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Biochemical and Spectroscopic Studies on (*S*)-2-Hydroxypropylphosphonic Acid Epoxidase: A Novel Mononuclear Non-heme Iron Enzyme[†]

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ABSTRACT: The last step of the biosynthesis of fosfomycin, a clinically useful antibiotic, is the conversion of (*S*)-2-hydroxypropylphosphonic acid (HPP) to fosfomycin. Since the ring oxygen in fosfomycin has been shown in earlier feeding experiments to be derived from the hydroxyl group of HPP, this oxirane formation reaction is effectively a dehydrogenation process. To study this unique C−O bond formation step, we have overexpressed and purified the desired HPP epoxidase. Results reported herein provided initial biochemical evidence revealing that HPP epoxidase is an iron-dependent enzyme and that both NAD(P)H and a flavin or flavoprotein reductase are required for its activity. The 2 K EPR spectrum of oxidized iron-reconstituted fosfomycin epoxidase reveals resonances typical of $S = {}^5/_2$ Fe(III) centers in at least two environments. Addition of HPP causes a redistribution with the appearance of at least two additional species, showing that the iron environment is perturbed. Exposure of this sample to NO elicits no changes, showing that the iron is nearly all in the Fe(III) state. However, addition of NO to the Fe(II) reconstituted enzyme that has not been exposed to O_2 yields an intense EPR spectrum typical of an $S = {}^3/_2$ Fe(II)−NO complex. This complex is also heterogeneous, but addition of substrate converts it to a single, homogeneous $S = {}^3/_2$ species with a new EPR spectrum, suggesting that substrate binds to or near the iron, thereby organizing the center. The fact that NO binds to the ferrous center suggests O_2 can also bind at this site as part of the catalytic cycle. Using purified epoxidase and ^{18}O isotopic labeled HPP, the retention of the hydroxyl oxygen of HPP in fosfomycin was demonstrated. While ether ring formation as a result of dehydrogenation of a secondary alcohol has precedence in the literature, these catalyses require α-ketoglutarate for activity. In contrast, HPP epoxidase is α-ketoglutarate independent. Thus, the cyclization of HPP to fosfomycin clearly represents an intriguing conversion beyond the scope entailed by common biological epoxidation and C−O bond formation.

Fosfomycin ((1*R*,2*S*)-1,2-epoxypropylphosphonic acid, **1**) is a clinically useful antibiotic against a broad spectrum of Gram positive and Gram negative bacteria (*1*). It exhibits very low toxicity and few side effects and is currently used for the treatment of acute uncomplicated lower urinary tract bacterial infections (*2*). The target of fosfomycin has been identified as UDP-GlcNAc-3-*O*-enolpyruvyltransferase (MurA)[1] (*3*), which catalyzes the first committed step in bacterial cell wall peptidoglycan biosynthesis (*4−6*). The antibacterial action of fosfomycin results from the alkylation of an active site cysteine residue (Cys115 in the *Escherichia coli* enzyme) of MurA (*7, 8*). Such a covalent modification irreversibly inhibits the catalytic function of MurA (*9*).

Structurally, fosfomycin belongs to a steadily growing family of natural products containing a C−P bond (*10*). Members of this family, such as fosfomycin (*11*), phosphinothricin (*12*), and bialaphos (*13*), are all derived from phosphoenolpyruvate (PEP, **2**). The C−P bonds in these compounds are formed through an intramolecular rearrangement reaction catalyzed by PEP mutase resulting in the

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[1] Abbreviation: ADP, adenine diphosphate; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; CAS, clavaminic acid synthase; DAOCS, deacetoxycephalosporin C synthase; DEAE, diethylaminoethyl; DTT, dithiothreitol; E_3, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FPLC, fast protein liquid chromatography; H6H, hyoscyamine 6β-hydroxylase; HPLC, high-performance liquid chromatography; HPP, 2-hydroxypropylphosphonic acid; IPNS, isopenicillin N synthase; IPTG, isopropyl-β-D-thiogalactopyranoside; LB medium, Luria−Bertani medium; ICP, inductively-coupled plasma spectrometer; MurA, UDP-GlcNAc-3-*O*-enolpyruvyl transferase; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; PnPy, phosphopyruvate; PnAA, phosphonoacetaldehyde; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TauD, taurine dioxygenase; TyrH, tyrosine hydroxylase.

Scheme 1
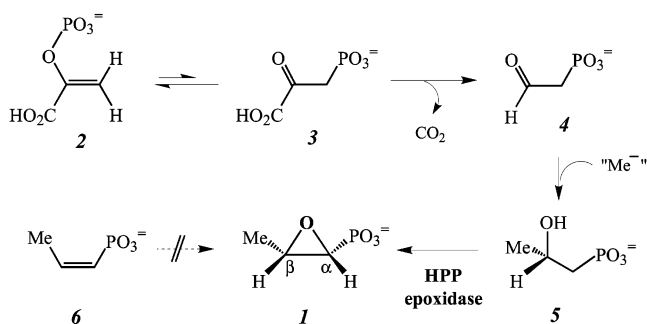
conversion of PEP (**2**) to phosphopyruvate (PnPy, **3**) (*14–16*) (Scheme 1). Since the equilibrium between PEP and PnPy highly favors the cleavage of the C−P bond, the decarboxylation catalyzed by a second enzyme, PnPy decarboxylase, which converts PnPy to phosphonoacetaldehyde (PnAA, **4**), provides the driving force to shift the equilibrium to favor the C−P bond formation (*17*). Unlike these two early transformations that have been well established and characterized, little is known about the remaining steps in the biosynthesis of fosfomycin (*18*), especially the mechanism of its oxiranyl ring formation.

In nature, most epoxide rings are generated via oxidation of the corresponding alkenes by either heme-dependent cytochrome P450s (*19, 20*) or non-heme iron-dependent monooxygenases (*21*). Therefore, a similar biosynthetic route from *cis*-propenylphosphonic acid (**6**) to fosfomycin had been proposed. However, attempts to convert **6** to fosfomycin, both in vivo and in vitro, were futile (*22*). In contrast, fosfomycin was produced upon feeding the producing strain, *Streptomyces fradiae*, with 2-hydroxypropylphosphonic acid (HPP, **5**) (*22*). It was later determined that (*S*)-HPP (**5-*S***) was the preferred substrate (*23, 24*). More importantly, feeding experiments showed the incorporation of ^{18}O from ^{18}O-labeled (*S*)-HPP into the epoxide ring of fosfomycin (*23, 25*). On the basis of these findings, a minimum of four enzymatic steps has been proposed for the biogenesis of fosfomycin (Scheme 1) (*7*).

Recently, the entire fosfomycin biosynthetic gene cluster has been cloned from *Streptomyces wedmorensis* (*26*) and *Pseudomonas syringae* PB-5123 (*27*). Expression of *orf3* from the *Pseudomonas* cluster in *E. coli* and a preliminary activity assay led to the tentative assignment of *orf3* as the encoding gene for HPP epoxidase (*27*). In a separate effort, we had expressed the *orf3* equivalent in *S. wedmorensis* (*fom4*) and purified the encoded protein (Fom4) (*28*). Since the isolated Fom4 was determined to be only the apoprotein, considerable work was devoted to find conditions for reconstitution of the enzyme activity and confirmation of its identity as the desired epoxidase. Through this effort, we have found that Fom4 is indeed the HPP epoxidase and is an NADH-dependent mononuclear non-heme iron catalyst. We have also demonstrated its competence to catalyze the epoxidation reaction in vitro and established the requirement of an exogenous electron mediator for its activity. Reported herein is a full account of the biochemical characterization of this enzyme and the implications for its mode of catalysis.

MATERIALS AND METHODS

General. Protein concentrations were determined by the procedure of Bradford (*29*) using bovine serum albumin as

the standard. The NMR spectra were acquired on a Varian Unity 300 spectrometer, and chemical shifts (δ in ppm) are given relative to those for Me$_4$Si (for ^1H and ^{13}C) and aqueous 85% H_3PO_4 (external, for ^{31}P), with coupling constants reported in hertz (Hz). The UV−vis absorption spectra were recorded on a HP 8453A diode array spectrophotometer or a Beckman DU 650 spectrophotometer. N-Terminal sequencing of purified enzyme was carried out by the University of Minnesota Microchemical Facility in the Institute of Human Genetics. ICP analysis was carried out at the Research Analytical Laboratory of the Department of Soil, Water, and Climate, University of Minnesota.

Materials. The plasmid pFBG5000 containing the HPP epoxidase gene and the recombinant strain (*E. coli*-M15/pREP4/pQE5110*)* containing a gene construct encoding the N-terminal His-tagged HPP epoxidase were kindly provided by Drs. Haruo Seto and Tomohisa Kuzuyama of the University of Tokyo, Japan. Enzyme E$_3$ (AscD) used in the assay was purified from the *E. coli* JM105/pOPI cultures on the basis of a procedure published earlier (*30*). Culture medium ingredients were purchased from Difco (Detroit, MI). DNA minipreps were performed using Wizard DNA purification kits from Promega (Madison, WI). All oligonucleotide primers for PCR amplification of the desired inserts were customly prepared by Gibco BRL (Grand Island, NY) and used without further purification. Restriction endonucleases were products of Amersham (Arlington Heights, IL), Gibco BRL, or Promega. All electrophoresis materials were purchased from Gibco BRL or Bio-Rad (Hercules, CA). All chemicals were analytical grade or the highest quality commercially available. Biochemicals including fosfomycin disodium salt standard were purchased from Sigma (St. Louis, MO), unless noted otherwise. The substrate of HPP epoxidase, 2-hydroxypropylphosphonic acid (**5**) in its racemic form, was chemically synthesized according to a procedure reported by Hammerschmidt (*31*), and used in the bioassay.

Construction of Expression Plasmid for HPP Epoxidase. Two oligonucleotide primers were designed to amplify the *fom4* gene: the start primer, 5′-GGC **CAT ATG** AGC AAC ACC AAG-3′, containing an *Nde*I restriction site (in bold), and the halt primer, 5′-GGC **AAG CTT** TCA GAA GTT GAC-3′, containing a *Hind*III restriction site (in bold). The PCR mixture (100 μL) contained 46.0 μL of H$_2$O, 8.0 μL of deoxyribonucleotidyltriphosphate mix (dNTP, 2.5 mM each), 4 μL of DMSO, 4 μL of 40% glycerol, 10 μL of each of the two primers (20 pmol/μL), 6 μL of template DNA (pFBG5000, 0.1 μg), 10.0 μL of Pfu polymerase buffer (10×), and 2.0 μL of cloned Pfu polymerase (5.0 units) (Stratagene, Madison, WI). Amplification was carried out under the following conditions: (1) 1 cycle of incubation at 94 °C for 5 min; (2) 30 cycles of incubation at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The PCR product was purified from agarose gel (Gibco BRL) using a Geneclean kit (Bio 101, La Jolla, CA) and ligated into the *Nde*I/*Hind*III sites of the transcription vector, pET24b(+) (Novagen, Madison, WI), to give pPL1001. This recombinant plasmid was isolated and was used to transform *E. coli* HB101. Positive clones were identified by digestion of the plasmid DNA with *Nde*I/*Hind*III and visualization of the excised insert by staining an agarose gel of the DNA with ethidium bromide after electrophoresis. The plasmid DNA

from positive clones was used to transform *E. coli* BL21(DE3). The resulting construct (pPL1001) was further confirmed by DNA sequencing. The general methods and protocols for recombinant DNA manipulations were as described by Sambrook et al (*32*).

Growth of E. coli BL21(DE3)/pPL1001 Cells. An overnight culture of *E. coli* BL21(DE3)/pPL1001 grown at 37 °C in LB medium supplemented with kanamycin (50 μg/mL) was used, in a 200-fold dilution, to inoculate 6 L of the same medium supplemented with 0.1 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$. When the OD$_{600}$ reached 0.4−0.6, the incubation temperature was lowered to 18 °C and IPTG was added to a final concentration of 0.1 mM to induce gene expression. After incubation for an additional 15 h at 18 °C, cells were harvested by centrifugation (8000*g*, 5 min) at 4 °C, washed with Tris·HCl buffer (20 mM, pH 7.5), collected again by centrifugation (8000*g*, 5 min), and stored at −80 °C for future use. The typical yield was 6 g of wet cells per liter of culture.

Purification of Recombinant Wild-Type HPP Epoxidase. All purification operations were carried out at 4 °C except for the FPLC step, and all buffers were degassed and saturated with nitrogen before use. Thawed cells were resuspended in a 5-fold (w/v) excess of lysis buffer (20 mM Tris·HCl, pH 7.5, 0.1 mM DTT; 1 mM EDTA was included to remove trace amounts of iron ion when necessary) and subjected to 5 × 40 s ultrasonic bursts, with a 1 min cooling interval between each blast. Cellular debris was removed by centrifugation at 15000*g* for 20 min. The supernatant was fractionated by ammonium sulfate, and the 30−65% ammonium sulfate precipitate was collected. The protein pellet was resuspended in a minimal amount of Tris·HCl buffer (20 mM, pH 7.5). The resulting protein solution was dialyzed against 2 L of the same buffer for 4 h with three buffer changes.

The dialysate was applied to a DEAE-Sepharose CL-6B column (2.5 cm × 24 cm) pre-equilibrated with 20 mM Tris·HCl, pH 7.5. After loading, the column was washed with 500 mL of the wash buffer (20 mM Tris·HCl, pH 7.5, 0.15 M KCl). The elution was then continued with a linear gradient of KCl from 0.15 to 0.35 M in 20 mM Tris·HCl buffer, pH 7.5 (2 L total volume). The flow rate was 2 mL/min, and fractions of 15 mL were collected throughout the gradient elution. The fractions containing the desired HPP epoxidase, as determined by SDS−PAGE, were pooled, concentrated to about 10 mL by ultrafiltration on an Amicon concentrator using a YM 10 membrane (Millipore, Bedford, MA), and desalted by dialyzing against 20 mM Tris·HCl buffer, pH 7.5.

The protein from the last step was further purified at room temperature by FPLC equipped with a Mono Q HR 10/10 (Amersham Biosciences, Uppsala, Sweden) using the solvent systems A (20 mM Tris·HCl buffer, pH 7.5) and B (A plus 0.6 M NaCl). The elution profile included a linear gradient of 0 to 60% B from 0 to 25 min, followed by a linear gradient of 60 to 100% B from 25 to 26 min, and concluded with a 5 min wash at 100% B. The flow rate was 3 mL/min, and the detector was set at 280 nm. A sharp peak with a retention time of about 19 min was collected, concentrated by ultrafiltration as described before, desalted with 20 mM Tris·HCl buffer (pH 7.5), and stored at −80 °C.

Molecular Mass Determination. The molecular mass of HPP epoxidase was determined by size exclusion chroma-

Scheme 2
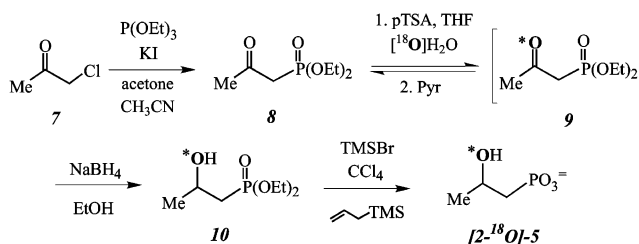
tography performed on a Pharmacia FPLC Superdex 200 HR 10/30 column with 20 mM Tris·HCl, 0.15 M NaCl, pH 7.5, at a flow rate of 0.8 mL/min. Calibration of the column was achieved using the following protein standards (Sigma): cytochrome C (14.2 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa). The void volume (V_0) of the column was measured using blue dextran. A linear fit to a plot of the molecular weight versus V_e/V_0 was used to estimate the native molecular mass (M_r) of the protein sample (*33*).

ICP Analysis and Iron Titration. Protein samples for ICP analysis were prepared by diluting 1 mg of HPP epoxidase with HCl solution to 5 mL (final concentration of HCl was 10%). For iron titration analysis (*34*), protein samples (1 mL each) were mixed with 500 μL of reagent A (1:1 of 4.5% KMnO$_4$:1.2 N HCl) and incubated at 60 °C for 2 h. To these samples were added 100 μL of reagent B (8.8 g of ascorbic acid, 9.7 g of ammonium acetate, 80 mg of ferrozine, 80 mg of neocuprione, ddH$_2$O to 25 mL total volume) followed by immediate vortexing. The samples were read at 562 nm after the color was fully developed by a 1 h incubation period at room temperature.

Preparation of (S)-2-Hydroxypropylphosphonic Acid ((S)-HPP, 5-S). The substrate of HPP epoxidase in its enantiomerically pure form was chemically synthesized according to a sequence reported by Hammerschmidt (*31*) (Scheme 2). The **5**-*S* enantiomer is used in the NMR assay. The spectral data of the final product are listed below. (*S*)-2-Hydroxypropylphosphonic acid (**5**-*S*): ^{1}H NMR (500 MHz, D$_2$O) δ 3.80 (1H, m, J = 6.5, 2-H), 1.39 (2H, dd, J = 18.0, 6.6, 15.3, 1-H), 0.97 (3H, d, J = 6.5, 3-H); ^{13}C NMR (75.5 MHz, D$_2$O) δ 65.0 (d, J = 2.0, C-2), 37.9 (d, J = 132.0, C-1), 22.1 (C-3); ^{31}P NMR (D$_2$O) δ 19.9 (s).

Disk Diffusion Assay. An activity assay was developed to monitor the conversion of HPP (**5**) to fosfomycin (**1**) by HPP epoxidase on the basis of the ability of fosfomycin to inhibit the biosynthesis of the bacterial cell wall (*27*). A typical assay mixture (100 μL) contained 10.5 mM HPP (**5**), 82 μM HPP epoxidase, 21.8 mM NADH, and 120 μM FMN (or 60 μM E$_3$) in 20 mM Tris·HCl buffer, pH 7.5. A variety of other compounds were also used as possible ingredients in our early screening to define the assay conditions, but they did not result in higher activity. Each incubation was carried out at room temperature for 2 h. Meanwhile, an LB agar plate was freshly prepared by mixing *E. coli K12 HW8235* LB culture (1 mL) with 20 mL of melted LB agar medium at about 40−45 °C. After the agar was solidified, a sterilized paper disk, 10 mm in diameter, was placed on the top of the agar. A 100 μL aliquot of the above reaction mixture was applied to the paper disk. After incubation at 37 °C for 10−16 h, the amount of fosfomycin produced in the reaction mixture was estimated by measuring the size of the inhibition

zone in comparison with that of a fosfomycin standard (10 μg).

NMR Assay. To directly determine the percentage of HPP conversion to fosfomycin catalyzed by HPP epoxidase, an NMR assay was developed. A typical assay mixture (100 μL) contained 10.5 mM (*S*)-HPP (**5-S**), 21.6 μM HPP epoxidase, 21.8 mM NADH, and 60 μM E_3 in 20 mM Tris·HCl buffer, pH 7.5. The reaction was carried out at room temperature with vigorous shaking and was quenched at an appropriate time by adding EDTA to a final concentration of 100 mM, followed by freezing with liquid nitrogen. The frozen sample was thawed immediately before NMR analysis. The amount of fosfomycin produced was determined on the basis of integration of the appropriate NMR peaks. Spectral data of fosfomycin (**1**): ^{1}H NMR (500 MHz, D_2O) δ 3.03 (1H, m, $J = 5.0$, 2-H), 2.57 (1H, d, $J = 5.0$, 14.0, 1-H), 1.21 (3H, d, $J = 5.5$, 3-H); ^{13}C NMR (75.5 MHz, D_2O) δ 54.2 (s, C-2), 54.3 (d, $J = 176.0$, C-1), 13.2 (C-3); ^{31}P NMR (D_2O, 121 MHz) δ 10.9 (s).

HPP Epoxidase Activity Dependence on FMN. To study the effect of FMN on the activity of HPP epoxidase, samples (100 μL) containing 10.5 mM HPP (**5-S**), 108 μM HPP epoxidase, 21.8 mM NADH, 140 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, and varied amounts of FMN (0−270 μM) in 20 mM Tris·HCl buffer, pH 7.5, were prepared. The reaction was run for 2 h at room temperature and quenched by adding EDTA to a final concentration of 100 mM, followed by freezing with liquid nitrogen. The amount of product formation was determined by ^{1}H NMR spectroscopy as described above.

HPP Epoxidase Activity Dependence on E_3. The competence of E_3 as an alternative electron carrier for the reaction catalyzed by HPP epoxidase was studied. A typical assay mixture (100 μL) contained 10.5 mM HPP (**5-S**), 15.4 μM HPP epoxidase, 21.8 mM NADH, 140 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, and a varied amount of E_3 (0−120 μM) in 20 mM Tris·HCl buffer, pH 7.5. The reaction was performed at room temperature and quenched after 2 min by the addition of EDTA to a final concentration of 100 mM, followed by freezing with liquid nitrogen. The amount of fosfomycin formation was determined by ^{1}H NMR spectroscopy as described above.

Effect of EDTA on the Activity of HPP Epoxidase. To assess the metal requirement for the enzyme activity, the effect of metal chelators, such as EDTA, on the catalysis of HPP epoxidase was also examined. A typical assay mixture (100 μL) contained 9.0 mM NADH, 4.5 mM HPP (**5-S**), 78 μM HPP epoxidase, and 156 μM FMN in 20 mM Tris·HCl buffer, pH 7.5. The reaction was allowed to proceed at room temperature for 2 h in the presence of up to 10 mM EDTA. The percentage of HPP conversion was determined by ^{1}H NMR spectroscopy as described above.

Effects of Metal Ions on the Activity of HPP Epoxidase. A systematic investigation to determine the metal ion(s) requirement for the activity of HPP epoxidase was performed by including different amounts of redox-active metal ions in the reaction mixture and assessing their effects on enzyme activity. A typical reaction mixture (100 μL) contained 8.0 mM NADH, 4.0 mM HPP (**5-S**), 80 μM HPP epoxidase, 160 μM FMN, and an appropriate amount of metal ion (0−80 μM) being tested in 20 mM Tris·HCl buffer, pH 7.5. The following five redox-active metals were used in this study: Fe^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+}. The relative activity of

each sample was determined by ^{1}H NMR spectroscopy after a 2 h incubation period at room temperature.

HPP Epoxidase Activity Dependence on Oxygen. In a sealed vessel, an assay mixture (1 mL) containing 10.5 mM HPP (**5-S**), 15.4 μM HPP epoxidase, and 140 μM Fe(NH$_4$)$_2$-(SO$_4$)$_2$ in 20 mM Tris·HCl, pH 7.5, was made anaerobic by 15 repeated cycles of subjecting the mixture to vacuum and purging with argon. The mixture was kept under argon for 20 min followed by another 15 cycles of vacuum and argon purging to ensure anaerobic conditions. In a separate sealed vessel, a solution of NADH and E_3 in the same buffer was made anaerobic by the same strategy. The NADH and E_3 solution was transferred anaerobically via a gastight syringe to the above assay mixture of HPP epoxidase, HPP, and Fe(II) to give a final concentration of 21.8 mM for NADH and 60 μM for E_3. After incubation for 2 min at room temperature, the reaction was quenched by the addition of anaerobic EDTA solution to give a final concentration of 100 mM. Subsequently, the reaction mixture was frozen using liquid nitrogen. The production of fosfomycin was analyzed by ^{1}H NMR spectroscopy as described earlier.

EPR Spectroscopy. EPR first derivative spectra of HPP epoxidase were collected at X-band microwave frequency with 100-kHz field modulation using a Bruker Elexsys E500 spectrometer. A calibrated frequency counter and a Bruker ER035M NMR Gauss meter were used for the *g*-value determinations. The cryotemperature measurements were achieved with an Oxford Instruments ESR-10 continuous flow liquid helium cryostat and a digitalized temperature controller. The EPR parameters were obtained using an EPR program written by Dr. Frank Neese (*35*) and were further verified in Bruker SimFonia. The isolated, Fe(II)-loaded (reduced), Fe(III)-reconstituted (oxidized), and (*S*)-HPP (**5-S**) incubated epoxidase samples were examined by EPR spectroscopy at 2−50 K. In the nitric oxide (NO) experiments, oxygen was removed prior to NO exposure to the oxidized HPP epoxidase samples. Nitric oxide experiments with the reduced enzyme were carried out as follows: the as-isolated HPP epoxidase was reconstituted with ferrous ion under anaerobic conditions, and the Fe(II)-loaded sample was split into two portions. Nitric oxide gas was passed over NaOH pellets to remove any acid impurities and then introduced to one portion of the sample through a gastight Hamilton syringe under argon in EPR tubes and then frozen by slow immersion in liquid nitrogen for later EPR analysis. The other portion was exposed to air for 2 h at room temperature and then transferred into an EPR tube as a control. Spin quantification was performed by double integration of the EPR spectra recorded under nonsaturating conditions at 20 K for comparison with a Cu-EDTA standard (1 mM).

Preparation of [2-^{18}O]-2-Hydroxypropylphosphonic Acid. Chemical synthesis of [2-^{18}O]-HPP ([2-^{18}O]-**5**) was carried out according to the reaction sequence shown in Scheme 2. The ^{18}O labeling was introduced into the C-2 ketone oxygen in an acid-catalyzed hydration/dehydration raction. The labeled product ([2-^{18}O]-**5**) has the following spectral data: ^{1}H NMR (300 MHz, CDCl$_3$): 4.04−4.20 (5H, m), 3.32 (1H, brs), 1.89 (1H, d, $J = 17.4$), 1.87 (1H, dd, $J = 17.3$, 2.9), 1.29 (6H, t, $J = 7.1$), 1.23 (3H, dd, $J = 2.3$, 6.2, 3-Hs). ^{13}C NMR (75.5 MHz, D_2O) δ 65.0 (t, $J = 2.0$, C-2), 37.9 (d, $J = 132.0$, C-1), 22.1 (C-3).
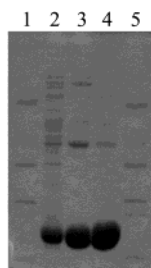
FIGURE 1: SDS−PAGE of purified *S. wedmorensis* HPP epoxidase isolated from *E. coli* BL21(DE3)/pPL1001: lane 1, the molecular markers; lane 2, after 35−60% $(NH_4)_2SO_4$ fractionation; lane 3, after a DEAE−sepharose column; lane 4, after an FPLC Mono Q column.

Table 1: Reconstitution of HPP Epoxidase Activity under Various Conditions

no.	reagents	activity
1	cell crude extract/(S)-HPP	positive
2	fosfomycin (10 μg)	positive
3	HPP epoxidase/(S)-HPP	negative
4	HPP epoxidase/NAD(P)$^+$/(S)-HPP	negative
5	HPP epoxidase/H$_2$O$_2$/(S)-HPP	negative
6	HPP epoxidase/metal ions/(S)-HPP	negative
7	HPP epoxidase/metal ions/ catalase/(S)-HPP	negative
8	HPP epoxidase/ascorbate/(S)-HPP	negative
9	HPP epoxidase/α-ketoglutarate/ (S)-HPP	negative
10	HPP epoxidase/NAD(P)H/(S)-HPP	positive

Incubation of [2-¹⁸O]-2-Hydroxypropylphosphonic Acid ([2-¹⁸O]-5) with HPP Epoxidase. A mixture of 15 mM [2-¹⁶O]- and [2-¹⁸O]-HPP (1:1 molar ratio of **5**/[2-¹⁸O]-**5**) was mixed with 21.8 mM NADH, 140 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, and 100 μM purified enzyme in 10 mL of 20 mM Tris·HCl buffer (pH 7.5). This mixture was incubated at room temperature and monitored by ¹H NMR spectroscopy. After all of the substrate had been consumed, the proteins were removed by ultrafiltration on an Amicon concentrator using a YM 10 membrane. The resulting solution was loaded onto a DEAE-Sepharose column and eluted with a linear gradient of 0−0.5 M NH$_4$HCO$_3$ buffer, pH 7.8. The fractions containing the desired product were combined and lyophilized. Spectral data of the product, which is a 1:1 mixture of [2-¹⁶O]- and [2-¹⁸O]-fosfomycin, are as follows:¹³C NMR (75.5 MHz, D$_2$O) δ 54.2 (s, C-2), 54.3 (d, J = 176.0, C-1), 13.2 (C-3); ³¹P NMR (121 MHz, D$_2$O) δ 10.9 (s).

RESULTS AND DISCUSSIONS

Cloning, Overexpression, and Purification of HPP Epoxidase. The gene, *fom4*, coding for HPP epoxidase, which catalyzes the last step of fosfomycin biosynthesis in *S. wedmorensis* (see Scheme 1), was amplified by PCR and cloned into the *Nde*I and *Hind*III sites of the expression vector pET24b(+). The resulting construct, pPL1001, was used to transform *E. coli* BL21(DE3) cells. The induction of *fom4* expression by IPTG was conducted at 18 °C to minimize the formation of inclusion bodies. After HPP epoxidase was found to be an iron-depending enzyme, the growth broth was supplemented with 0.1 mM Fe(NH$_4$)$_2$(SO$_4$)$_2$.

As shown in SDS−PAGE (Figure 1), the desired protein was isolated in nearly homogeneous form after ammonium sulfate fractionation and two anion exchange chromatographic steps (DEAE-Sepharose and MonoQ). N-Terminal sequence analysis confirmed that the first 10 amino acid residues (S-N-T-K-T-A-S-T-G-F) of HPP epoxidase are identical to the translated *fom4* sequence (*26*), except that the N-terminal Met was post-translationally removed. The subunit molecular mass of 21 kDa, assessed by SDS−PAGE, correlates well with the predicted value of 21 210 Da calculated from the deduced amino acid sequence. The purified recombinant HPP epoxidase exists as a homotetramer having a mass of 89 kDa as determined by gel filtration chromatography.

Reconstitution of Epoxidase Activity Based on a Two-Electron Redox Model. An activity-based disk diffusion assay

was developed in order to screen conditions for the reconstitution of HPP epoxidase activity. When HPP (**5**) was incubated with the crude cell extracts of *E. coli* BL21(DE3)/pPL1001, a clear inhibition zone was observed, indicating the existence of detectable amounts of fosfomycin (microgram quantity) produced by the recombinant strain. However, when purified HPP epoxidase was used, instead of the crude cell extracts, no inhibition zone was discernible. Evidently, some essential components present in the crude extracts were missing in the latter assay mixture to reconstitute the epoxidase activity in vitro. As shown in Table 1, the addition of NAD(P)$^+$ or H$_2$O$_2$, which are commonly used as two-electron oxidants in reactions catalyzed by dehydrogenases (*36*) and peroxidases (*37*), exhibited no effect on the activity assay. The incorporation of metal ions in the incubation mixture to test whether the conversion of HPP to fosfomycin mimics those of copper-dependent amine oxidase or galactose oxidase (*38*) was also pursued. A series of metal ions commonly found in biological oxidoreductive systems, such as Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Mn^{2+}, were used in these experiments. As shown in Table 1, negative effects were obtained in all cases including those coincubated with catalase, which was added to deplete the possible byproduct H$_2$O$_2$, thereby preventing it from inactivating the enzyme. The failure to detect H$_2$O$_2$ formation in the assay mixture also argues against a two-electron oxidation model.

Reconstitution of Epoxidase Activity Based on a Four-Electron Redox Model. Since the reaction catalyzed by HPP epoxidase is oxygen-dependent (vide infra), it should be accompanied by the reduction of dioxygen to water, if catalysis involves an overall four-electron redox event. However, reduction of O$_2$ to H$_2$O requires four electrons and conversion of HPP to fosfomycin alone can only provide two electrons, indicating that two more electrons must be supplied by an exogenous source. Two common two-electron donors in biological catalysis, ascorbate, which is a cosubstrate of 1-aminocyclopropane-1-carboxylate oxidase (*39*, *40*) and dopamine β-monooxygenase (*41*), and α-ketoglutarate, which is used by many non-heme iron enzymes (*42*, *43*), were used as the required electron source to test the four-electron redox model for HPP epoxidase. Once again, the results were negative (Table 1). Interestingly, when NADH alone was included in the assay mixture in a molar ratio of 1:1 to HPP, a small but discernible inhibition zone appeared. This observation provided preliminary evidence indicating that NADH is a necessary component for HPP epoxidation and the overall catalysis is a four-electron redox reaction.
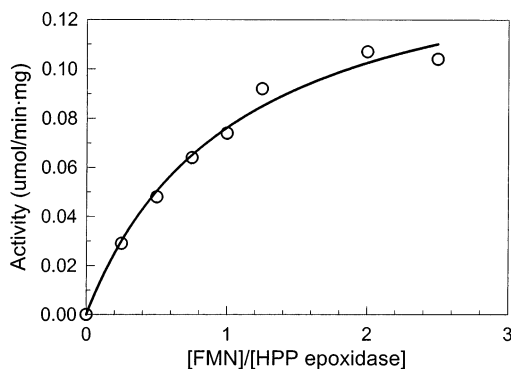
FIGURE 2: Effect of FMN on the activity of HPP epoxidase. Assay conditions: 10.5 mM (*S*)-HPP, 108 μM HPP epoxidase, 21.8 mM NADH, 140 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, and varied amounts of FMN in 20 mM Tris·HCl buffer, pH 7.5. See Materials and Methods for experimental details.

FIGURE 3: Effect of E$_3$ on the activity of HPP epoxidase. Assay conditions: 10.5 mM (*S*)-HPP, 15.4 μM HPP epoxidase, 21.8 mM NADH, 140 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, and varied amounts of E$_3$ in 20 mM Tris·HCl buffer, pH 7.5. See Materials and Methods for experimental details.

Oxygen Requirement. Because no chemical oxidant was added in the preceding experiments and the experiments were carried out aerobically, O$_2$ must be the final electron acceptor. Indeed, no fosfomycin could be detected when the reactions were conducted under anaerobic conditions. Because oxidation of HPP and NADH can each furnish two electrons during turnover, O$_2$ must be reduced to H$_2$O by HPP epoxidase, a prediction further supported by the fact that no H$_2$O$_2$ is produced in the assay mixture. The involvement of O$_2$ and NADH in an overall four-electron redox process is well precedented in enzyme reactions. However, in all known cases, an electron mediator, such as a transition metal or an organic cofactor, capable of mediating single electron transfer is required for oxygen activation. This is because O$_2$ has a triplet ground state while all stable organic compounds are at singlet state. This electron mediator(s) may be embedded in HPP epoxidase or in an exogenous reductase, whose absence may account for the weak activity observed under the above assay conditions. Reason that weak activity could be detected in the presence of NAD(P)H may be attributed to the presence of some exogenous electron mediators in the agar media.

Effect of Metal Ions on the Activity of HPP Epoxidase. To assess whether HPP epoxidase is a metalloprotein, the metal content of purified epoxidase was determined by ICP as well as iron titration analysis. Only a minute amount of Fe ion (<0.05% relative to enzyme monomer) was found in the isolated HPP epoxidase. The fact that epoxidase was totally inactive in the presence of 10 mM EDTA is a strong indication that metal ions are involved in catalysis. Reconstitution of the apo-epoxidase with a variety of metal ions, such as Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+}, was also attempted. Only ferrous ion was found to have a significant effect on the enzyme activity (in the presence of NADH and O$_2$), which was enhanced by nearly 100-fold. Moreover, ICP analysis revealed a stoichiometry of one iron per enzyme monomer after reconstitution of the apo-epoxidase with Fe(NH$_4$)$_2$(SO$_4$)$_2$. These results strongly suggest that HPP epoxidase is an iron-dependent enzyme.

Effects of FAD and FMN on the Activity of HPP Epoxidase. Interestingly, when FMN or FAD was introduced into the assay mixture, the level of fosfomycin production was greatly increased (Figure 2). The effect of FMN is slightly better than that of FAD. It is well-known that flavin coenzymes can func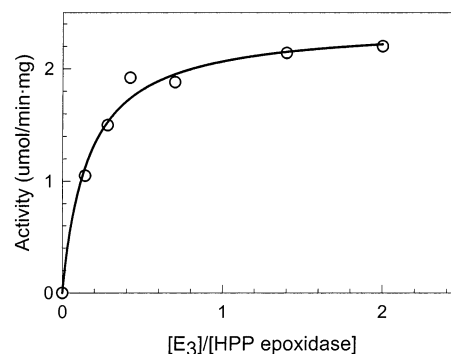tion as a two-electron/one-electron switch by receiving a hydride from NAD(P)H and transferring the two-electron reducing equivalents in a stepwise manner via a semiquinone intermediate. The observed activity enhancement suggested that FMN or FAD might be the missing electron mediator for HPP epoxidase. However, the translated *fom4* sequence lacks the Rossman's ADP-binding fold (*44*), which is characteristic for flavoproteins, and attempts to reconstitute apo-HPP epoxidase with FMN or FAD were unsuccessful. Thus, the flavin coenzyme is not likely to be an integral part of the epoxidase itself, but it may serve as a surrogate for the putative electron mediator in the in vitro assay.

Effects of E$_3$ on the Activity of HPP Epoxidase. To explore whether the putative electron mediator could be replaced by an exogenous protein reductase, E$_3$, an NADH-dependent [2Fe-2S]-containing flavoprotein reductase found in the 3,6-dideoxyhexose biosynthetic pathway from *Yersinia pseudotuberculosis* (*30*) was included in the assay mixture to replace FMN. Our data showed that E$_3$ is a more effective electron mediator than FMN (Figure 3). The activity of HPP epoxidase determined in the presence of E$_3$ is 2.1−2.2 μmol/min·mg, which is about 20-fold higher than that measured with FMN under the same assay conditions. Since the function of E$_3$ is to catalyze electron transfer from NADH to reduce the metal center of a dehydrase (E$_1$) which, along with E$_3$, catalyzes the C-3 deoxygenation step in the biosynthesis of 3,6-dideoxysugars (*45*), it may assume a similar role in HPP epoxidation. Hence, the electron mediator required for HPP epoxidase activity is likely a protein reductase capable of mediating single electron transfer to reduce the iron center in HPP epoxidase.

Electronic Absorption Spectrum of HPP Epoxidase. When the isolated HPP epoxidase was reconstituted with Fe(NH$_4$)$_2$(SO$_4$)$_2$ under anaerobic conditions, the resulting protein was colorless. However, when this sample was exposed to air, it slowly turned green in color. The optical spectrum of this green protein exhibits a broad band around 650−700 nm with a molar absorption coefficient of approximately 450 (M of Fe)$^{-1}$·cm^{-1} (Figure 4). This absorption is likely due to a ligand-to-iron (III) charge-transfer transition. Detailed characterization of this chromophore is in process and will be reported separately.

EPR Characterization of HPP Epoxidase. As mentioned above, HPP epoxidase as isolated contains little iron and thus is effectively EPR-silent (data not shown). Stoichiometric
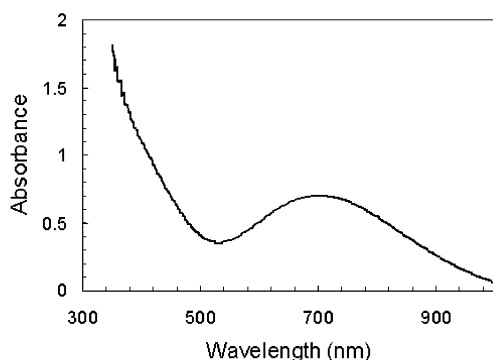
FIGURE 4: Electronic absorption spectrum of HPP epoxidase reconstituted aerobically with a stoichiometric amount of Fe(NH$_4$)$_2$-(SO$_4$)$_2$. The protein concentration is 1.4 mM.
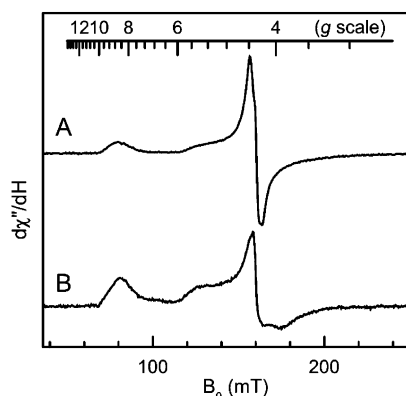

FIGURE 5: EPR spectra of HPP epoxidase: (A) 250 μM reconstituted (oxidized) HPP epoxidase; (B) 250 μM reconstituted (oxidized) HPP epoxidase in the presence of a ten times× excess of (*S*)-HPP. Instrumental parameters: temperature, 2 K; microwave frequency, 9.6 GHz; microwave power, 0.6 mW; modulation amplitude, 5 G; time constant, 0.02 s; sweep rate, 50 G/s. The *g*-scale is plotted on the top of the spectrum.

addition (1.0 Fe per enzyme monomer) of Fe(NH$_4$)$_2$(SO$_4$)$_2$ to the as-isolated epoxidase under anaerobic conditions led to an Fe(II)-loaded holoprotein. As expected, this Fe(II)-loaded epoxidase is EPR-inactive. Upon oxidation, the EPR spectrum of the sample displays sets of resonances at $g = 4.4, 4.27, 4.17,$ and 8.59 and 5.4 characteristic for S $= ^5/_2$ mononuclear Fe(III) centers with E/D values of 0.31 and 0.145, respectively (Figure 5A). The E/D value is a measure of the electronic symmetry and varies from a value of 0 for a center with axial symmetry to a value of $^1/_3$ for a center with maximal rhombic distortion. The appearance of centers with different E/D values suggests that there are at least two ligand symmetries and/or compositions possible in the active site. However, the species with $E/D = 0.31$ accounts for only a minor fraction of the iron centers because the observed intensity per spin is much greater for centers with resonances with similar g-values. Upon binding with (*S*)-HPP (**5-S**), the S $= ^5/_2$ EPR signal changes (Figure 5B). In addition to the two species seen in the resting reconstituted enzyme, two new species with resonances at 7.0 ($E/D = 0.05$) and 4.17 and 3.9 ($E/D = 0.27$) are resolved. This shows that the environment of the iron is perturbed and suggests that the substrate binds to or near the iron center.

EPR Characterization of HPP Epoxidase in the Presence of NO. To assess the Fe(II) content of the samples, nitric oxide was added. Since NO is known to complex with non-heme ferrous ion to produce species with electronic spin
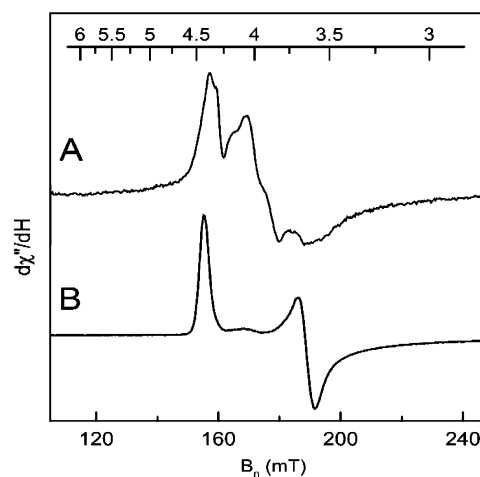

FIGURE 6: EPR spectra of reduced HPP epoxidase nitrosyl complexes at 2 K: (A) 250 μM Fe(II)-loaded HPP epoxidase in the presence of NO; (B) the ternary complex of Fe(II)−HPP epoxidase, substrate (10-fold excess over active sites), and NO. Instrumental parameters: temperature, 2 K; microwave frequency, 9.6 GHz; microwave power, 0.6 mW; modulation amplitude, 5 G; time constant, 0.02 s; sweep rate, 50 G/s. The *g*-scale is plotted on the top of the spectrum.

S $= ^3/_2$, a new spectral feature is expected if Fe(II) is present in the active site (*46*). The EPR spectrum of the oxidized Fe(III) epoxidase is unaffected by the anaerobic addition of NO to the sample (data not shown), showing that the iron can be completely oxidized. Anaerobic addition of NO to the Fe(II) loaded sample led to the formation of at least two major S $= ^3/_2$ species with resonances at $g = 4.32, 3.7,$ and 1.98 ($E/D = 0.055$) and 4.14, 3.92, and 1.99 ($E/D = 0.013$) (Figure 6A). Simulations of the spectra show that the $E/D = 0.055$ accounts for about 70% of the iron. In addition, a signal derived from a small amount of ferric ion is also discernible at $g = 4.3$. It is much more straightforward to quantitate these EPR spectra because they arise from the ground state and all of the resonances can be observed. Double integration of the spectra show that greater than 95% of the active sites of HPP epoxidase are occupied by Fe(II) in the sample. These results also clearly demonstrated that the ferrous center of the reduced HPP epoxidase can react with NO, an O$_2$ analogue.

As shown in the spectrum of Figure 6B, addition of **5-S** to the HPP epoxidase nitrosyl complex (order of addition is not critical) results in a significant change in the EPR spectrum. Most of the heterogeneity that characterizes the ferric complex and the nitrosyl complex of substrate free enzyme disappears, and a single species predominates ($g = 4.42, 3.63,$ and 1.97, $E/D = 0.066$). Quantification of the spectrum by double integration shows that >95% of the iron centers bind substrate and NO. These results suggest that substrate binds near, and perhaps to, the active site Fe(II) and in doing so organizes the center so that effectively one species is present.

Origin of the Oxiranyl−Oxygen in Fosfomycin. Another interesting question in this reaction is the origin of the epoxide ring oxygen. Early experiments had shown that when the fosfomycin producing strain *S. fradiae* was grown in an ^{18}O$_2$ enriched atmosphere, no ^{18}O was incorporated into fosfomycin (*25*). In contrast, using [2-^{18}O]-hydroxyethyl phosphonic acid as the precursor, 15−20% of the isolated fosfomycin derivative was found to be ^{18}O-labeled (*23*). To

```
S. wedmorensis  (1)   MSNTKTASTGFAELLKDRREQVKMDHAALASLLGETPE
   P. syringae  (1)   -MDVRTLAVGKAHLEALLAT----RKMTLEHLQDVRHD
    Consensus  (1)       T AGAL              L L

S. wedmorensis (39)   TVAAWENGEGGELTLTQLGRIAHVLGTSIGALTP-PA
   P. syringae (34)   ATQVYFDG------LEHLQNVAQYLAIPLSEFFVGQT
    Consensus (39)         G      L  L  A  L

S. wedmorensis (75)   GNDLDDGVIIQMPDERPILKGVRDNVDYYVYNCLVRT
   P. syringae (65)   QSDLDDGVKIARRNGGFKREEIRGGVHYYTYEHLVTT
    Consensus (76)      D DDGV I           R  V YY Y   V T

S. wedmorensis (112)  KRAPSLVPLVVDVLTDNPDDAKFNSGHAGNEFLFVL
   P. syringae (102)  NQDPGLMALRLDLHSDDEQPLRLNGGHGSREIVYVT
    Consensus (113)       P L  L  D    D         GH   E   V

S. wedmorensis (148)  EGEIHMKWG-DKENPKEALLPTGASMFVEEHVPHAF
   P. syringae (138)  RGAVRVRWVGDNDELKEDVLNEGDSIFILPNVPHSF
    Consensus (149)           W  D    KE L            VPH F

S. wedmorensis (183)  TAAKGTGSAKLIAVNF---
   P. syringae (174)  TNHVGGAKSEIIAINYG--
    Consensus (185)   T   G      IA N
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FIGURE 7: Alignment of the deduced amino acid sequences of HPP epoxidases isolated from *S. wedmorensis* and *P. syringae* PB-5123. The conserved 2-His-1-carboxylate triads of His-138, Glu-142, and His-180 are highlighted with an asterisk (∗).

verify these in vivo results, a 1:1 mixture of [2-^{18}O]- and [2-^{16}O]-HPP ([2-^{18}O]-**5**-*S* and **5**-*S*) was incubated with purified epoxidase and the fosfomycin product was analyzed by ^{13}C NMR spectroscopy. Unlike the case of unlabeled fosfomycin, whose C_β and C_α signals appear as a singlet and doublet ($J_{C-P} = 176$ Hz), respectively, the corresponding signals derived from the mixed sample are further split. The additional splitting is in fact a result of overlapping of two sets of signals, one for the unlabeled fosfomycin and the other for the labeled fosfomycin. The upfield shift by 0.018−0.025 ppm for the signals of the labeled species is due to the well-known ^{18}O isotope substitution effect (*47*). This result provided unequivocal evidence that the oxygen in the epoxide ring derives from the C-2 hydroxyl group of HPP. Thus, the reaction catalyzed by HPP epoxidase is an overall dehydrogenation reaction.

Mechanistic Implications. The expression, purification, and biochemical characterization of HPP epoxidase have shown that it is a mononuclear non-heme iron-dependent enzyme. This epoxidase belongs to the cupin superfamily, since it contains two sequence motifs conserved among members of this family: motif 1, **GX$_5$HXHX$_{3,4}$EX$_6$G**, and motif 2, **GX$_5$PXGX$_2$HX$_3$N** (*48*). Structural study of germin, an archetypal cupin member, had identified two histidine residues and one glutamate residue as ligands for binding manganese in the active site (*49*). So far, quite a few mononuclear non-heme iron(II)-dependent enzymes have been characterized by X-ray crystallography and shown to have such a 2-His-1-carboxylate facial triad (*50*). Examples include 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) (*51*, *52*), pterin-dependent tyrosine hydroxylase (TyrH) (*53*), isopenicillin N synthase (IPNS) (*54*), and the α-ketoglutarate-dependent deacetoxycephalosporin C synthase (DAOCS) (*55*), clavaminic acid synthase (CAS) (*56*), and taurine dioxygenase (TauD) (*57*). Since a conserved triad of His-138, Glu-142, and His-180 can also be found in HPP epoxidase isolated from *S. wedmorensis* (*26*) and *P. syringae* (*27*) (Figure 7), it is reasonable to propose these residues in a 2-His-1-carboxylate coordination for the iron center in HPP epoxidase. Since enzymes with 2-His-1-carboxylate coordi-

nation for the iron center typically activate molecular oxygen for biosynthetic reactions, its presence implicates a similar role played by iron in the molecular mechanism of HPP epoxidase.

The epoxidation catalyzed by HPP epoxidase is a unique reaction. The conversion consumes NADH, and the ultimate oxidant is dioxygen. Although a reductase is required to mediate the transfer of the reducing equivalents, alternative electron carriers, such as FMN and FAD, or generic exogenous reductases, such as E$_3$, appear to be competent substitutes. The ^{18}O labeling results confirmed that the overall reaction is, in effect, a dehydrogenation of a secondary alcohol (**5**-*S* → **1**). The oxiranyl ring formation catalyzed by hyoscyamine 6β-hydroxylase (H6H) in the biosynthesis of scopolamine (**11**) appears to be a closely related example (Scheme 3) (*58*). However, H6H is α-ketoglutarate dependent, while HPP epoxidase is not. Moreover, H6H can also catalyze the hydroxylation of hyoscyamine (**12**) to 6β-hydroxyhyoscyamine (**13**) and the epoxidation of 6,7-dehydrohyoscyamine (**14**) to scopolamine (**11**). Both activities are lacking in the case of HPP epoxidase. Thus, the catalytic mode of action of H6H must be distinct from that of HPP epoxidase. The transformation of (*S*)-HPP (**5**-*S*) to fosfomycin (**1**) clearly represents an intriguing cyclization beyond the scope entailed by common biological epoxidation and C−O bond formation.

Scheme 3

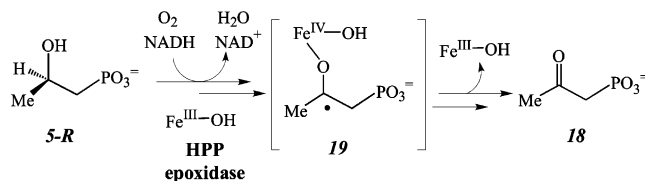R = (*S*)-3-hydroxy-2-phenylpropanoyl
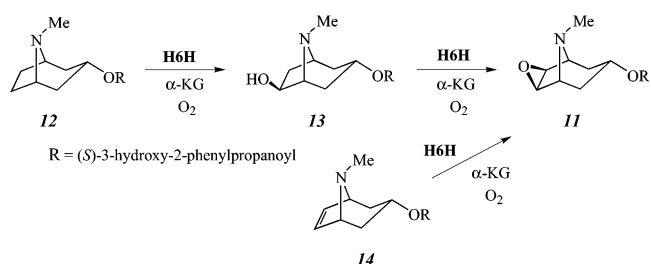
Recently, Hammerschmidt and co-workers reported that (1*S*,2*S*)-1,2-epoxypropylphosphonic acid (**15**) may be a

Scheme 4
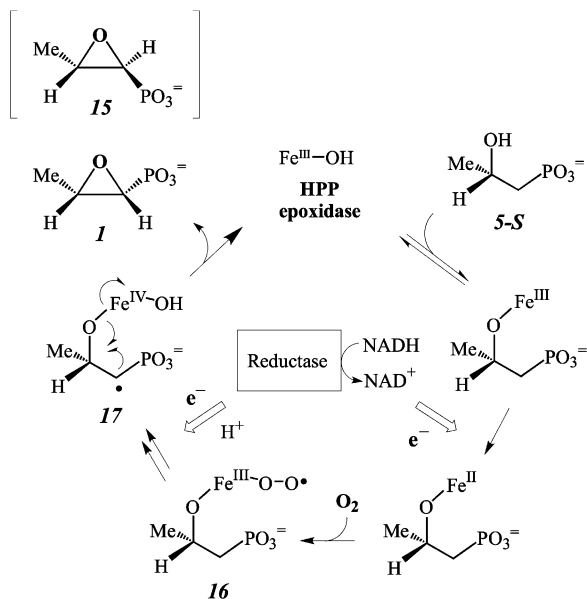
Scheme 5

cometabolite of fosfomycin (**1**), and both **1** and **15** are products of the oxidation of (*S*)-HPP (**5-*S***) by HPP epoxidase (*59*). Several mechanisms can be envisioned to account for the above observations based on the biochemical properties of HPP epoxidase, in which an iron-oxo intermediate is proposed to be the reactive species that triggers the reaction (*60*). Shown in Scheme 4 is an example wherein the Fe(III) center is reduced after substrate binding to Fe(II) and then reacts with dioxygen to generate the yet-elusive reactive iron-oxo species (**16**). Abstraction of an α-H of **5-*S*** followed by a radical induced homolytic cleavage of the Fe−O bond in **17** will produce **1**. The formation of *trans*-epoxide may be a result of the rotation around the C_1−C_2 bond at the α-C radical (**17**) stage. It should be mentioned that HPP epoxidase converts not only **5-*S*** to **1** but also the *R*-isomer of HPP (**5-*R***) to 2-oxopropylphosphonic acid (**18**) with nearly equal efficiency (*24*). The oxidation of the *R*-isomer of HPP by HPP epoxidase most likely involves the formation of a carbon-centered β-ketyl radical (**19**) (Scheme 5), which is distinct from the α-radical intermediate proposed for the catalysis of (*S*)-HPP (**5-*S***). Such findings lend further credence to the presence of a highly reactive species, such as an iron-oxo intermediate, capable of abstracting a hydrogen atom from HPP in the catalytic mechanism.

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