O-H Activation by an Unexpected Ferryl Intermediate during Catalysis by 2-Hydroxyethylphosphonate Dioxygenase

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SUPPORTING INFORMATION

Materials and Methods

General

Wild-type (wt) HEPD and HEPD-E176A were overexpressed and purified as previously described. For protein used for multi-turnover steady-state kinetic assays, an additional gel-filtration purification step was used to isolate the active dimer fraction. For transient-state experiments, the protein was prepared as before but then concentrated to a stock concentration of ~ 4 mM by using a 30 kDa centrifuge filter (EMD Millipore). The protein stock solution was then rendered anoxic by repeated cycles of gentle evacuation (~250 Torr) and refilling with argon while stirring on ice, as previously described. All substrates were prepared as previously described. Reagents were obtained from Sigma Aldrich and used as received unless otherwise noted. All reactions were carried out in 50 mM sodium HEPES, pH/D 7.5, unless specified otherwise.

Construction and initial characterization of HEPD-E176A and rationale for its investigation

The active site glutamate residue (E176) that ligates the iron was chosen for mutagenesis because E176 is cis to the putative O₂ binding site in the HEPD crystal structure. We hypothesized that its removal might disrupt the hydrogen-bonding network...
responsible for binding H\textsubscript{2}O to the metal at this site. A less-tightly anchored H\textsubscript{2}O ligand might render the active site more reactive toward O\textsubscript{2}, which could unmask a substrate KIE under multi-turnover conditions. Thus, HEPD-E176A was constructed by using the QuikChange Mutagenesis Kit (Stratagene) and the following primers (the altered codon is in red):

Forward: \textit{\texttt{5'}ggc
cagctctacgtg
c\texttt{3'}}

Reverse: \textit{\texttt{5'}gacc
cgtgatc
c\texttt{3'}}

The identity of the products generated from HEP by HEPD-E176A were confirmed to be HMP by \textsuperscript{31}P NMR spectroscopy and formate by LC-MS analysis following derivatization, as described previously.\textsuperscript{6} To confirm production of HMP, apo HEPD-E176A was converted to its Fe(II) complex as before,\textsuperscript{5} except that 3 rather than 1 equiv. (NH\textsubscript{4})\textsubscript{2}Fe(SO\textsubscript{4})\textsubscript{2}•6H\textsubscript{2}O [hereafter referred to as Fe(II)] was added to the enzyme (10 \mu M final concentration). The enzyme was then incubated in O\textsubscript{2}-saturated buffer (25 mM sodium HEPES, pH 7.5) with HEP (2 mM). After a 2-h incubation, EDTA (25 mM final concentration), dithionite [10 mM final concentration, to reduce line broadening from Fe(III)], and D\textsubscript{2}O (20% final v/v) were added, and the \textsuperscript{31}P NMR spectrum was recorded.\textsuperscript{6} The identities of the signals were confirmed by amending the NMR sample with the authentic compounds. To confirm formate production, the Fe(II)-treated HEPD-E176A (2 \mu M final concentration) was incubated with 2-[2-\textsuperscript{13}C]-HEP (2 mM, to avoid spurious formate contamination) in O\textsubscript{2}-saturated buffer (25 mM sodium HEPES, pH 7.5). After a 5-min incubation, the solution was quenched, derivatized, and analyzed as previously described.\textsuperscript{5} A positive control with wt HEPD under identical conditions confirmed that HEPD-E176A produced \textsuperscript{13}C-formate under these conditions.
**Mössbauer spectroscopy**

Simulations of Mössbauer spectra were performed with the WMOSS spectral analysis software package (www.wmoss.org, WEB Research, Edina, MN). The strong-field reference spectra of the Fe(IV)-oxo intermediate were analyzed according to the $S = 2$ spin Hamiltonian (Equation 1)

$$H = \beta S \cdot g \cdot B + D \left( S_z^2 - \frac{S(S+1)}{3} \right) + E \left( S_z^2 - S_y^2 \right) + \frac{eQV}{12} \left[ 3I_z^2 - I(I+1) + \eta \left( I_z^2 - I_y^2 \right) \right] + S \cdot A \cdot I - g_s \beta_n \cdot B \cdot I$$ (Eq. 1)

in which the first three terms describe the electron-Zeeman effect and the axial and rhombic zero-field splittings of the electron-spin ground state, the fourth term describes the electronic quadrupole interaction of the $^{57}$Fe nucleus induced by the electric field gradient, the fifth term represents the magnetic hyperfine interaction between the electronic spin and the $^{57}$Fe nucleus, and the last term represents the nuclear Zeeman interaction. All Mössbauer analysis was carried out with the assumption that fluctuation rate of the electron spin is much less than the Larmor frequency of the $^{57}$Fe nuclei.

**Rapid Chemical Quench**

The freeze-quench Mössbauer and SF-abs time courses were obtained by mixing an anaerobic solution of HEPD-E176A:Fe(II):2-HEP with an O$_2$-saturated solution. Attempts to conduct rapid chemical quench experiments in the same way were unsuccessful since oxygen contamination repeatedly showed much higher amounts of product at the very first time point than possible based on the kinetic parameters of the enzyme.
18O Labeling Experiments

To quantify exchange of oxygen derived from solvent and O2 gas into or out of the hydroxyl group of the HMP product, 18O2 (97% 18O, Sigma Aldrich) and H218O (97% 18O, Cambridge Isotope Labs) and D218O (97.6% D, 97% 18O, Cambridge Isotope Labs) were employed. In a typical assay using 18O2, a stock solution of HEPD-E176A was rendered anoxic by using a NAP-5 column equilibrated with O2-free buffer (50 mM sodium HEPES, pH 7.5, rendered anoxic on a vacuum line by repeated evacuation and refill with N2). The O2-free apo HEPD-E176A (40 µM final concentration in 50 mM sodium HEPES, pH 7.5) was converted to its Fe(II) complex by treatment with 3 equiv. Fe(II), and D2-HEP was then added (1 mM final concentration). For the reaction in D2O, the same conditions were used, except that the HEPD-E176A stock solution was exchanged into buffer made in D2O (50 mM sodium HEPES, pD 7.5; pD = pH reading + 0.41) using a 0.5 mL 30 kDa MWCO Amicon centrifuge filter. This process ultimately allowed a deuterium content of ~99% to be reached in the final reaction. A third set of samples was prepared in which equal parts of the anaerobic protein-phosphonate solution in H2O and in D2O were mixed.

Each of the three samples (500 µL each) was transferred under N2 to a 15 mL round-bottom flask equipped with a stir-bar and sealed tightly with a septum and Parafilm. A canister of 18O2 was fitted with the appropriate regulator and septum, and the regulator headspace was purged with 18O2 several times. A 10 mL sample lock syringe was used to remove 18O2 from the canister and charge each of the three reaction flasks (~10 mL of 18O2, pressure unknown). The reactions were allowed to stir in an anoxic chamber at room temperature for 2 h. The reactions were quenched by removing the
protein in each sample with a 0.5 mL 30 kDa centrifuge filter that had been rinsed with O2-free water to remove any traces of contaminating 16O2.

The filtrates (1 µL) were separated by liquid chromatography coupled to multi-reaction mode MS monitoring. The components of the filtrate were separated by using LC (Thermo Scientific Hypercarb column, 100 x 4.6 mm, 5 µm mesh) with a gradient of 25 mM ammonium acetate in H2O (buffer A) and acetonitrile (buffer B). The column was equilibrated with a 98:2 mixture of buffer A:buffer B. Following injection, this ratio was maintained for 2 min, after which time the mobile phase was gradually changed to a 50:50 mixture of buffer A:buffer B over the course of 8 min. The mobile phase was returned to a 98:2 mixture of A:B over 1 min and held at that composition for a further 6 min to re-equilibrate the column. At all stages, the flow rate was maintained at 0.3 mL/min. After chromatographic separation, the samples were analyzed by in-line detection with a 5500 QTrap (AB Sciex) MS by monitoring the 113 → 62.9 m/z (18O-HMP) and 111 → 62.9 m/z (16O-HMP) transitions in negative mode (curtain gas: 32 psi; voltage: 4,500 V; temperature: 450 °C; N2 gas one: 50 psi; N2 gas two: 65 psi). Percent retention of the label was corrected based on the isotopic composition of the reaction.

For reactions using H218O and/or D218O, wt apo HEPD or HEPD-E176A (final concentration of 40 µM) was reconstituted to its Fe(II) complex (by addition of 1 equiv Fe(II) for WT HEPD or 3 eq. for HEPD-E176A) directly in H2O, or first exchanged into buffer made in D2O and then treated with Fe(II), depending on the desired final D2O content of the reaction. The buffer used in all cases was 25 mM sodium HEPES, pH/pD 7.5. The protein solution (5 µM final concentration in the reaction) was removed from the anoxic chamber, and additional H218O, H2O, D2O, and/or D218O were added as needed to
adjust the total deuterium content of the solution to the target value (0-98%) while maintaining the $^{18}$O content of the reaction at 48.5%. Substrate (2-HEP or 2-[2-$^2$H$_2$]-HEP) was added (1 mM final concentration), and the solution (150 µL) was allowed to react under atmospheric conditions for 2 h at room temperature. The reaction was quenched by filtering with a 0.5 mL 30 kDa MWCO centrifuge filter and analyzed by LC-MRM as before.
Figure S1: Identification of the products generated by HEPD-E176A. a, $^{31}$P NMR spectroscopy was used to confirm that HEPD-E176A produced HMP (17.5 ppm) from HEP (20 ppm). Other phosphorus-containing products were not observed. b, Formate was produced by HEPD-E176A in the reaction with HEP, as revealed by single ion monitoring of the formyl-nitrophenylhydrazide adduct as previously described⁵ (black: wt HEPD; blue: HEPD-E176A; red: HEPD-E176A control without substrate).

Figure S2: Dependence of the activity of HEPD and HEPD-E176A on Fe(II) concentration. Apo wt HEPD (blue) or HEPD-E176A (red) was treated with varying equivalents of Fe(II) followed by assaying the activity of each protein (2 µM final concentration in the reaction) toward HEP by using an O₂ electrode. All subsequent experiments with HEPD-E176A were conducted with saturating concentrations of Fe(II).
Figure S3: Dependence of the rate of product formation by HEPD-E176A on substrate concentrations. 

a and b, Kinetic parameters were obtained by reacting HEPD-E176A with HEP (red) or D2-HEP (blue) at constant, saturating O2 concentration in two independent experiments. The data shown within each panel was obtained with an identical purified stock of HEPD-E176A. 

c and d, The \( K_m \) of O2 was determined by reacting HEPD-E176A with constant, saturating HEP (red) or D2-HEP (blue) and varying O2 concentration in two independent experiments. The data shown within each panel was obtained with an identical purified stock of HEPD-E176A. HEPD-E176A used in panels b and d was from the same purification, whereas protein used for panels a and c were from a different batch purified on a different day. The data indicate a small but reproducible KIE on \( k_{cat} \).
Figure S4. Determination of the Mössbauer parameters for the ternary HEPD-E176A•Fe(II)•D2-HEP reactant complex, Fe(IV)-oxo intermediate, and HEPD-E176A•Fe(II)•D2-HMP product complex. The [25 ms − 0 ms] and the [500 ms − 25 ms] difference spectra are shown as vertical bars. The blue, red and greens lines represent the contributions from the HEPD-E176A•Fe(II)•D2-HEP reactant complex, Fe(IV)-oxo intermediate, and HEPD-E176A•Fe(II)•D2-HMP product complex, respectively. The black lines are the simulation of the difference spectra. Importantly, this procedure unmasked the low-energy line of the Fe(IV) intermediate (see line at -0.13 mm/s pointing downward in the [25 ms−0 ms] and upward in the [500 ms−25 ms] difference spectra), allowed for accurate determination of the parameters of the intermediate, and removes ambiguity in assignment of the transient species as Fe(IV).
Figure S5: 4.2-K/variable-field Mössbauer spectra of the 25-ms FQ sample from the HEPD-E176A reaction with D₂-HEP shown in Figure 2. The magnetic fields were applied parallel to the γ-beam, and the field strengths are indicated in the figure. (Left panel) Experimental spectra of the 25-ms FQ sample are shown as vertical bars. Spectra of the (effectively t = 0) O₂-free control sample collected under the same conditions are scaled to 66% of total iron absorption and shown as solid black lines. The fractional contribution of the reactant complex was determined from the 53-mT spectrum. (Right panel) Experimental "reference" spectra of the Fe(IV) intermediate (vertical bars) were generated by subtracting the 66% contribution of the spectrum of the reactant complex from the spectrum of the 25-ms sample. The overlaid solid red lines are spin-Hamiltonian simulations with the parameters listed in Table S1.
Figure S6: 4.2-K/variable-field Mössbauer spectra of the 10 ms and 25 ms FQ samples from the HEPD-E176A reaction with D2-HEP shown in Figure 2. (Top) Spectra of the 10 ms FQ sample recorded in zero-field (vertical bars) and 53 mT (orange line). (Bottom) Spectra of the 25 ms FQ sample recorded in zero-field (vertical bars) and 53 mT (orange line). For both samples, the observed differences between the zero-field and 53-mT spectra can be attributed to the field-strength dependence of Fe(II) species. As a result of its unique ground-state electronic structure \([S = 2\) due to antiferromagnetic coupling between a high-spin \((S_l = 5/2)\) Fe(III) to an \(S_2 = 1/2\) O\(2^-\) radical and a small axial ZFS parameter \((D_{S=2})\) for the spin-coupled system due to small \(D_{Fe(III)}\), the Fe(III)-superoxocomplex is expected to display a quadrupole doublet in zero-field and noticeable broadening in an externally applied field of 53 mT.\(^7\text{-}^{10}\) The fact that, for early reaction time points (10 and 25 ms) the \([0 \text{ mT} – 53 \text{ mT}]\) difference spectrum does not show substantial changes that can be associated with a high-spin Fe(III) species argues against accumulation of a putative, C–H cleaving Fe(III)-superoxocomplex intermediate to a detectable level.
Figure S7: (A) 4.2-K/53-mT Mössbauer spectra from a time course of the reaction of wt HEPD with D$_2$-HEP. A solution of wt HEPD, Fe(II), and D$_2$-HEP was reacted with O$_2$ (generated \textit{in situ} by using the enzyme chlorite dismutase and chlorite) and quenched after 10, 25, 80, and 2,000 ms after mixing. Raw experimental spectra are shown as vertical bars. The red solid lines are simulations of the ferryl complex using the
parameters quoted in the text, scaled to 10% (25-ms spectrum) and 8% (80-ms spectrum) of the total intensity of the spectrum. **(B)** 4.2-K/53-mT Mössbauer spectrum of a sample prepared by mixing a solution of wt HEPD, Fe(II), and D2-HEP with an O2-saturated buffer and freeze-quenching 20 ms after mixing. **(C)** 4.2-K/53-mT Mössbauer spectrum of a sample of E176A-HEPD with HEP. A solution of the E176A-HEPD:Fe(II):HEP complex was reacted with an equal volume of an O2-saturated buffer solution for 10 ms at 4 °C. The resulting spectrum is shown as black vertical bars. A simulation of the Fe(IV)-oxo intermediate with parameters quoted in the main manuscript (red solid line, 10% of total intensity) is overlaid.
Figure S8: Extent of retention of oxygen from O$_2$ in HMP increases as the deuterium content of the solvent increases. All experiments were conducted with HEPD-E176A and D$_2$-HEP. The results from using the label in the gas (O$_2$, blue) agree well with those obtained with labeled water (red, H$_2^{18}$O and/or D$_2^{18}$O as appropriate, in which case $^{16}$O-HMP corresponds to the label arising from molecular O$_2$).
Table S1. Comparison of the Mössbauer parameters of high-spin Fe(IV)-oxo complexes from mononuclear nonheme-iron enzymes.

| Fe(IV)-oxo complex | \( \delta \) (mm/s) | \( \Delta E_Q \) (mm/s) | \( \eta \) | \( A/\gamma\beta_N \) (T) | \( D \) (cm\(^{-1}\)) | \( (E/D) \) | Ref. |
|-------------------|---------------------|---------------------|----------|---------------------|---------------------|---------------------|-----|
| Fe(IV) site of E176A | 0.22 | −0.69 | −0.8 | (−15.0, −16.0, n.d.) | 12.3 | 0.02 | This work |
| J of TauD | 0.31 | −0.88 | 0 | (−18.0, −18.0, n.d.) | 10.5 | 0.03 | \(^2,^{11}\) |
| J of P4H | 0.30 | −0.82 | −0.2 | (−18.0, −18.0, n.d.) | 15.5 | 0.02 | \(^{12}\) |
| Fe(IV) sites of CytC3 | 0.30 (\( \delta_1 \)) 0.22 (\( \delta_2 \)) | −1.09 (\( \Delta E_{Q1} \)) −0.70 (\( \Delta E_{Q2} \)) | 0 | (−18.0, −18.0, n.d.) | 8.1 | 0.02 | \(^{13}\) |
| Fe(IV) site of TyrH | 0.25 | −1.27 | −0.5 | (−18.0, −18.0, n.d.) | 12.5 | 0.05 | \(^{14}\) |
References

(1) Peck, S. C.; Cooke, H. A.; Cicchillo, R. M.; Malova, P.; Hammerschmidt, F.; Nair, S. K.; van der Donk, W. A. *Biochemistry* **2011**, *50*, 6598.

(2) Price, J. C.; Barr, E. W.; Tirupati, B.; Bollinger, J. M., Jr.; Krebs, C. *Biochemistry* **2003**, *42*, 7497.

(3) Dassama, L. M. K.; Yosca, T. H.; Conner, D. A.; Lee, M. H.; Blanc, B.; Streit, B. R.; Green, M. T.; DuBois, J. L.; Krebs, C.; Bollinger, J. M., Jr. *Biochemistry* **2012**, *51*, 1607.

(4) Cicchillo, R. M.; Zhang, H.; Blodgett, J. A. V.; Whitteck, J. T.; Li, G.; Nair, S. K.; van der Donk, W. A.; Metcalf, W. W. *Nature* **2009**, *459*, 871.

(5) Whitteck, J. T.; Malova, P.; Peck, S. C.; Cicchillo, R. M.; Hammerschmidt, F.; van der Donk, W. A. *J. Am. Chem. Soc.* **2011**, *133*, 4236.

(6) Whitteck, J. T.; Cicchillo, R. M.; van der Donk, W. A. *J. Am. Chem. Soc.* **2009**, *131*, 16225.

(7) Mbughuni, M. M.; Chakrabarti, M.; Hayden, J. A.; Bominaar, E. L.; Hendrich, M. P.; Münck, E.; Lipscomb, J. D. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 16788.

(8) Tamanaha, E.; Zhang, B.; Guo, Y.; Chang, W. C.; Barr, E. W.; Xing, G.; St Clair, J.; Ye, S.; Neese, F.; Bollinger, J. M., Jr.; Krebs, C. *J. Am. Chem. Soc.* **2016**, *138*, 8862.

(9) Stout, H. D.; Kleespies, S. T.; Chiang, C. W.; Lee, W. Z.; Que, L., Jr.; Munck, E.; Bominaar, E. L. *Inorg. Chem.* **2016**, *55*, 5215.

(10) Chiang, C. W.; Kleespies, S. T.; Stout, H. D.; Meier, K. K.; Li, P. Y.; Bominaar, E. L.; Que, L., Jr.; Munck, E.; Lee, W. Z. *J. Am. Chem. Soc.* **2014**, *136*, 10846.
(11) Krebs, C.; Price, J. C.; Baldwin, J.; Saleh, L.; Green, M. T.; Bollinger, J. M., Jr.

*Inorg. Chem.* **2005**, *44*, 742.

(12) Hoffart, L. M.; Barr, E. W.; Guyer, R. B.; Bollinger, J. M., Jr.; Krebs, C.

*Proc. Natl. Acad. Sci. USA* **2006**, *103*, 14738.

(13) Galonić, D. P.; Barr, E. W.; Walsh, C. T.; Bollinger, J. M., Jr.; Krebs, C.

*Nat. Chem. Biol.* **2007**, *3*, 113.

(14) Eser, B. E.; Barr, E. W.; Frantom, P. A.; Saleh, L.; Bollinger, J. M., Jr.; Krebs, C.; Fitzpatrick, P. F.

*J. Am. Chem. Soc.* **2007**, *129*, 11334.