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Evidence that the Fosfomycin-producing Epoxidase, HppE, is a Non-heme-iron Peroxidase[‡]

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Abstract

The iron-dependent epoxidase, HppE, converts (*S*)-2-hydroxypropyl-1-phosphonate (*S*-HPP) to the antibiotic, fosfomycin [(1*R*, 2*S*)-epoxypropylphosphonate], in an unusual 1,3-dehydrogenation of a secondary alcohol to an epoxide. HppE has been classified as an oxidase, with proposed mechanisms differing primarily in the identity of the O₂-derived iron complex that abstracts hydrogen (H•) from C1 of *S*-HPP to initiate epoxide ring closure. We show here that the preferred co-substrate is actually H₂O₂ and that HppE therefore almost certainly employs an iron(IV)-oxo complex as the H• abstractor. Reaction with H₂O₂ is accelerated by bound substrate and produces fosfomycin catalytically with a stoichiometry of unity. The ability of catalase to suppress the HppE activity previously attributed to its direct utilization of O₂ implies that reduction of O₂ and utilization of the resultant H₂O₂ were actually operant.

The drug fosfomycin [(1*R*,2*S*)-epoxypropylphosphonate, Fos; see Fig. 1 for structure] kills pathogenic bacteria by inactivating UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and thereby blocking synthesis of peptidoglycan, which constitutes the backbone of the cell wall (1–2). The chemical “warhead” of Fos is its strained epoxide ring, which is opened upon attack by a cysteine residue in the active site of MurA, resulting in an inactivating covalent modification (1–2). The epoxide of Fos is installed in the last step of its biosynthesis by the non-heme-iron(II) enzyme, (*S*)-2-hydroxypropyl-1-phosphonate (*S*-

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HPP) epoxidase (HppE), which mediates an unusual 1,3-dehydrogenation of the secondary alcohol in the substrate to form the new carbon-oxygen bond of the three-membered ring (3).

HppE has been described as an oxidase, i.e., purported to use O₂ as its oxidizing co-substrate (Fig. 1A, reaction I) (3). This formulation of the epoxidation reaction would require that two electrons, in addition to the two provided by *S*-HPP in forming the epoxide, be transferred to the HppE active site to achieve redox balance in the four-electron reduction of O₂. The source of these electrons and the manner in which they might be delivered have remained unclear, as published structures of the enzyme do not reveal an obvious reductase domain (4–6) and gene clusters specifying the Fos-biosynthetic enzyme machinery do not encode a readily identifiable, dedicated reductase protein (7–8). In vitro investigations of the HppE reaction have relied on either a heterologous reductase protein (called E₃) from the 3,6-dideoxyhexose biosynthetic pathway of *Yersinia pseudotuberculosis* with its reducing co-substrate NADH (E₃/NADH) or the combination of the biochemical reductants, NADH and flavin mononucleotide (NADH/FMN) (3, 9). The fact that these reducing systems support only very slow Fos production (~1 min⁻¹) has reinforced the notion that additional components (e.g., the natural reducing system) might remain to be identified (9).

By contrast to simpler chemical and enzymatic epoxidation reactions involving addition of an oxygen atom (e.g., from a high-valent metal-oxo complex) to a carbon-carbon double bond (10–11), the HppE reaction involves cleavage of a carbon-hydrogen bond of *S*-HPP (the *pro-R* C1–H bond) and formation of a new carbon-oxygen single bond to C1. In the product, the epoxide oxygen takes up the position on C1 originally occupied by the *pro-S* rather than the abstracted *pro-R* hydrogen, implying that C1 is inverted in the ring-closure step (3, 12). Although the mechanisms by which the C1–H-bond is cleaved and the new C1–O-bond is formed remain unknown, the observation by X-ray crystallography that *S*-HPP chelates the Fe^{II} cofactor via the C2 hydroxyl and the phosphonate (5) and precedent from studies on other O₂ activating iron-enzymes (13–14) led to the suggestion of three alternative pathways (Fig. 1B, blue arrow) (4, 15). These hypothetical O₂-dependent mechanisms are distinguished by the nature of the C1–H-cleaving iron complex and the order of bond-breaking, bond-forming, and electron-injection steps.

As depicted in Fig. 1B, abstraction of the *pro-R* hydrogen atom (H•) from C1 could be mediated by a Fe^{III}-superoxo (Fe^{III}–O₂^{-•}) complex formed by simple addition of O₂ to the Fe^{II} cofactor (pathway I, beige arrows); a Fe^{III}-hydroperoxo complex, formed by addition of O₂, an electron, and a proton to the cofactor (pathway II, green arrows); or a Fe^{IV}-oxo (ferryl) complex, formed by addition of O₂, transfer of two electrons and two protons, and cleavage of the O–O bond (pathway III, red arrows). Of these possibilities, a ferryl complex was favored in a recent computational study (16). The mechanism of epoxide-ring closure following formation of the C1 radical is equally uncertain. The most straightforward possibility, suggested by studies on other iron enzymes that mediate formation of new carbon-heteroatom bonds, would be a radical group transfer of the Fe-coordinated C2–O atom to the C1 radical (17–22). However, the inversion of C1 remains challenging to explain according to this mechanism. On the basis of a remarkable oxidative 1,2-phosphonate migration mediated by HppE upon the substrate analog, (*R*)-1-

hydroxypropyl-1-phosphate (Fig. 1A and C, reactions II), a recent study posited a different ring-closure mechanism, involving formation of a C1 carbocation by electron transfer from the C1 radical to the iron cofactor and subsequent nucleophilic capture of the C1 carbocation by the C2 alkoxide (Fig. 1C, reaction I) (23). In this mechanism, neighboring group participation (anchimeric assistance) by the phosphonate (with retention of the C1 stereochemistry, dashed red line) could promote formation of the formal carbocation and then also dictate the net inversion of C1 following subsequent attack by the C2-O.

Precedent suggests that the H•-abstracting complex might be identified by use of rapid-kinetic and spectroscopic methods to detect it and demonstrate a kinetic isotope effect on its decay upon use of [1,1-²H₂]-*S*-HPP as the substrate (24). However, related O₂-activating iron oxidases and oxygenases have catalytic rate constants typically in the range of 1 to 100 s⁻¹ (24–25). Therefore, if generation of the C1-H-cleaving intermediate requires one or two electrons, a reductant considerably more efficient than the reported E₃/NADH and NADH/FMN systems [which support maximum turnover rates of ~0.01 s⁻¹ (9)] might be required to gain entry into the reaction sequence fast enough to accumulate the C1-H-cleaving complex (24). In seeking a more suitable reductant, we determined that sodium dithionite (Na₂S₂O₄) can support multiple turnovers at rates more than a thousand times greater (10 to 100 s⁻¹) than those supported by the NADH-based reducing systems. Dithionite is itself reactive toward O₂ on this same timescale (26), creating a conundrum as to how it could deliver electrons to HppE without first being oxidized by O₂. Closer examination of the reaction led to the new hypothesis that dithionite might actually function by reducing O₂ directly rather than donating electrons to the HppE active site during an O₂-initiated catalytic cycle. We therefore evaluated whether the expected O₂-reduction product, H₂O₂, is capable of serving as the oxidant for Fos production by HppE.

To test this hypothesis, varying quantities of H₂O₂ were added slowly to solutions of Fe^{II}-HppE, *L*-ascorbate, and [2,3,3,3-²H₄]-(*S*)-2-hydroxypropyl-1-phosphonate (d₄-*S*-HPP), remotely labeled to distinguish the enzymatic product ([2,3,3,3-²H₄]-(*1R,2S*)-epoxypropyl-1-phosphonate; d₄-Fos) from the commercially available Fos standard. Reaction samples were analyzed by liquid chromatography with mass-spectrometric detection (LC-MS). As shown in Fig. S1, the intensity of the peak arising from the d₄-*S*-HPP substrate at *m/z* = 143 (negative-ion mode) with an elution time of ~3 min is diminished (panel A), and a new peak at *m/z* = 141 (consistent with the mass of the d₄-Fos product) co-eluting at ~2 min with the commercial Fos (*m/z* = 137; dotted orange trace) grows in (panel B) as more H₂O₂ is added. The identity of the new product was established to be Fos by ¹H-NMR analysis of a reaction sample to which H₂O₂ was added to a final 1:1 molar ratio with respect to the unlabeled *S*-HPP substrate (Fig. 2). Although line-broadening, possibly arising from the presence of iron from the enzyme in the NMR sample, is evident, comparison of the spectrum of the reaction product (spectrum B) to that of commercial Fos (spectrum A) reveals features with the proper chemical shifts and integrated intensities at ~3.05 ppm (multiplet from C2-H), ~2.62 ppm (doublet of doublets from C1-H), and ~1.25 ppm (doublet from C2-Me). None of these features is present in the spectrum of the *S*-HPP starting material (spectrum C). Additional minor features at ~2.72, ~2.66, and ~2.43 ppm in the spectrum of the product sample (B) arise from the 2-keto oxidation product known to be

generated by HppE from the (*R*)-2-hydroxypropyl-1-phosphonate (*R*-HPP) enantiomer (27), which is present as a minor (~ 6%) contaminant in our synthetic *S*-HPP substrate.

The nearly complete consumption of *S*-HPP by one molar equivalent of H₂O₂ evident in the NMR spectra implies an experimental Fos:H₂O₂ stoichiometry close to the predicted value of unity. The experimental stoichiometry was accurately determined by LC-MS using commercial Fos as an internal standard (Fig. 3A, gray traces) to quantify the d₄-Fos product from the HppE peroxidase reaction (black traces). The stoichiometry was determined to be 1.00 ± 0.05 (Fig. 3B). Coupling between H₂O₂ reduction and Fos production is thus extremely tight.

The requirements for the productive HppE peroxidase reaction were defined and its catalytic nature verified by analysis of reactions carried out with high H₂O₂/HppE ratios from which individual reaction components were serially omitted (Fig. S2 and associated text). With all components present, the enzyme could effect at least 50 turnovers. The kinetics of a single turnover of H₂O₂-driven Fos production were then determined at 4 °C. With 0.096 mM HppE•Fe^{II}, 0.050 mM H₂O₂, and 0.50 mM d₄-*S*-HPP, d₄-Fos production was complete in ~ 0.1 s (Fig. 4). Simulation of the kinetic data requires a second-order rate constant of at least $1.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for reaction of the HppE•Fe^{II}•*S*-HPP complex with H₂O₂ (— — —), and the data are simulated best with a value of $4.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for this rate constant (——). It is possible that the first-order steps following addition of H₂O₂ to the enzyme could be slower than the addition step itself; in this case, the second-order rate constant for H₂O₂ addition could be significantly underestimated by the analysis. Regardless, the determined rate constant is only ten to a hundredfold less than the value of $k_{\text{cat}}/K_{\text{M}}$ reported for bovine liver catalase, an enzyme for which H₂O₂ is the established physiological substrate and which is often touted as having evolved almost to catalytic perfection (i.e., to operate at the diffusion limit) (28). Quantitative assessment of the ability of commercial bovine liver catalase to compete with HppE for reaction with added H₂O₂ yielded an estimate of $5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for the $k_{\text{cat}}/K_{\text{M}}(\text{H}_2\text{O}_2)$ of HppE at 21 °C (Fig. S3 and associated text). It is likely that the experimental error in the rate constants obtained at 4 °C and 21 °C (we estimate a factor of two for each) coincidentally obscures the actual temperature dependence of the reaction rate.

Both reducing systems used in previous in vitro studies on HppE (E₃/NADH and NADH/FMN) are known to generate H₂O₂ from O₂ (29–30). The demonstration that HppE is an efficient peroxidase therefore raises the possibility that its reported oxidase activity in the presence of the NADH-based reducing systems might actually have resulted from reduction of O₂ by the reducing system and utilization of the resultant H₂O₂ by HppE. As a test of this possibility, HppE reactions with O₂ and one of the two reducing systems were carried out in the presence of varying concentrations of catalase. As shown in Fig. S3, increasing the concentration of catalase diminishes the Fos yield in these reactions with [catalase] dependencies (blue and green points) essentially identical to that for the direct reaction with H₂O₂ (red). These data strongly suggest that the Fos production previously attributed to HppE oxidase activity actually reflects its peroxidase activity. Consistent with this conclusion, use of H₂¹⁸O₂ as oxidant and H₂¹⁸O-enriched solvent does not result in incorporation of a detectable quantity of ¹⁸O into the Fos product. This observation confirms that the epoxide is formed from the oxygen atom already present in the substrate, as

previously demonstrated when the reaction was thought to involve O_2 . In addition, when added directly, H_2O_2 also supports the alternative oxidations of substrate analogs that were previously attributed to O_2 , including the aforementioned oxidative 1,2-phosphonate migration with (*R*)-1-hydroxypropyl-1-phosphonate [Fig. 1A, reaction II (23)] and the C2 dehydrogenation of (*R*)-2-hydroxypropyl-1-phosphonate to the corresponding ketone [Fig. 1A, reaction III (27)]. The observations suggest that all HppE activities reported in previous studies arose from the enzyme's reaction with H_2O_2 generated by the reducing systems rather than its reaction directly with O_2 .

Additional evidence that HppE is not an oxidase is the failure of the $Fe^{III}\text{-}O_2^{\bullet-}$ complex that should result from addition of O_2 to its Fe^{II} cofactor (17, 31–34) to accumulate in the absence of a reducing system. Freeze-quench Mössbauer spectroscopic experiments employing an efficient enzymatic O_2 -generation system that can drive formation of even dissociable O_2 adducts (35) revealed that O_2 adds either very slowly or with low affinity (or both) to the HppE cofactor, irrespective of the presence of bound substrate (Fig. S4 and associated text). The apparent failure of substrate binding to promote O_2 addition contrasts with the behavior of most proven oxidases and oxygenases that employ non-heme-iron cofactors (24, 36–40). It also contrasts with the behavior of HppE toward H_2O_2 : reaction of the substrate-free enzyme with H_2O_2 was found to be ~ fiftyfold slower (Fig. S5 and associated text) than the productive reaction in the presence of *S*-HPP, implying that substrate binding does indeed promote reaction with H_2O_2 . Conversely, the inability of 2-hydroxyethyl-1-phosphonate (HEP) dioxygenase (HEPD), the non-heme-iron enzyme that is structurally most similar to HppE (Fig. S6) (41), to use H_2O_2 also suggests that the peroxidase activity seen with HppE reflects its natural function (Fig. S7 and associated text). HppE and HEPD share the ability to oxidize HEP (Fig. S7A, blue bars and Figure S7B, green bars) [also *R*-HPP (42)], but HEPD unequivocally uses O_2 as its co-substrate for this oxidation (41). It does not use H_2O_2 for catalytic oxidation of HEP (Fig. S7A, green bars) under the conditions that support efficient consumption of *S*-HPP by HppE (red bars). Even in its cross-reactivity toward HEP, HppE is still an efficient peroxidase (blue bars) but has no activity with O_2 as the oxidant in the absence of a reducing system to first convert the O_2 to H_2O_2 (Fig. S7B, blue bars). The enzymes' nearly orthogonal use of the oxidants, despite their very similar structures and overlapping substrate profiles, further supports the hypothesis that HppE is an authentic peroxidase rather than an oxidase capable of an adventitious “peroxide-shunt” reaction.

Unlike the previously postulated O_2 -dependent epoxidation reaction, in which the iron complex responsible for the crucial abstraction of the *pro-R* $H\bullet$ from C1 of *S*-HPP could potentially have been in any of three different overall oxidation states (the three pathways in Fig. 1B), reaction of the Fe^{II} cofactor with H_2O_2 is redox balanced to bypass the first two oxidation states and proceed directly to a Fe^{IV} -oxo (ferryl) complex (magenta and red arrows). Formation of inorganic ferryl complexes from Fe^{II} precursors and H_2O_2 is well preceded [e.g., (43–44)], and the abstraction of $H\bullet$ by a ferryl complex has been demonstrated in the reactions of a number of other non-heme-iron enzymes (13, 18, 38, 40, 45–47). The use of H_2O_2 , the literature precedents, and the recent computational study (16) all point to a ferryl complex as the most likely initiator of the HppE reaction.

The conclusion that HppE utilizes a ferryl complex generated directly from H₂O₂ rather than O₂ to initiate its epoxidation reaction suggests that the Fos-biosynthetic machinery should not require a specific HppE reductase. Instead, the epoxidation could be supported by any endogenous or exogenous reaction that generates H₂O₂. Conceivably, Fos-synthesizing *Streptomyces* might encounter H₂O₂ in their environments, turning a general toxin produced by competing organisms to their advantage by using it to produce a counter-toxin of their own. Such a scenario would add to the growing list of known uses of H₂O₂, which had previously been considered primarily as a metabolic toxin, in productive physiological reactions related to intercellular interactions (48–49).

Supplementary Material

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Acknowledgments

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Figure 1.

Proposed and demonstrated reactions of HppE and their possible mechanisms. **A**, Putatively O_2 -dependent reactions purportedly catalyzed by HppE on *S*-HPP (**I**) and its stereo- and structural isomers (**II–IV**). **B**, mechanisms previously proposed for the putatively O_2 -dependent fosfomycin-generating epoxidation reaction (**blue arrow**) and the mechanism indicated by the finding that H_2O_2 rather than O_2 is the oxidizing co-substrate (**magenta and red arrows**). **C**, proposed mechanisms for H_2O_2 -driven (**I**) epoxide ring closure to produce fosfomycin and (**II**) phosphonate migration with the (*R*)-1-hydroxypropyl-1-phosphonate analog (23).

Figure 2.

¹H-NMR spectra demonstrating production of Fos by HppE upon its reaction with H₂O₂. **A**, commercial Fos standard. **B**, products following slow addition of H₂O₂ (final concentration of 5.0 mM) to a solution of HppE (final concentration of 0.10 mM) Fe^{II} (0.080 mM), S-HPP (5.0 mM) and L-ascorbic acid (4.0 mM). The reaction was carried out at 21 °C in the absence of O₂. **C**, the S-HPP substrate. Details of sample preparation in B and spectral acquisition are provided in supplementary materials.

Figure 3.

Determination of the Fos:H₂O₂ reaction stoichiometry. **A**, LC-MS chromatograms monitoring $m/z = 141$ for the HppE-generated d₄-Fos product (**black**) and $m/z = 137$ for the commercial Fos internal standard (**gray**). **B**, quantity of d₄-Fos produced by HppE as a function of [H₂O₂] added. The error bars are the standard deviations from the mean values of six measurements at each concentration. The slope gives the Fos:H₂O₂ reaction stoichiometry (1.00 ± 0.05). Reaction conditions were the same as in Figure 2, with the exception of the [H₂O₂] added.

Figure 4.

Kinetics of d₄-Fos production in a single turnover with limiting H₂O₂. Samples were prepared by mixing a solution containing 0.24 mM HppE, 0.19 mM Fe^{II}, and 1.0 mM d₄-S-HPP with an equal volume of 0.10 mM H₂O₂ and allowing the reaction to proceed at 4 °C for the indicated time before terminating by mixing the reaction solution with an equal volume of the quench solution (80% isopropanol/20% acetic acid on a volume basis). All data points from three independent trials, with duplicate points in each trial, are shown. A quench time of 5 ms has been assumed. The points at zero reaction time, which represent samples of the reactant complex that were quenched without exposure to H₂O₂, are plotted with an x-value of 10⁻⁵ s for display on the logarithmic time axis. The traces are simulations with the second-order rate constants given in the legend.