Protein Purification and Function Assignment of the Epoxidase Catalyzing the Formation of Fosfomycin

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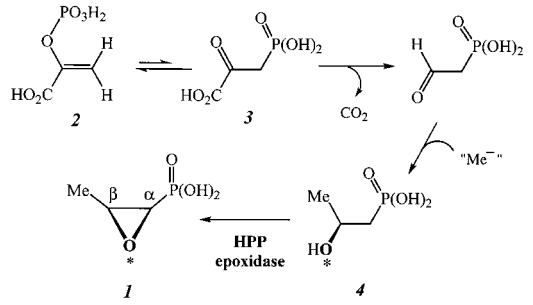
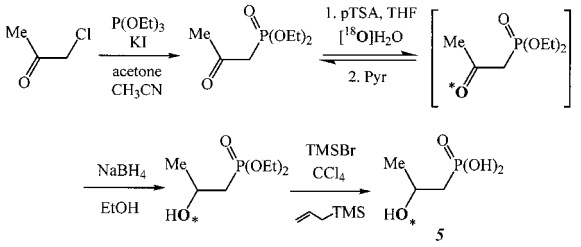
Received December 4, 2000

(1*R*,2*S*)-Epoxypropylphosphonic acid (**1**), also known as fosfomycin, is a clinically useful antibiotic.[1] Its biological target has been identified as UDP-Glc*N*Ac-*O*-enolpyruvoyl transferase,[2] which catalyzes the attachment of phosphoenolpyruvate (PEP) to UDP-Glc*N*Ac,[3] a key step in the assembly of the peptidoglycan layer in bacterial cell wall. Early studies had shown that the biosynthesis of fosfomycin begins with isomerization of PEP (**2**) to phosphonopyruvate (**3**) (Scheme 1).[4] Feeding experiments also established that the immediate precursor of fosfomycin is (*S*)-2-hydroxypropylphosphonic acid (HPP, **4**).[5] On the basis of these findings, a minimum of four enzymatic steps had been proposed for the biogenesis of fosfomycin (Scheme 1).[6] Recently, Seto and co-workers had cloned the entire fosfomycin biosynthetic gene cluster from *Streptomyces wedmorensis*,[7] and part of the cluster from *Pseudomonas syringae* PB-5123.[8] Expression of *orf3* of *P. syringae* in *Escherichia coli* and a preliminary activity assay led to the tentative assignment of *orf3* as encoding for the HPP epoxidase.[8] In a joint effort, we have expressed the *orf3* equivalent in *S. wedmorensis* (*fom4*) and purified the encoded protein (Fom4). In addition, we have also developed an efficient assay for Fom4, which now allows the first unambiguous assignment of Fom4 as the desired HPP epoxidase. Reported herein are the initial characterization of this enzyme and the implications for its mode of catalysis.

Scheme 1



Scheme 2



The gene for Fom4 was amplified by polymerase chain reaction (PCR) and cloned into a pQE30 expression vector. The ensuing plasmid, pQE5110, was used to transform *E. coli* M15 host cells. Growth of the recombinant strain in LB medium led to overproduction of Fom4. This *N*-terminal His-tagged enzyme was purified and exhibited no apparent absorption above 300 nm. Inductive coupled plasma (ICP) analysis of this purified protein also failed to detect any redox-active metal ions in significant quantities. To determine whether Fom4 is the desired epoxidase, (*S*)-HPP (**4**) was synthesized,[9,10] and tested for its competence as the substrate for Fom4. To our disappointment, no fosfomycin could be detected by NMR in this incubation. A bioassay[8] was then used to evaluate whether various metal ions and common biological reducing agents are required for Fom4 to function as an epoxidase.[11] In this experiment, a paper disk soaked with the assay mixture was placed in direct contact with a lawn of *E. coli* K12 HW8235 grown on nutrient (LB) agar. When fosfomycin was present in the assay mixture, an inhibition zone was visible after a few hours of incubation. Using this sensitive bioautography method, it was found that both $Fe(NH_4)_2(SO_4)_2$ and NAD(P)H are essential for Fom4 to convert **4** to fosfomycin. These findings provided initial evidence revealing that Fom4 is the desired epoxidase, and its catalysis is iron-dependent. However, no fosfomycin production was discernible by NMR even when all of the above components were included in the incubation.[12]

Since this epoxidase is iron-dependent, to avert any complication that may be associated with the fused His_6-tag, the *fom4* gene was cloned into a pET24b vector to express the epoxidase in its wild-type form.[13] The purified protein was eluted as a single peak with an apparent M_r of 89 kDa from an FPLC S200 column, and was calculated based on a monomeric M_r of 21210 Da to be

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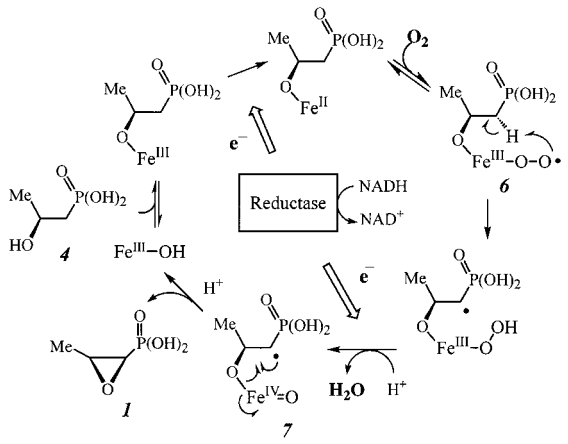
(10) Spectral data of **4**: ^{1}H NMR (500 MHz, D_2O) δ 0.97 (3H, d, J = 6.5 Hz, 3-H), 1.39 (2H, ddd, J = 18.0, 6.6, 15.3, 1-H), 3.80 (1H, dm, J = 6.5, 2-H); ^{13}C NMR (75.5 MHz, D_2O) δ 22.1 (C-3), 37.9 (d, J = 132 Hz, C-1), 65.0 (d, J = 2, C-2); ^{31}P NMR (121 MHz, D_2O) δ 19.9 (s).

(11) The redox active metals that had been tested include Fe, Cu, Co, Ni, and Mn. Ascorbate, NADH or NADPH were used as reducing agents in the incubation mixture.

(12) The reaction mixture contained 21.8 mM NADH, 1.0 mM $Fe(NH_4)_2(SO_4)_2$, 10.5 mM HPP (**4**), and 0.8 mM purified enzyme in 100 μL of 20 mM Tris·HCl buffer (pH 7.5). The reaction was run at room temperature for 4 h, and then the mixture was lyophilized. The resulting powder was redissolved in D_2O and analyzed by ^{1}H and ^{31}P NMR.

10.1021/ja004153y CCC: $20.00
Published on Web 04/21/2001

Scheme 3



tetrameric. *N*-terminal amino acid sequencing confirmed its identity.[14] Similar to its His-tagged homologue, the as-isolated protein is transparent above 300 nm and requires NAD(P)H and exogenous ferrous ion for activity. Upon treatment with $Fe(NH_4)_2(SO_4)_2$, the apo-enzyme could be reconstituted to have one iron per monomer. However, the catalytic efficiency of the reconstituted protein remained poor. Considering that additional electron mediators may be required, the effect of a variety of redox-active cofactors on the catalysis of this protein was investigated. Interestingly, it was discovered that the addition of FMN or FAD greatly enhanced the production of fosfomycin (100 nmol·min^{-1}·mg^{-1}).[15] The effect, however, was catalytic, and reconstitution failed to incorporate FMN/FAD into the epoxidase. Hence, the flavin coenzyme is unlikely an integral part of the epoxidase itself, but may act as a surrogate for an electron-transfer protein involved in the overall catalysis in vivo.

Indeed, E_3, an NADH-dependent [2Fe-2S]-containing flavoprotein reductase from the 3,6-dideoxyhexose biosynthetic pathway in *Yersinia pseudotuberculosis*,[16] was found to be a better electron mediator than FMN (activity increased >20-fold).[17] This result clearly indicated that inclusion of a reductase is necessary to fully reconstitute the epoxidase activity. Hence, conversion of **4** to fosfomycin is most likely catalyzed by a complex of HPP epoxidase and its reductase with NAD(P)H as the reductant (see Scheme 3). Interestingly, OrfD, the encoded product of another gene (*orfD*) just downstream of *fom4* in this cluster,[7] was found by the yeast two-hybrid system[18] to form a complex with Fom4 in vivo. Since all other open reading frames with unknown function in this cluster gave negative results in the yeast two-hybrid experiment, the physiological reductase for Fom4 is likely encoded by *orfD*. Although study of the catalytic details of epoxidation must await the isolation and characterization of the Fom4 reductase, the fact that FMN or E_3 is a convenient substitute for the reductase has greatly facilitated the initial investigation of HPP epoxidase. It should be noted that no fosfomycin was produced when the reaction was conducted anaerobically, and no H_2O_2 formation could be detected during the reaction. It is thus concluded that the oxidant of this reaction is O_2, which is reduced by four electrons to water during the catalysis.

To test whether the oxiranyl ring formation in the biosynthesis of fosfomyin proceeds via a net dehydrogenation as implicated by earlier feeding experiments,[5b,19] the ^{18}O-labeled HPP (**5**) was prepared (Scheme 2). A 1:1 mixture of unlabeled **4** and ^{18}O-labeled **5** was incubated with purified epoxidase in the presence of FMN. The product was purified by DEAE Sepharose eluted with a linear gradient of 0−0.5 M NH_4HCO_3, and analyzed by ^{13}C NMR. Unlike the unlabeled fosfomycin whose C_β and C_α signals appear as a singlet and doublet (J_{C-P} = 176 Hz), respectively,[20] the corresponding signals in fosfomycin derived from this mixed sample were further split. The complication derives from the 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 ^{18}O isotope substitution effect.[21] Thus, these data firmly establish the retention of ^{18}O label in the product.

Most enzymes capable of catalyzing epoxidation rely on a mechanism of oxygen insertion to an olefinic substrate to form the oxirane.[22] The epoxidation catalyzed by HPP epoxidase is unusual since it is effectively a dehydrogenation of a secondary alcohol (**4**→**1**). Two closely related examples are the epoxide ring formation catalyzed by hyoscyamine 6β-hydroxylase involved in the biosynthesis of scopolamine,[23] and the ring closure of proclavaminic acid catalyzed by clavaminate synthase.[24] However, both enzymes belong to the family of α-ketoglutarate-dependent non-heme iron oxygenases.[25] In contrast to these two examples, HPP epoxidase is α-ketoglutarate-independent, and its activity is adversely sensitive to ascorbate.[26] Thus, the cyclization of HPP (**4**) to fosfomycin (**1**) clearly represents an intriguing conversion beyond the scope entailed by common biological epoxidation and C−O bond formation. One of several plausible mechanisms that are consistent with the available data is shown in Scheme 3. The reaction may be initiated with an electron transfer from the putative reductase followed by oxygen activation to generate an iron-coordinated superoxide radical **6**. Abstraction of an α-H atom of **6** in conjunction with a proton-coupled electron transfer may lead to a high valent iron species **7**.[27] Radical-induced homolytic cleavage of the Fe−O bond in **7** will produce **1** and also regenerate the iron core. Future research on this enzyme is aimed at further elucidation of the mechanism of this unique epoxidase and isolation as well as characterization of its putative reductase.

Acknowledgment. We thank Professors Lawrence Que, Jr. and John Lipscomb and Dr. Aimin Liu for helpful discussion.This work was supported in part by the National Institutes of Health grant GM40541.

JA004153Y

(13) The ensuing construct, pPL001, was used to transform BL21 (DE3) cells, and the recombinant strain was grown in LB medium at 37 °C under IPTG induction (0.1 mM). The overproduced Fom4 was purified to near homogeneity by a sequence of 40−60% $(NH_4)_2SO_4$ fractionation, DEAE-Sepharose, and FPLC Mono Q chromatography.

(14) *N*-terminal amino acid sequencing confirmed that the first 10 residues, SNTKTASTGF, of the purified protein are identical to those of the translated *fom4* sequence.

(15) The reaction mixture contained 21.8 mM NADH, 140 μM $Fe(NH_4)_2(SO_4)_2$, 10.5 mM HPP, 200 μM FMN, and 108 μM purified enzyme in 100 μL of 20 mM Tris·HCl buffer (pH 7.5). The reaction was run at room temperature for 30 min, quenched by adding 100 mM EDTA, and frozen with liquid N_2. The sample was later thawed and analyzed by ^1H NMR. The activity of HPP epoxidase was determined to be 100 nmol·min^{-1}·mg^{-1}.

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(17) The contents of the reaction mixture were identical to those described in ref 15 except for the amount of epoxidase (21.6 μM) and $Fe(NH_4)_2(SO_4)_2$ (28 μM), and the addition of 60 μM E_3. The reaction was run at room temperature for 5 min, quenched by adding 100 mM EDTA, and frozen with liquid N_2. The analysis was also the same as described in ref 15.

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(20) Spectral data of **1**: ^1H NMR (500 MHz, D_2O) δ 1.21 (3H, d, J = 5.5.Hz, 3-H), 2.57 (1H, dd, J = 5.0, 14.0, 1-H), 3.03 (1H, dm, J = 5.0, 2-H); ^{13}C NMR (75.5 MHz, D_2O) δ 13.2 (C-3), 54.3 (C-1, d, J = 176), 54.2 (C-2, s); ^{31}P NMR (121 MHz, D_2O) δ 10.9 (s).

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(26) Addition of α-ketoglutarate up to 1 equiv of the substrate in the incubation mixture showed no effect on the production of fosfomycin. However, complete inactivation of Fom4 was observed when 5−10-fold of ascorbate relative to enzyme concentration was added.

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