Supporting Information

for

Oxygen-18 Kinetic Isotope Effects of Non-heme Iron Enzymes HEPD and MPnS Reveal Iron(III) Superoxide as the Hydrogen Abstraction Species

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Experimental Procedures

HEPD[1] and MPnS[2] were expressed and purified as described previously. 2-HEP was purchased from Sigma-Aldrich and [2-$^2H_2$]-2-HEP was synthesized as described previously.[3]

^{18}O KIE measurements

^{18}O kinetic isotope effects (KIEs) were measured competitively as previously described.[4-7] The isotopic ratios of ^{18}O and ^{16}O were measured using isotopic ratio mass spectrometry (IRMS, Laboratory for Environmental and Sedimentary Geochemistry, Department of Earth and Planetary Science, University of California, Berkeley). ^{18}O KIEs were obtained by nonlinear curve fitting of Eq S1, where R is the $^{18}O/^{16}O$ isotopic ratio at fractional conversion F, and R_0 is the isotopic ratio at time 0, and errors on KIEs are reported as the standard error from the nonlinear curve fitting, except for that of HEPD on H_2-HEP, where the KIEs are calculated for each individual experiment and averaged, and the error is reported as the standard deviation of three measurements.

$$1 - F = (R/R_0)^{\frac{\mathrm{KIE}}{1-\mathrm{KIE}}} \quad \text{(S1)}$$

Reactions of HEPD and MPnS were carried out in 25 mM HEPES, pH 7.5 at 20 °C with 0.5 – 1 mM O_2 and 0.5 mM HEP. The enzymatic reactions were initiated with addition of 1 – 2 μM enzyme that was freshly reconstituted by addition of 1.2 eq of $Fe(NH_4)_2(SO_4)_2$. The fractional conversions used for the ^{18}O KIE measurements were between 10% and 60%.

Re-analysis of the relationship between rates for hydrogen atom transfer and oxygen KIEs in DβM implicates nuclear tunneling of oxygen

The close similarity of the magnitude and deuterium sensitivity of the oxygen KIEs for HEPD and MPnS to the properties of the copper-based enzyme DβM led us to reexamine previously reported trends in ^{18}O KIEs for DβM.[7] In the earlier study, it was possible to calculate rate constants for the hydrogen transfer from a series of substrates to a copper-activated oxygen complex and to relate these rate constants to the size of the experimental ^{18}O KIEs. A surprising feature is that the trends in ^{18}O KIEs are opposite to expectations when the origin of the KIE is restricted to semi-classical changes in force constants (see below and **Figure S4**). A similar dilemma arose in the glucose oxidase catalyzed reduction of molecular oxygen to superoxide anion by electron tunneling, necessitating a treatment of environmental reorganization of the oxygen motions quantum mechanically.[4] We now extend the treatment applied by Roth *et al*[4] to the nuclear tunneling of hydrogen from substrate to the activated oxygen of DβM.

Following on the work of Jortner, the rate of electron tunneling can be expressed by the following expression:[8,9] $k = (2\pi/\hbar)|V|^2\exp(F)$ (S2)

where $\hbar$ is reduced Planck's constant, $|V|^2$ is the electronic coupling term in the unit of energy, and $\exp(F)$ is the Frank-Condon factor. F is expressed by the following equation:

$$F = -\lambda_{out}(1 - y^2)/(4k_BT) - \Delta G^0(y + 1)/(2k_BT) - \lambda_{in}[\coth(2v) - \cosh(2vy)/\sinh(2v)]/(\hbar\omega) \quad \text{(S3)}$$

According to the above, λ_{in} and λ_{out} are inner- and outer-sphere reorganization energy, ΔG^0 is the driving force of the reaction, ω and v are the average frequency [$\omega = 2\omega_{react}\omega_{prod}/(\omega_{react} + \omega_{prod})$] and reduced frequency ($v = \hbar\omega/k_BT$) of the vibrational mode treated quantum mechanically, and y is the solution from the following equation:

$$\Delta G^0 = \lambda_{out}y + \lambda_{in}[\sinh(2vy)/\sinh(2v)] \quad \text{(S4)}$$

In this quantum mechanical treatment, λ_{in}, λ_{out} and ΔG^0 are considered isotopic insensitive. Therefore, the isotope effect on the rate of ET is solely from the change of ω, and consequently v and y.

$$^{18}k_{ET} = \exp[F(\lambda_{in}, \lambda_{in}, \Delta G^0, \omega_{16}) - F(\lambda_{in}, \lambda_{in}, \Delta G^0, \omega_{18})] \quad \text{(S5)}$$

According to the mechanism for DβM as well as HEPD and MPnS, the measured ^{18}O KIE [$^{18}(V/K)$] is the product of the equilibrium KIE for the formation of the H abstraction species and the KIE on the transfer step.

$$^{18}(V/K) = {}^{18}K{}^{18}k \qquad \text{(S6)}$$

where ^{18}K is 1.0054 from myoglobin.[6] The O-O stretching frequency of Cu(II)-$O_2^{\bullet-}$ species has been calculated as 1120 cm^{-1},[10] which is very close to the calculated O-O stretching frequency of Fe(III)-$O_2^{\bullet-}$ (1136 cm^{-1}).[5] When fitting the measured rate and KIE data for DβM, we thus used the averaged frequency of Fe(III)-$O_2^{\bullet-}$ (1136 cm^{-1}) and Fe(III)-OOH (844 cm^{-1}),[5] and the frequency terms for ^{16}O-^{16}O and ^{16}O-^{18}O are calculated as: ω_{16} = 968 cm^{-1}, and ω_{18} = 925 cm^{-1} . Using Eq (S5) and a reasonable set of values of λ_{in} = 5.3 kcal·mol^{-1}, λ_{out} = 19 kcal·mol^{-1}, $(2\pi/\hbar)|V|^2 = 10^8$ s^{-1}, the data for DβM are well fitted by the model (**Figure S4**).

As a comparison, we also present the trend that a semi-classical approach predicts. According to Eq 7 from Ref[4], the KIE from the change on the driving force obeys the following equation:

$$\ln\left(\frac{k_{16}}{k_{18}}\right) = \left(\frac{\Delta G^0_{18} - \Delta G^0_{16}}{2RT}\right)\left(1 + \frac{\Delta G^0_{18} + \Delta G^0_{16}}{2\lambda}\right) \qquad \text{(S7)}$$

From the definition of equilibrium isotope effect, we have:

$$\text{EIE} = K_{16}/K_{18} \qquad \text{(S8)}$$

We then relate the equilibrium constants to the driving forces:

$$\text{EIE} = K_{16}/K_{18} = \exp(\Delta G^0{}_{16}/RT) \,/\, \exp(\Delta G^0{}_{18}/RT) = \exp[(\Delta G^0{}_{16} - \Delta G^0{}_{18})/RT] \qquad \text{(S9)}$$

$$\ln(\text{EIE}) = (\Delta G^0{}_{16} - \Delta G^0{}_{18})/RT \qquad \text{(S10)}$$

Plugging Eq S10 into the first term of Eq S7 to the right of the equal mark:

$$\ln\left(\frac{k_{16}}{k_{18}}\right) = \left(\frac{\ln(\text{EIE})}{2}\right)\left(1 + \frac{\Delta G^0_{18} + \Delta G^0_{16}}{2\lambda}\right) \qquad \text{(S11)}$$

For illustrative purpose, we used the EIE value 1.034 of O_2 and HO_2^- equilibrium from Ref. 6. The trend is opposite to what is observed experimentally (**Figure S4**), and, as shown, the opposite trend is seen irrespective of the assigned value for EIE.

An alternative explanation for the magnitude of the KIE observed for DβM has been discussed in Ref. 10, arguing that the KIE results mainly from the equilibrium isotope effect (EIE) for the formation of the Cu(II)-$O_2^{\bullet-}$ species. While it is possible that the EIE for the formation of the Cu(II)-$O_2^{\bullet-}$ species is larger than the EIE for the formation of a Fe(III)-$O_2^{\bullet-}$ species, the EIE argument cannot explain the trend[7] where the KIE becomes smaller as the driving force becomes less favored. Further, the EIE argument cannot explain the magnitude of similar KIEs measured for the iron enzymes HEPD and MPnS, as discussed in the main text.

We conclude first, that while the quantum mechanical theory of Jortner[9] was derived for electron tunneling mechanisms, it is able to accommodate the hydrogen tunneling mechanism for DβM and second, that the environmental reorganization at oxygen is occurring predominantly via heavy atom tunneling.

Steady-state Michaelis-Menten kinetics with WT HEPD

HEPD (40 μM) was reconstituted anaerobically on ice with 1 eq $Fe(NH_4)_2(SO_4)_2 \bullet 6\ H_2O$ in buffer (50 mM HEPES pH 7.5). After 10 min, the protein solution was removed from the anaerobic glovebag. Buffer (50 mM HEPES pH 7.5) was sparged with N_2 from a house line or O_2 from an O_2 tank to adjust the dissolved O_2 concentration (10-500 μM). HEPD (1 μM final concentration) was reacted in this buffer with a saturating concentration of either 2-HEP or [2-$^2H_2$]-HEP (250 μM) and with varying initial O_2 concentrations (10-500 μM). The [O_2] in solution was monitored with a Clark-type O_2 electrode (Hansatech, Inc.), and the initial rate of O_2 consumption as a function of O_2 concentration was determined in triplicate for each O_2 concentration. Data were fit to the standard Michaelis-Menten curve. For 2-HEP oxidation, the kinetic parameters were k_{cat} = 0.27 ± 0.01 s^{-1} and $K_{m,O2}$ = 19 ± 4 μM. For [2-$^2H_2$]-2-HEP oxidation, the kinetic parameters were k_{cat} = 0.31 ± 0.02 s^{-1} and $K_{m,O2}$ = 110 ± 20 μM. As previously reported,[11] no KIE on k_{cat} due to deuteration of substrate was observed, but deuteration of substrate engendered a substantial KIE on $k_{cat}/K_{m,O2}$ of 4.4 ± 1.4.

Table S1. Summary of ^{18}O Kinetic Isotope Effects

HEPD				MPnS			
2-HEP		[2-$^2H_2$]-2-HEP		2-HEP		[2-$^2H_2$]-2-HEP	
1 - F	R/R_0	1 - F	R/R_0	1 - F	R/R_0	1 - F	R/R_0
0.664	1.00527	0.731	1.00948	0.438	1.01469	0.670	1.00799
0.664	1.00616	0.400	1.01844	0.435	1.01420	0.502	1.01525
0.645	1.00689	0.533	1.01380	0.438	1.01181	0.750	1.00720
KIE = 1.0147(15)		KIE = 1.0231(26)		0.425	1.01232	0.476	1.01161
				0.945	1.00169	0.481	1.01249
				0.884	1.00270	0.920	1.00106
				0.935	1.00101	0.505	1.00984
				0.694	1.00376	0.549	1.00877
				KIE = 1.0158(10)		0.793	1.00488
						0.752	1.00625
						0.785	1.00667
						KIE = 1.0189(13)	

Figure S1. Isotope fractionation plots for HEPD reactions on unlabeled 2-HEP (blue) and [2-$^{2}H_2$]-2-HEP (red). The R/R_0 is the ^{18}O enrichment compared to time 0 while the 1-F is the mole fraction of O_2 remaining in solution. Curves are fitted to $R/R_0 = (1\text{-}F)^{(1/KIE-1)}$.

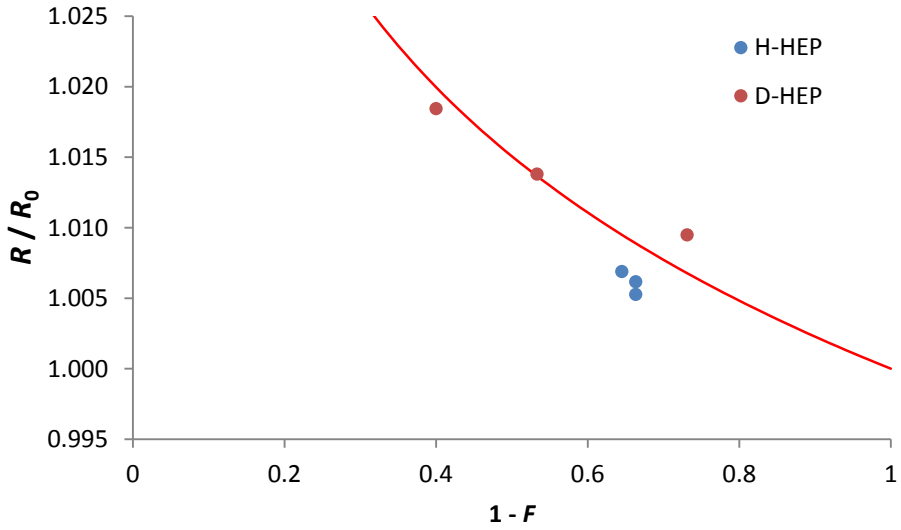


Figure S2. Isotope fractionation plots for MPnS reactions on unlabeled HEP (blue) and [2-$^{2}H_2$]-2-HEP (red). The R/R_0 is the ^{18}O enrichment compared to time 0 while the 1-F is the mole fraction of O_2 remaining in solution. Curves are fitted to $R/R_0 = (1\text{-}F)^{(1/KIE-1)}$.

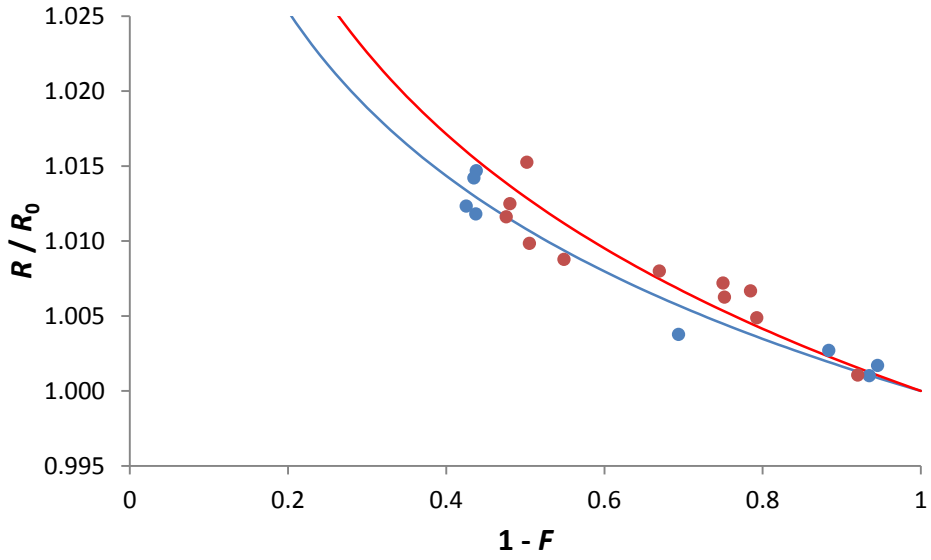


Figure S3. Michaelis-Menten plot of HEPD reacted with saturating concentrations of 2-HEP (blue) or [2-$^{2}H_2$]-2-HEP (red) (250 μM in each case) with varying initial O_2 concentrations (10-500 μM).

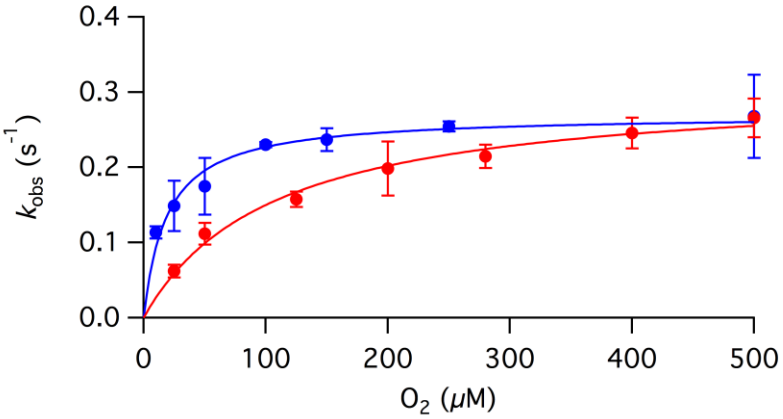
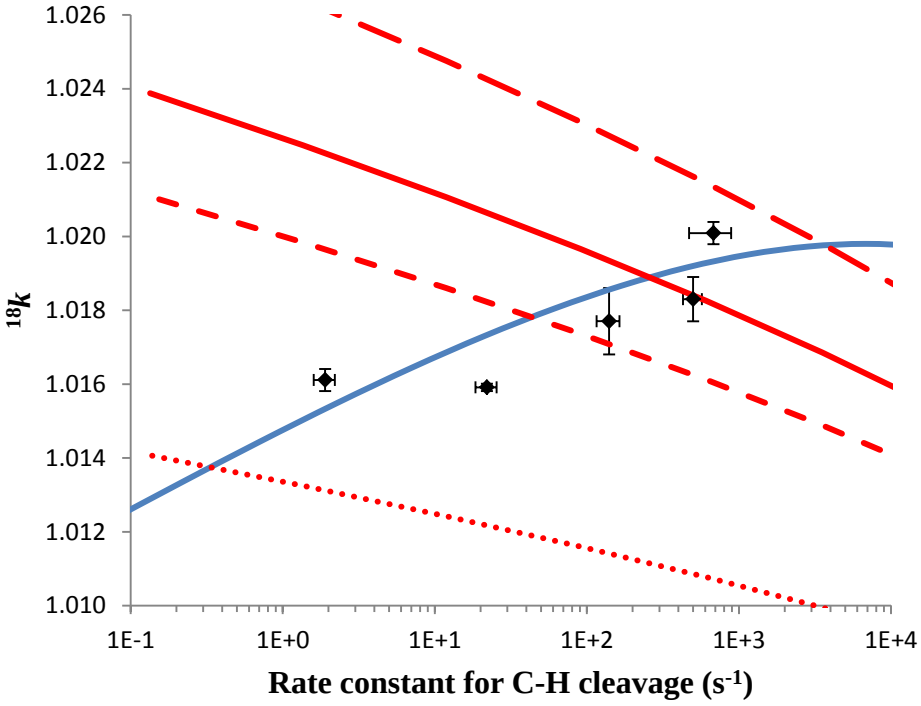


Figure S4. Analysis of the trend in ^{18}k for DβM[a] as a function of the rate constant for C-H cleavage according to Eq S5 (blue line),[b] compared to the same trend calculated by a semi-classical model (red lines).[c]



[a] The data of KIEs and rates come from Ref[7]. The ^{18}k is the $^{18}(V/K)_D$ measured in Ref[7] divided by the estimated ^{18}K (1.0054, Ref[6]) for the formation of metal-superoxide complex.

[b] These data as shown can be fit with the following set of reasonable values (λ_{in} = 5.3 kcal·mol^{-1}, λ_{out} = 19 kcal·mol^{-1}, $(2\pi/\hbar)|V|^2 = 10^8$ s^{-1}.

[c] Parameters used are: $\lambda = \lambda_{in} + \lambda_{out}$ = 24.3 kcal·mol^{-1}, $(2\pi/\hbar)|V|^2 = 10^8$ s^{-1}, and different EIE values: EIE = 1.034 (the formation of HO_2^- from O_2, Ref[6], solid red line), EIE = 1.02 (dot red line), EIE = 1.03 (short dash red line), and EIE = 1.04 (long dash red line).

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