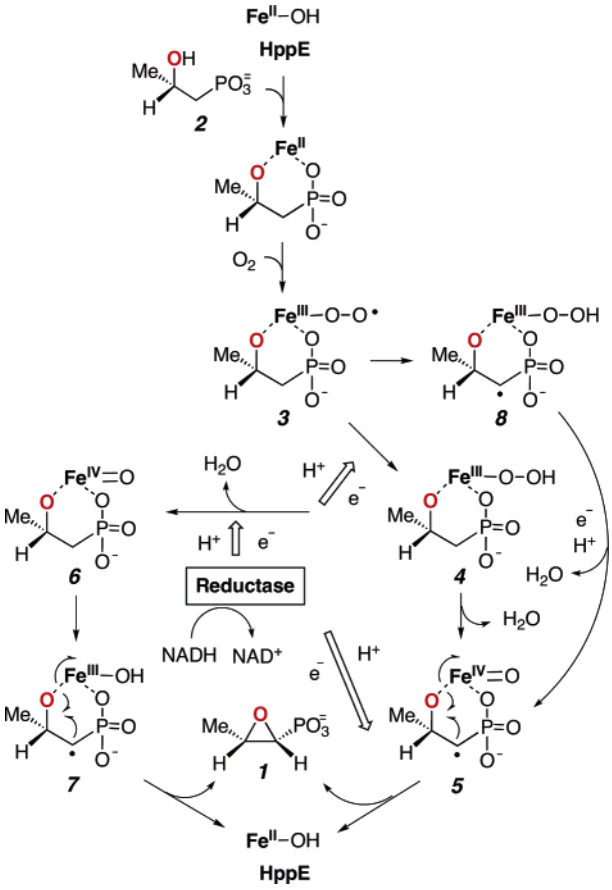
Scheme 1

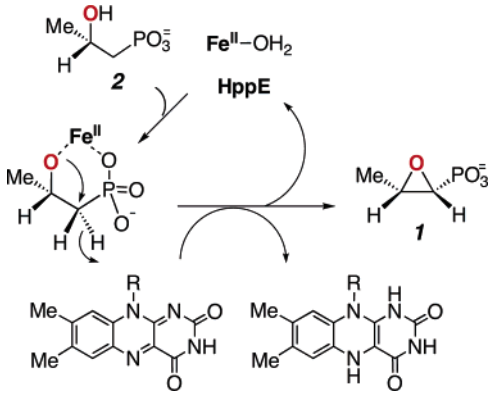


color. This green chromophore has been assigned, on the basis of UV−vis and resonance Raman spectral analyses, to a catecholate-to-Fe(III) charge transfer complex (*20*).

Unlike the self-hydroxylation reactions observed for other non-heme iron-dependent enzymes, such as the ribonucleotide reductase (RNR) R2 F208Y mutant (*21*), taurine dioxygenase (TauD) (*22*), and 2,4-dichlorophenoxyacetic acid dioxygenase (TfdA) (*23*), where the newly introduced oxygen atom in DOPA is derived from H_2O, the oxygen atom incorporated into DOPA105 of HppE originates from dioxygen (*20*). The discovery of such oxygenase activity for HppE is significant because the same reactive intermediate responsible for self-hydroxylation may also participate in the reaction cycle for the formation of fosfomycin.

The mechanism of HppE-catalyzed HPP epoxidation has been proposed to parallel those of alkane hydroxylation catalyzed by cytochrome P450 (*24*) and non-heme iron oxygenases (*25*, *26*). Accordingly, the reaction likely invokes an Fe^{III}−OOH intermediate (**4**) derived from the one-electron reduction of an initial Fe(II)−O_2 adduct. As shown in Scheme 1, this peroxo intermediate (**4**) may either directly abstract a hydrogen atom from C-1 of (*S*)-HPP (**2**) to generate a transient substrate radical intermediate (**5**) or be converted to a high-valent iron−oxo species (such as **6**) that carries out the oxidation (**6** → **7**). A direct attack by the iron−superoxide complex (**3**) to generate radical **8** is also possible. Evidence for the involvement of an iron−superoxide species in the myoinositol oxygenase reaction has been reported (*27*). The subsequent cyclization to yield fosfomycin (**1**) and concomitant reduction of the metal center is reminiscent of oxygen rebound in cytochrome P450 (*24*).

Scheme 2



In a recent study, McLuskey et al. found that the purified recombinant HppE is active when reconstituted with Fe^{2+} or Zn^{2+} (*28*). They also showed that HppE exhibits modest affinity for FMN which is required for activity. Since Zn^{2+} is redox inert, these findings suggested that the previously proposed mechanism, which relies on metal ion redox chemistry, is incorrect. Hence, McLuskey et al. proposed a new mechanism in which the active site-bound divalent metal ion (Fe^{2+} or Zn^{2+}) serves as a Lewis acid to activate the C-2 hydroxyl group, and the epoxide ring is formed by the attack of the 2-hydroxyl group at C-1 coupled with the transfer of the C-1 hydrogen as a hydride ion to the bound FMN (Scheme 2, a "nucleophilic displacement−hydride transfer" mechanism).

To resolve the mechanistic discrepancies, we characterized the Zn^{2+}-reconstituted HppE and re-evaluated the NADH as well as the oxygen dependence of HppE activity. We also compared the efficiency of various enzyme activity assays and determined the binding affinity of HppE for several flavin derivatives. The results support an exclusive role for iron in HppE catalysis. We also found that FMN is unlikely a hydride acceptor, but an artificial electron mediator for the in vitro HppE activity. It serves the role of accepting a hydride from NADH and then passes electrons on to reduce the iron center of HppE. On the basis of these lines of evidence, we conclude that the "iron redox chemistry" mechanism stands as the more likely mechanism for the HppE-catalyzed reaction.

MATERIALS AND METHODS

General. Enzyme E_3 (AscD, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase) used in the assay was purified from the *Escherichia coli* JM105/pOPI cultures based on a procedure published earlier (*29*). Culture medium ingredients were purchased from Difco (Detroit, MI). Biochemicals, including the fosfomycin disodium salt standard (**1**), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The substrate, (*S*)-2-hydroxypropylphosphonic acid [(*S*)-HPP, **2**], used in the bioassay for HppE was chemically synthesized according to a literature procedure (*11*, *12*). All other reagents and solvents were purchased from commercial sources and were used without further purification unless otherwise noted. Protein concentrations were determined by the procedure of Bradford (*30*) using bovine serum albumin as the standard.

Purification of HppE with and without Bound Metals. Recombinant HppE was overproduced from *E. coli* BL21-(DE3)/pPL1001. The metal-free HppE (apo-HppE) was purified according to the published procedure (*12*). The metal-bound HppE was obtained by an identical purification procedure, except DTT and EDTA were omitted from the buffers. SDS-PAGE was used to monitor column fractions during purification.

Metal Content Analysis. Protein samples for metal analysis were prepared by diluting the metal-bound HppE (1 mM) 2000-fold and the apo-HppE (1 mM) 20-fold with ddH_2O to 1 mL. The metal ion content in each sample was measured using inductively coupled plasma mass spectrometry (ICP-MS) performed by J. M. Lansdown of the Department of Geological Sciences, Jackson School of Geosciences, University of Texas.

^{31}P NMR Spectroscopy Assay. To directly determine the percentage of conversion of (*S*)-HPP (**2**) to fosfomycin (**1**) catalyzed by HppE, a ^{31}P NMR spectroscopy assay developed previously (*11*, *12*) was used. A typical assay mixture (200 μL) contained 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 90 μM FMN, 22.5 mM NADH, and 150 μM metal ions in 20 mM Tris-HCl buffer (pH 7.5). The reaction was carried out at room temperature for 2 h, followed by rapid freezing with liquid nitrogen. The frozen sample was thawed immediately before ^{31}P NMR analysis. The amount of fosfomycin produced was determined on the basis of the integration of the appropriate ^{31}P NMR peaks. To test the newly proposed "hydride transfer" mechanism, the assay conditions were modified to include 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 900 μM FMN, and 150 μM metal ions in 200 μL of 20 mM Tris-HCl buffer (pH 7.5). The incubation was allowed to proceed at room temperature for 2 h and then flash-frozen with liquid nitrogen. The frozen sample was thawed immediately before NMR analysis.

Bioautography Assay. The conversion of (*S*)-HPP (**2**) to fosfomycin (**1**) by HppE was also monitored by a previously developed bioautography assay, which is based on the ability of fosfomycin to inhibit the biosynthesis of the bacterial cell wall (*12*). A typical assay mixture (200 μL) contained 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 90 μM FMN, 22.5 mM NADH, and 150 μM metal ions in 20 mM Tris-HCl buffer (pH 7.5). The reaction was carried out at room temperature for 2 h. Meanwhile, 20 mL of LB agar medium was autoclaved, cooled to 40−45 °C, mixed with 1 mL of *E. coli* K12 strain HW8235 precultivated in LB medium, and poured into a Petri dish. After the agar solidified, sterilized paper disks (10 mm in diameter) were placed on the top of the agar. Each reaction mixture was divided into two portions, one of which was filtered through a Y-10 membrane to remove HppE. Subsequently, an aliquot of 20 μL was applied to the paper disk, and the plate was incubated at 37 °C for 12−16 h. The amount of fosfomycin produced in the reaction mixture was estimated by measuring the diameter of the inhibition zone and comparing it with that of a fosfomycin standard (10 μL of a 5 mg/mL solution). To test the proposed hydride transfer mechanism, a separate set of reaction mixtures containing 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 900 μM FMN, and 150 μM metal ions in 200 μL of 20 mM Tris-HCl buffer (pH 7.5) was prepared and incubated at room temperature for 2 h. The subsequent treatment was the same as that described above.

Oxygen Dependence. To study the effect of oxygen on the activity of HppE, the same sets of reactions used for the NMR and bioautography assays were repeated under anaerobic conditions A typical assay mixture (200 μL) contained 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 90 μM FMN, 22.5 mM NADH, and 150 μM metal ions in 20 mM Tris-HCl buffer (pH 7.5). Each component of the reaction mixture, including the apo-HppE solution, (*S*)-HPP, FMN, NADH, and metal ions, was individually made anaerobic by cycles of vacuum and argon purging. The organic compounds and metal ions were then dissolved in argon-saturated buffer or water. The mixing of components was carried out in the glovebox under a nitrogen atmosphere for 2 h at room temperature. The reactions were quenched by the addition of ascorbate to a final concentration of 50 mM. The production of fosfomycin was analyzed by ^{31}P NMR spectroscopy.

NADH Stoichiometry. The NADH dependence of the conversion of (*S*)-HPP to fosfomycin was also studied. The reaction mixture (100 μL) contained 4.5 mM (*S*)-HPP (**2**), 78 μM apo-HppE, 120 M $Fe(NH_4)_2(SO_4)_2$, 156 μM FMN, and various amounts of NADH (0−0.9 mM). The incubation was carried out at room temperature for 2 h, and the reaction was quenched by the addition of EDTA to a final concentration of 100 mM, followed by freezing with liquid nitrogen. Product formation was assessed by ^{31}P NMR spectroscopy. The percentage of conversion was calculated on the basis of the integration of the corresponding peaks [fosfomycin vs the sum of fosfomycin and (*S*)-HPP] from the spectra.

Binding Affinity of Flavin Derivatives. The binding affinity between HppE and various flavin derivatives was determined by the change in fluorescence intensity of flavin resulting from binding to HppE. Since flavin fluorescence is linear only up to ~5 μM, HppE was titrated into the flavin solution [1 μM in 2 mL of 20 mM Tris-HCl buffer (pH 7.5)], as opposed to the standard method of titrating the ligand into the enzyme solution. The excitation and emission wavelengths were 450 and 525 nm, respectively, and the excitation and emission slit widths were set at 1 and 3 nm, respectively. The titrations were carried out at 25 °C. The fluorescence data were adjusted so that the *y*-axis represented the net decrease in fluorescence. These data were then fit, using Grafit 5.0.1, to eq 1 with F_o, F, and K_D as parameters

$$\Delta F = F_o + \left[\frac{E_o + L_o + K_D - \sqrt{(E_o + L_o + K_D)^2 - 4E_oL_o}}{2L_o}\right](F_\infty - F_o) \quad (1)$$

where F_o is the initial fluorescence, F_∞ is the final fluorescence, E_o is the total HppE concentration, and L_o is the total ligand concentration (*31*).

HPLC Activity Assay. Enzyme assays were carried out in 50 μL of 20 mM Tris-HCl buffer (pH 7.5) in triplicate. The reactions were initiated by the addition of a mixture of apo-HppE, $Fe(NH_4)_2(SO_4)_2$, and an electron mediator (flavin cofactors, benzyl viologen, or E_3) to a mixture of (*S*)-HPP (**2**) and NADH. The final concentrations were as follows: 50 μM HppE, 50 μM Fe^{2+}, 75 μM flavin cofactors/benzyl viologen/E_3, 10 mM (*S*)-HPP (**2**), and 15 mM NADH. The

reactions were quenched by the addition of 2 M acetic acid (50 μL) and then the mixtures centrifuged through a Nanosep centrifugal device with a 10K Omega Membrane (Pall Life Sciences, Ann Arbor, MI) to remove protein. The samples (77 μL each) were analyzed with a HPLC system equipped with a Dionex CarboPac PA-1 (4 mm × 250 mm) column. The fosfomycin product was eluted with a gradient of water as solvent A and 500 mM NH_4OAc as solvent B where the following gradient was used: 5% B for 2 min, from 5 to 25% B over 30 min, from 25 to 100% B over 1 min, 100% B for 9 min, from 100 to 5% B over 2 min, and then 5% B for 13 min. The flow rate was 1 mL/min. The detector used is the Corona charged aerosol detector (CAD) (ESA, Inc., Chelmsford, MA), which is designed to detect nonvolatile compounds. The amount of fosfomycin produced was determined by peak integration and then converted into micromoles on the basis of a calibration curve.

RESULTS AND DISCUSSION

Protein Purification and Metal Ion Contents. The HppE used by McLuskey et al. is a fusion protein with an N-terminal His_6 tag and was purified by Ni−NTA affinity chromatography (*28*). In contrast, the HppE protein used in all our work does not contain an affinity tag and was purified as an apoprotein because DTT and EDTA were added to purification buffers to remove all metal ions (*12*). The apo-HppE was then reconstituted with various metal ions to examine their effects on enzyme activity. Among the divalent metal ions that were examined [Fe(II), Co(II), Ni(II), Cu(II), and Mn(II)], only Fe(II) could reconstitute HppE activity (*12*). Thus, HppE is believed to be iron-dependent, and the Fe(II)-reconstituted HppE has been used in subsequent activity assays, spectroscopic analysis, and mechanistic studies (*14*, *20*). The Fe(II)-reconstituted enzyme was colorless until it was exposed to air. After 1 h, a green color fully developed in the protein solution, which has been assigned to a catecholate-to-Fe(III) ligand−metal charge transfer (LMCT) complex (*20*). It should be pointed out that HppE purified in the absence of DTT and EDTA is at least partially iron-bound, because it has a grayish green color arising from the same LMCT complex in the active site (*20*).

Interestingly, the His_6-tagged HppE purified by McLuskey et al. was a mixture of 80% apoenzyme and 20% Zn(II)-containing enzyme (*28*). No iron was found in their protein, and no color development was noted. Similar observations were also made in our early study of the N-terminally His_6-tagged HppE (*11*). The fusion of a His_6 tag to HppE and the use of imidazole during purification, both entities having metal chelating properties, may have contributed to the low metal content in the purified His_6-tagged protein. To compare the His_6-tagged and nontagged (wild type) HppE in more detail, we decided to re-examine the metal contents of the non-tagged HppE used in our study. Both metal-bound and metal-free HppE (apo-HppE) were prepared and subjected to ICP-MS analysis. The results are summarized in Table 1.

As expected, no divalent metal ion is present at a level of more than 4% per monomer in apo-HppE. Clearly, treatment with DTT and EDTA effectively removes all divalent metal ions from HppE. In contrast, HppE purified in the absence of DTT and EDTA (the metal-bound form) retains ~50% of the Fe per monomer. This is consistent with the fact that the purified metal-bound HppE is colored green and is distinct from the metal ion binding characteristics reported for the His_6-tagged HppE by McLuskey et al. However, the ICP-MS data also revealed a high affinity of HppE for Zn, since nearly 40% of HppE was found to be bound to Zn (Table 1). Apparently, both the wild-type and His_6-tagged HppE bind zinc well but exhibit differences in iron binding. Although the high affinity of HppE for zinc is potentially interesting, it has not posed problems in our studies because only the Fe(II)-reconstituted HppE is used. A stoichiometry of one iron per subunit of the reconstituted HppE has been firmly established (*11*, *12*, *20*).

Table 1: Metal Content Analysis of Purified HppE by ICP-MS[a]

	^{24}Mg	^{55}Mn	^{56}Fe	^{59}Co	^{60}Ni	^{66}Zn
metal-bound	0.05	0	0.51	0	0	0.40
metal-free	0.01	0	0	0	0.03	0.03

[a] See Materials and Methods for details.

Enzyme Activity Determined by Bioautography Assay. Two assay methods had been developed in our early work to determine the activity of HppE (*11*, *12*). One is the bioautography assay, in which a paper disk soaked with the assay mixture is placed in direct contact with a lawn of *E. coli* K12 HW8235 grown on nutrient (LB) agar. When fosfomycin is produced in the assay mixture, an inhibition zone is visible after incubation for a few hours. Using this bioautography assay led to the identification of Fe(II) and NAD(P)H as two essential components for HppE activity (*11*, *12*). While this assay is convenient and sensitive, the same incubation mixture with all of the components mentioned above included failed to produce enough fosfomycin that can be discerned by NMR analysis (*11*). It was later found by a ^{31}P NMR assay that the addition of FMN or FAD greatly enhanced the production of fosfomycin (*11*, *12*). Because this NMR method allows direct detection of fosfomycin production in the assay mixture, it has been the assay of choice in our subsequent studies. However, since the conclusions of McLuskey et al. about HppE activity and mechanism were based mainly on the bioautography results (*28*), we decided to re-examine the assay conditions using both Fe(II)- and Zn(II)-reconstituted HppE. It should be pointed that FMN was introduced into the bioautography assay carried out by McLuskey et al. but was not included in our original bioautography assay (*11*, *12*).

As shown in Figure 1A, fosfomycin production was detected in the incubation mixture containing Zn(II)−HppE (disk 1) and Fe(II)−HppE (disk 4) complexes with excess FMN and (*S*)-HPP (**2**). The addition of NADH to the assay mixture led to more fosfomycin formation in both cases as indicated by the enlarged diameter of inhibition zones (Figure 1B, disks 1 and 4). These results are in agreement with those of McLuskey et al. (*28*) showing that the Zn(II)−HppE complex is catalytically active but less active than the Fe(II)−HppE complex. However, we were troubled by the positive response given by the metal-free apo-HppE sample, which was performed as a negative control (disk 3 in Figure 1A,B). The fact that the Fe(II)−HppE complex was active in the absence of NADH was also puzzling (Figure 1A, disk 4).

Since HppE was not removed prior to application of the reaction mixture to the disk, it should still be catalytically

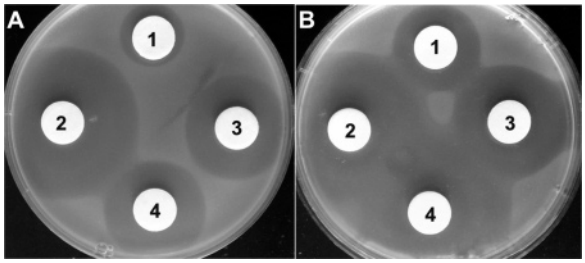


FIGURE 1: Bioautography assay results of unfiltered reaction samples. (A) (1) Apo-HppE, Zn(II), FMN, and (*S*)-HPP, (2) fosfomycin standard, (3) apo-HppE, FMN, and (*S*)-HPP, and (4) apo-HppE, Fe(II), FMN, and (*S*)-HPP. (B) (1) Apo-HppE, Zn(II), FMN, (*S*)-HPP, and NADH, (2) fosfomycin standard, (3) apo-HppE, FMN, (*S*)-HPP, and NADH, and (4) apo-HppE, Fe(II), FMN, (*S*)-HPP, and NADH. A typical assay mixture (200 μL) contained 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 90 μM FMN, and 150 μM metal ions, in 20 mM Tris-HCl buffer (pH 7.5) in the presence or absence of 22.5 mM NADH (see Materials and Methods for details).

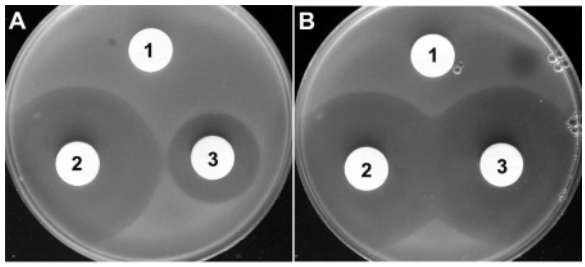


FIGURE 2: Bioautography assay results of filtered reaction samples. (A) (1) Apo-HppE, Zn(II), FMN, and (*S*)-HPP, (2) fosfomycin standard, and (3) apo-HppE, Fe(II), FMN, and (*S*)-HPP. (B) (1) Apo-HppE, Zn(II), FMN, (*S*)-HPP, and NADH, (2) fosfomycin standard, and (3) apo-HppE, Fe(II), FMN, (*S*)-HPP, and NADH. A typical assay mixture (200 μL) contained 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 90 μM FMN, and 150 μM metal ions, in 20 mM Tris-HCl buffer (pH 7.5) in the presence or absence of 22.5 mM NADH (see Materials and Methods for details).

active when exposed to the nutrient rich agar media where metal ions are plentiful. This "postincubation activation/activity" may be partially responsible for the positive response exhibited by the Zn(II)−HppE and apo-HppE samples. To eliminate this potential complication, the reaction mixture was passed through a membrane with a molecular mass cutoff of 10 kDa. After this treatment, the inhibition results should reflect the total amount of fosfomycin produced in the assay solution under defined reaction conditions because no HppE is available to make additional fosfomycin during the overnight incubation on the plate. As expected, no inhibition by the reaction mixture containing the Zn(II)−HppE sample was discernible (Figure 2A,B, disk 1), whereas the inhibitory effect of the Fe(II)−HppE sample remained very apparent (Figure 2B, disk 3). The addition of more FMN to the reaction mixture containing the Zn(II)−HppE complex (up to 200-fold more than the HppE concentration) showed no effect on the autobiography results (data not shown). It is thus evident that the Zn(II)−HppE sample is not active and Zn(II) cannot substitute for Fe(II) in reconstituting a catalytically active HppE. The activity observed for the unfiltered samples shown in Figure 1 must be an artifact and may be attributed to the ability of HppE on the disk to elicit iron from the LB agar to make fosfomycin.

While the observations described above clearly ruled out a role for Zn(II) in the HppE-catalyzed reaction, the

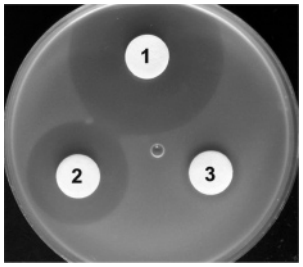


FIGURE 3: Bioautography assay results of filtered reaction samples carried out in dark and in sunlight: (1) fosfomycin standard, (2) apo-HppE, Fe(II), FMN, and (*S*)-HPP in sunlight, and (3) apo-HppE, Fe(II), FMN, and (*S*)-HPP in the dark. A typical assay mixture (200 μL) contained 20 mM (*S*)-HPP (**2**), 90 μM apo-HppE, 90 M FMN, and 150 μM metal ions, in 20 mM Tris-HCl buffer (pH 7.5) (see Materials and Methods for details).

inhibitory effect exhibited by the mixture containing Fe(II) and FMN but no NADH was unexpected (Figure 1A, disk 4, and Figure 2A, disk 3). We speculated that the photoreducible properties of FMN could play a role. This speculation was substantiated by the subsequent two parallel reactions, one carried out in the dark and another in sunlight. As for the reaction in the dark without NADH, no detectable level of fosfomycin was produced when the incubation mixture was filtered prior to being applied to the disk (Figure 3, disk 3). In sharp contrast, the same reaction performed in sunlight gave a large inhibition zone (Figure 3, disk 2). Thus, photoreduction of FMN is clearly the cause of the complication. Taken together, enzyme activity determined by the bioautography assay should be analyzed with caution, since the readout could be misleading if the assay conditions are not properly controlled.

Enzyme Activity Determined by NMR Spectroscopy. The HppE reactions conducted under various conditions as described above were also subjected to ^{31}P NMR analysis. In agreement with our previous observation, only the incubation mixture containing the Fe(II)−HppE complex in the presence of FMN and NADH produced NMR-discernible amounts of fosfomycin from (*S*)-HPP (**2**). The detection limit of fosfomycin is estimated to be 1 mM on the basis of ^{31}P NMR peak integration and is equivalent to 5% conversion of (*S*)-HPP (20 mM, total amount used) under our assay conditions. The fact that no fosfomycin formation was detected when the Zn(II)−HppE complex was used strongly suggested an exclusive role for iron in HppE catalysis. Clearly, the direct detection of product formation by NMR spectroscopy is a much more reliable activity assay.

Enzyme Activity Determined by the HPLC Assay. Although ^{31}P NMR spectroscopy is a highly reliable method of measuring fosfomycin production, it is not amenable to microanalysis due to its low sensitivity. To overcome this shortcoming, an HPLC assay coupled with a charged aerosol detector (CAD) to detect nonvolatile compounds in the eluent was developed. This HPLC assay allowed us to quantitatively analyze the conversion of (*S*)-HPP (**2**) to fosfomycin (**1**), both nonchromophoric, under different incubation conditions. As illustrated in Figure 4, baseline resolution of **1** and **2** was achieved using a Dionex CarboPac PA-1 (4 mm × 250 mm) column.

NADH Dependence of HppE Activity. A fundamental distinction between the iron-redox mechanism and the nucleophilic displacement−hydride transfer mechanism is the

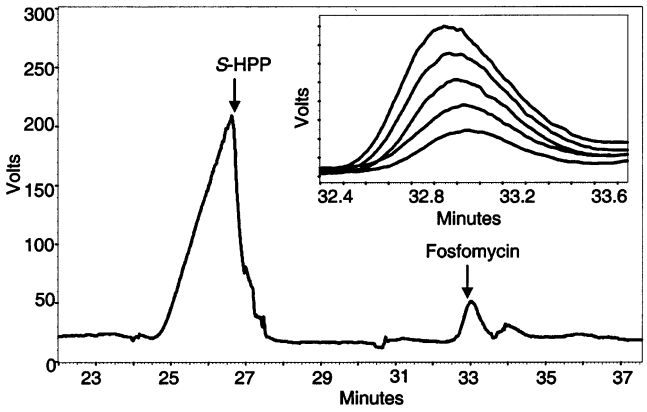


FIGURE 4: HPLC chromatogram of the HppE-catalyzed conversion of (*S*)-HPP (**2**) to fosfomycin (**1**). The inset shows the increase in the fosfomycin peak after 10, 20, 40, 60, and 80 min (from bottom to top). The starting concentrations were as follows: 50 μM HppE, 50 μM Fe^{2+}, 75 μM riboflavin, 10 mM (*S*)-HPP (**2**), and 15 mM NADH (see Materials and Methods for details).

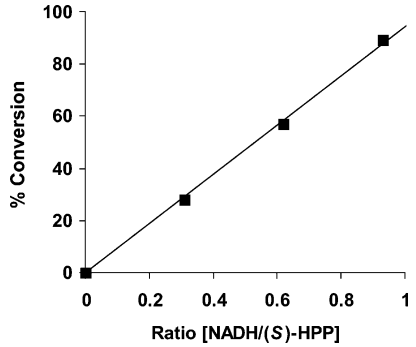
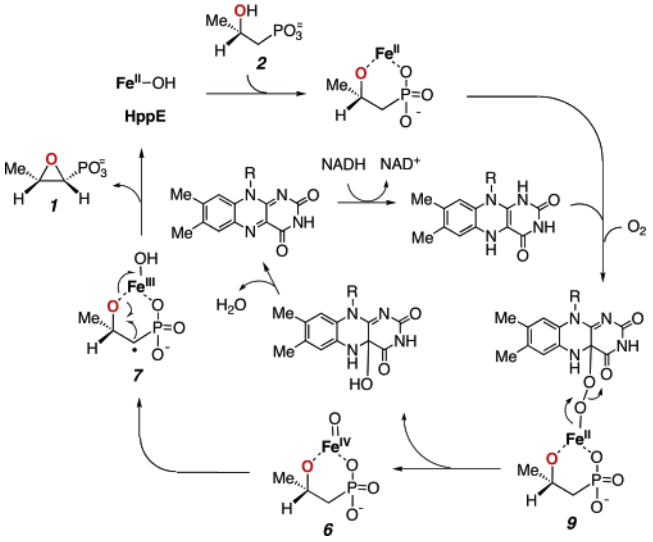


FIGURE 5: NADH dependence of the conversion of (*S*)-HPP (**2**) to fosfomycin (**1**) catalyzed by HppE (see Materials and Methods for details).

NADH dependence of the reaction. As depicted in Scheme 1, in the iron-redox mechanism, NADH plays an essential role by supplying two electrons to prime the iron center for oxygen activation and to reduce an iron−oxygen species during catalysis. In contrast, no NADH is needed for the nucleophilic displacement−hydride transfer mechanism (Scheme 2) since the transferred hydride is derived from (*S*)-HPP (**2**) and not from NADH. Although the activity assay results have clearly implicated NADH as the source of reducing equivalents, further analysis was performed to determine the stoichiometry of NADH required per catalytic turnover to better quantify the NADH dependence. As expected, a direct correlation between the percentage of conversion and the NADH/(*S*)-HPP ratio was found (Figure 5). Our data also established that 1 equiv of NADH is consumed per conversion of 1 equiv of (*S*)-HPP to fosfomycin. The direct involvement of NADH in the HppE reaction supports the iron-redox mechanism and is not consistent with a nucleophilic displacement−hydride transfer mechanism.

Oxygen Dependence of HppE Activity. The activation of dioxygen by the Fe(II) center, in the presence of NADH, to produce various oxidative iron−oxygen intermediates (**3**, **4**, and **6** in Scheme 1) responsible for the abstraction of a C-1 hydrogen atom from enzyme-bound HPP is the essence of the iron-redox mechanism for the HppE-catalyzed reaction. In support of this mechanism, no turnover was observed

Scheme 3



when the reaction was conducted in an oxygen-free environment. Although dioxygen is not directly involved in the nucleophilic displacement−hydride transfer mechanism, it is required for the regeneration of FMN. Thus, the observation that there is no turnover under anaerobic conditions is not sufficient to rule out the latter mechanism. However, a stoichiometric amount of fosfomycin formation was expected when a large amount of HppE was incubated with excess FMN in the absence of oxygen. The failure to detect any fosfomycin product under such a single-turnover condition does not support the nucleophilic displacement−hydride transfer mechanism.

Binding of Flavin Cofactors. While no flavin cofactor was found in the purified HppE, FMN had been shown to be an important component in the HppE activity assay (*11*, *12*). It serves as an electron mediator in the iron-redox mechanism and has been proposed to be the hydride acceptor in the nucleophilic displacement−hydride transfer mechanism (*28*). Since the translated sequence of HppE lacks an ADP $\beta\alpha\beta$-binding fold characteristic of flavin-dependent enzymes and reconstitution failed to incorporate FMN in the active site, FMN has not been considered to be a true coenzyme of HppE (*11*). Instead, it is believed to function as a surrogate in the iron-redox mechanism for an electron transfer protein involved in the catalysis in vivo. Interestingly, a K_d of 10 μM for dissociation of FMN from HppE was determined by McLuskey et al. (*28*), which was cited as an indication of a specific binding of FMN in HppE in support of the proposed nucleophilic displacement−hydride transfer mechanism.

If FMN is indeed a coenzyme of HppE, a non-heme iron pterin-dependent hydroxylase-like mechanism found for aromatic amino acid hydroxylase, where a Fe−O−O−pterin intermediate is involved (*17*, *18*), may also need to be considered. As depicted in Scheme 3, the reduced flavin, acting as a tetrahydrobiopterin mimic, may react with Fe(II)-activated dioxygen to form a Fe−O−O−flavin intermediate (**9**). Subsequent heterolytic breakdown of the O−O bond at the expense of two electrons leads to the generation of the reactive Fe(IV)−oxo intermediate (**6**). This iron−oxo species, capable of hydroxylating the substrate in the aromatic amino acid hydroxylase reaction, may be used to abstract the C-1 hydrogen of (*S*)-HPP (**2**) in the HppE

Table 2: Binding Affinity of HppE for Flavin Derivatives[a]

	apo-HppE	Zn(II)−HppE	Fe(II)−HppE	Fe(III)−HppE
FMN	NB[b]	3.2 ± 0.9 μM	5.3 ± 0.9 μM	9.0 ± 2.0 μM
FAD	NB[b]	300 ± 700 μM	50 ± 10 μM	−
riboflavin	NB[b]	NB[b]	NB[b]	−
FMN and (*S*)-HPP	−	NB[b]	NB[b]	−

[a] See Materials and Methods for details. [b] No binding. This is based on an estimated lower detection limit of a K_d equal to 1 mM.

Table 3: Rates of HppE-Catalyzed Epoxidation Using Different Electron Mediators[a]

	FMN	FAD	riboflavin	E_3	benzyl viologen
k_{obs} (min^{-1})	0.44 ± 0.05	0.22 ± 0.03	0.41 ± 0.03	1.3 ± 0.2	0.13 ± 0.01

[a] See Materials and Methods for details.

reaction (**6** → **7**). To gain more insight into flavin binding, the affinities of HppE for a series of flavin analogues were determined.

In this study, the K_d constants were determined by the change in the fluorescence intensity of flavin resulting from binding to HppE (Table 2). The K_d values for binding of FMN to the Zn(II)−HppE and Fe(II)−HppE complexes are 3.2 ± 0.9 and 5.3 ± 0.9 μM, respectively, similar to the reported values measured by isothermal titration calorimetry (*28*). A K_d of 9.0 ± 2.0 μM was also determined for the Fe(III)−HppE complex. However, when metal ions were absent (apo-HppE) or substrate, (*S*)-HPP (**2**), was present, no binding interaction between FMN and protein was detected (Table 2). These observations suggest that the binding of substrate and the binding of FMN are mutually exclusive and do not support specific binding of FMN to HppE. Interestingly, riboflavin, which is an effective FMN substitute (see Table 3), shows no affinity for the Zn(II)−HppE or Fe(II)−HppE complex (Table 2). Moreover, since the epoxidation reaction catalyzed by HppE proceeds at a comparable rate using FMN or riboflavin (Table 3), if HppE binds FMN in a specific manner, then one would expect a similar specific binding of riboflavin. Taken together, the measured binding interactions are unlikely catalytically relevant and may result from binding of the phosphate of FMN to the metal center. This would explain why the presence of substrate, which is known to bind to the active site metal (*15*), can block FMN binding and why riboflavin, which has no phosphate group, cannot bind to the enzyme. The probability of a flavoenzyme not having a defined flavin binding pocket is low. The fact that no flavin was found in the crystal structure of HppE (*15*, *28*) also suggests that a specific flavin binding site in HppE is not present.

Electron Mediators. Using our newly developed HPLC assay, we determined the rates for fosfomycin formation with FMN and other electron mediators, including FAD, riboflavin, benzyl viologen, and an electron transfer protein, E_3. As listed in Table 3, the rates of these reactions are within the same order of magnitude, suggesting that these compounds and/or proteins are all competent to enhance HppE reaction. As discussed above, riboflavin does not bind to HppE, yet it is as effective as FMN which binds to the Fe(II)−HppE complex with a K_d of 5.3 ± 0.9 μM (Table 2). This observation is at odds with the expectation of a flavin-dependent hydride transfer mechanism, because the lack of riboflavin binding should result in a significant reduction in enzyme activity. Interestingly, HppE remains active, albeit slightly slower, when FMN is replaced with benzyl viologen. This finding again supports an electron mediator role of FMN in catalysis because benzyl viologen is a well-known electron carrier and is not expected to bind to the putative FMN binding site due to lack of structural similarity with FMN. The reduced rate seen for benzyl viologen may be due to its $E_m°$ value of −370 mV which is lower than the midpoint potential of −211 mV for free FMN (*32*, *33*). In agreement with this analysis, replacing FMN with methyl viologen, which has a much lower $E_m°$ (−448 mV) (*32*), failed to reconstitute HppE activity.

The results obtained with E_3, an NADH-dependent [2Fe-2S]-containing flavoenzyme from *Yersinia pseudotuberculosis* (*34*, *35*), provide further support for the assigned electron mediator role of FMN in HppE catalysis. E_3, along with E_1 (CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase), catalyzes the C-3 deoxygenation reaction in the biosynthesis of 3,6-dideoxyhexoses (*36*). During turnover, the reducing equivalents are relayed from the E_3-bound NADH to the active site of E_1 via a chain of redox-active cofactors, including FAD and the iron−sulfur center in E_3 (*35*). The involvement of an iron−sulfur cluster, an obligatory one-electron carrier, defines E_3 as an electron transfer protein. No change in function is expected for E_3 in the HppE reaction where the FAD in E_3 is the immediate recipient of hydride from NADH and serves as a two-electron (hydride)/one-electron switch in the subsequent electron relay to reduce the ferric center in HppE. The fact that E_3 is more effective than any flavin analogues that were tested (Table 3) implies that the physiological electron mediator is likely a protein reductase. However, since no reductase gene exists in the two known fosfomycin biosynthetic gene clusters of *Streptomyces wedmorensis* (*10*) and *Pseudomonas syringae* (*37*), the electron transfer in the HppE reaction may not be mediated by a specific reductase but may rely on a promiscuous reductase within the cell.

CONCLUSION

Literature precedence for epoxide formation via a nucleophilic substitution mechanism comes from the halohydrin dehalogenase reaction in which the leaving group is a chloride (or a bromide) anion that is intramolecularly displaced by a vicinal hydroxyl group to yield the corresponding epoxide (*38*). A similar mechanism has recently been proposed for HppE where a divalent metal ion [Zn(II) or Fe(II)] acts as a Lewis acid and enhances the nucleophilicity of the attacking 2-hydroxyl group, and an active site-bound flavin coenzyme (FMN) receives the departing hydride to complete this energetically challenging epoxidation reaction (Scheme 2) (*28*). However, in this study, we have demonstrated that the Zn(II)-reconstituted HppE is catalytically inactive, and FMN cannot bind to the HppE−substrate binary complex. Both observations fail to support the nucleophilic displacement−hydride transfer mechanism. Likewise, a non-heme iron pterin-dependent hydroxylase-like mechanism (Scheme 3) can be ruled out on the basis of the preclusion of binding of FMN to the HppE−substrate complex and the competence of non-flavin electron mediators in the catalysis. Our results also confirmed that Fe(II) is the

only metal ion examined that is effective in reconstituting HppE activity, and NADH is an exogenous electron donor which is necessary for multiple turnovers by the Fe(II)−HppE complex. The requirement of an electron mediator, either an electron transfer protein or a small molecule electron carrier, for HppE activity is also firmly established. All of these results strongly support the iron-redox mechanism (Scheme 1). Clearly, the reaction catalyzed by HppE is beyond the scope encompassed by common biological epoxidation and C−O bond formation reactions. More studies aimed at uncovering details of the mechanism of HppE-catalyzed epoxidation are in progress. Insight gained from study of this unique non-heme iron-dependent enzyme will certainly enhance our understanding of this important and growing enzyme family.

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