Supporting Information

for

^{18}O Kinetic Isotope Effects in Non-Heme Iron Enzymes: Probing the Nature of Fe/O_2 Intermediates

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Protein Expression and Purification and Steady State Kinetics

General. All reagents and solvents were purchased from commercial sources and used without further purification unless otherwise indicated. (*S*)-2-Hydroxypropylphosphonic acid used in the studies of HppE was chemically synthesized according to a literature procedure.[1-3]

1-Aminocyclopropyl-1-carboxylic acid oxidase (ACCO). ACCO from *Lycopersicon esculentum* (ACO1) was produced in *E. coli* strain BL21(DE3)pLysS and purified by a two-column purification procedure as previously described.[4]

Initial velocities were measured by the rate of oxygen consumption at 25 °C, pH 7.2, using a Yellow Springs Instrument (YSI) biological oxygen monitor (model 5300) as previously described.[4] Temperature was maintained at 25 ± 0.1 °C with a Neslab circulating water bath. Standard reaction mixture (1 mL) contained 100 mM MOPS, pH 7.2, 100 mM NaCl, 20 mM $NaHCO_3$, 20 mM ascorbate and 1 mM ACC. Reactions were initiated with 2 μL of ACC oxidase reconstituted with equimolar $Fe(NH_4)_2(SO_4)_2$. Because of the loss of activity upon prolonged exposure to Fe(II) in the presence of oxygen,[5,6] ACCO was reconstituted in small aliquots and used within 30 min. Concentration of ACC oxidase is as indicated in figure legends. All initial rates were measured under conditions where less than 5% of any given substrate was consumed. All rates were calculated subtracting background oxygen consumption due to ascorbate and/or Fe(II) in the absence of enzyme. Data from initial velocity experiments with varying substrate concentrations were fitted to the Michaelis-Menten equation using the program Kaleidagraph. The kinetic parameters are reported with errors of ± 1σ.

Taurine α-ketoglutarate dioxygenase (TauD). TauD was cloned, overexpressed and purified as reported, with minor modifications.[7] The DNA template used to amplify the TauD gene was generated as follows. *E. coli* (JM109) genomic DNA was obtained using a Qiagen DNeasy kit and subsequently digested with BamHI and HpaI, generating a fragment of approximately 1400 bp containing the gene of interest. The restriction digest was subjected to agarose gel electrophoresis and fragments of between approximately 1200 and 1600 bp were excised from the gel, extracted with a Qiagen minelute gel extraction kit and used as template DNA. Protein purification was altered as follows: the cells were lysed using BugBuster with benzonase (Novagen) as per the manufacturer's instructions instead of using a French pressure cell, and the poly(ethyleneimine)

precipitation of DNA was omitted, as DNA was digested by the benzonase treatment.

Initial rates of oxygen uptake were measured with a YSI model 5300 biological oxygen electrode, and initial rates were seen to be linear for approximately 5 minutes. The fixed assay conditions were as follows: 50 mM Bis-Tris (pH 6.2), 100 μM $Fe(NH_4)_2(SO_4)_2$ and 100 μM sodium ascorbate in a volume of 1 mL at a temperature of 30 °C. αKG was varied from 2-1000 μM, taurine from 2-1000 μM and oxygen from 15-240 μM. TauD was present in concentrations between 0.1 and 1 μM. The apparent kinetic parameters k_{cat} and K_m were determined by fitting the kinetic data to the Michaelis-Menten equation using the program KaleidaGraph. The kinetic parameters determined for TauD purified and assayed by these methods do not differ substantively from those previously determined.[8,9]

S-(2)-Hydroxypropylphosphonic acid epoxidase (HppE). Recombinant HppE was overproduced from *E. coli* strain *BL21(DE3)/pLH01*. The metal-free HppE (apo-HppE) was purified according to the published procedure.[3]

Initial velocities were measured by the rate of oxygen consumption at 25 °C, pH 7.5, using a YSI biological oxygen monitor (model 5300) as previously described.[4] Standard reaction mixture (1 mL) contained 20 mM Tris-HCl buffer, pH 7.5, 1.5 mM S-HPP, 2.25 mM NADH, 27 μM FMN. Reactions were initiated with 3 μL of apo-HppE reconstituted with equimolar $Fe(NH_4)_2(SO_4)_2$, to give a HppE concentration of 16 μM. All initial rates were measured under conditions where less than 5% of any given substrate was consumed. All rates were calculated subtracting background oxygen consumption due to FMN, NADH and/or Fe(II) in the absence of enzyme.

^{18}O Kinetic Isotope Effects

^{18}O kinetic isotope effects (^{18}O KIEs) were measured competitively as described previously [10,11]. The $^{18}O/^{16}O$ ratios were measured using isotopic ratio mass spectrometry (Laboratory for Environmental and Sedimentary Geochemistry, Department of Earth and Planetary Science, UC Berkeley, CA). The ^{18}O KIEs were obtained by fitting the $^{18}O/^{16}O$ ratio of ratios versus fractional conversion according to eq (1), where R_f is the $^{18}O/^{16}O$ isotopic ratio at f fractional conversion and R_0 is the isotopic ratio prior to the enzymatic reaction. All KIEs are reported with errors of ± 1σ from the nonlinear regression fit to eq (1).

$$\frac{R_f}{R_0} = (1-f)^{1/(^{18}OKIE)-1} \qquad (1)$$

ACCO. Reactions with ACCO were carried out in 100 mM MOPS, pH 7.2, 20 mM $NaHCO_3$, 100 mM NaCl, 2 mM or 20 mM sodium ascorbate at 25 °C with 0.3–0.5 mM O_2 and 3 mM ACC. The enzymatic reactions were initiated with a pre-incubated mixture of ACCO:$Fe(NH_4)_2(SO_4)_2$ in a 1.75:1 ratio. This was done to minimize free iron in solution. Final concentrations were typically 3.5-8 μM ACCO and 2-4 μM Fe. The fractional conversions used for the ^{18}O KIE measurements were between 15% and 70%. The amount of O_2 consumed was corrected for the background O_2 consumption due to ascorbate and Fe in absence of enzyme. In all experiments the O_2 consumed in non-enzymatic reactions accounted for less than 10% of the total O_2 consumed. The ^{18}O KIE was also measured for the background oxygen consumption (at 1 μM Fe and 20 mM ascorbate), for comparison with the enzymatic reaction.

TauD. Reactions with TauD were carried out at 30 °C in 50 mM BisTris buffer, pH 6.2, 2 mM taurine, 2 mM αKG, and 0.4–0.6 mM O_2. Reactions were initiated by addition of ascorbate, $Fe(NH_4)_2(SO_4)_2$, and TauD to minimize the background oxygen consumption. Final concentrations were typically 200 μM ascorbate, 100 μM Fe and 0.2 μM TauD. The fractional conversions used for the ^{18}O KIE measurements were between 20% and 60%. The amount of O_2 consumed was corrected for the background O_2 consumption due to ascorbate and Fe in absence of enzyme. In all experiments the O_2 consumed in non-enzymatic reactions accounted for less than 2% of the total O_2 consumed. The ^{18}O KIE may be adjusted to 25 °C by employing the Arhenius equation, eq (2) and assuming E_a is temperature independent. However, the difference between the 30 °C and 25 °C values is within experimental error (1.0102 ± 0.0002 vs. 1.0104 ± 0.0002, respectively).

$$k = A * \exp^{-E_a/RT} \qquad (2)$$

HppE. Reactions with HppE were carried out in 20 mM Tris-HCl buffer, pH 7.5, 1 mM S-HPP, 1.5 mM NADH, and 0.4–0.6 mM O_2. Reactions were initiated with 3 μL of apo-HppE reconstituted with equimolar $Fe(NH_4)_2(SO_4)_2$, followed by addition of FMN to minimize the background oxygen consumption. Final concentrations were typically 8–12 μM HppE and 9–13 μM FMN. The fractional conversions used for the ^{18}O KIE measurements were between 20% and 60%. The amount of O_2 consumed was corrected for the background O_2 consumption due to FMN, NADH and/or Fe(II) in the absence of enzyme. In all experiments the O_2 consumed in non-enzymatic reactions accounted for less than 10% of the total O_2 consumed. The ^{18}O KIE was also measured for the background oxygen consumption (at 11 μM FMN and 1.5 mM NADH), for comparison with the enzymatic reaction.

Calculation of ^{18}O Equilibium Isotope Effects

The $^{18}O/^{16}O$ equilibrium isotope effects (^{18}O EIEs) for reactions with O_2 (such as eq 3) can be expressed as a product of three terms, contributed from the zero-point energies (ZPE), excited vibration states (EXC), and the mass and moments of inertia (MMI): ^{18}O EIE = ZPE x EXC x MMI.[12,13] All three terms are related to vibrational frequencies (ν) of ^{18}O– and ^{16}O–containing reactants (R ≡ O_2) and products (P), as shown in eqs (4)-(6), where the asterisk denotes the ^{18}O-containing reactants or products, T is temperature in K, *h* is Planck's constant. and *k* is Boltzmann's constant.[10] Experimentally determined frequencies for Fe^{III}-$OO^{\bullet-}$,[14] Fe^{III}-OOH,[15] Fe^{III}-$OO^{t}Bu$,[16,17] and Fe^{IV}=O species[18] were used for calculation of the ^{18}O EIE (Table S1).[18] For the asymmetric Fe^{III}-$O_2^{\bullet-}$, Fe^{III}-OOH, and Fe^{III}-$OO^{t}Bu$ species, the ^{18}O label can be at either the central or terminal position. The populations of the two isotopic products are expected to be close to each other, hence the ^{18}O EIE was calculated using the formula: $^{18}EIE_{calc} = 2/(^{18,16}K^{-1} + {}^{16,18}K^{-1})$.[10]

$$^{16,16}O_2 + \begin{matrix} Fe\text{–}^{18}O^{16}O(H) \\ \text{or} \\ Fe\text{–}^{16}O^{18}O(H) \end{matrix} \rightleftharpoons[\text{}^{18}O\text{ EIE}]{} {}^{18,16}O_2 + Fe\text{–}^{16}O^{16}O(H)$$

R **P*** **R*** **P** (3)

$$ZPE = \frac{\prod_j^{3N-6} \frac{\exp^{(h\nu_j^{P*}/2kT)}}{\exp^{(h\nu_j^{P}/2kT)}}}{\prod_j^{3N-5} \frac{\exp^{(h\nu_j^{R*}/2kT)}}{\exp^{(h\nu_j^{R}/2kT)}}} \quad (4)$$

$$EXC = \frac{\prod_j^{3N-6} \frac{1-\exp^{-(h\nu_j^{P*}/kT)}}{1-\exp^{(h\nu_j^{P}/kT)}}}{\prod_j^{3N-5} \frac{1-\exp^{(h\nu_j^{R*}/kT)}}{1-\exp^{(h\nu_j^{R}/kT)}}} \quad (5)$$

$$MMI = \frac{\prod_j^{3N-6} (\nu_j^{P}/\nu_j^{P*})}{\prod_j^{3N-5} (\nu_j^{R}/\nu_j^{R*})} \quad (6)$$

Table S1. Vibrational frequencies (cm^{-1}) of O_2, H_2O, and Fe/O_2 species.

molecule	Frequency (cm^{-1})				
	mode	$\nu_{16\text{-}16}$ [a]	$\nu_{18\text{-}18}$ [a]	$\nu_{18\text{-}16}$ [a]	$\nu_{16\text{-}18}$ [a]
O_2	O–O [b]	1556	1467	1512	1512
H_2O	H–O [b]	3824	- [c]	3824	-
	H–O [b]	3939	-	3922	-
	H-O-H [b]	1654	-	1644	-
Fe^{III}-$OO^{\bullet-}$	Fe–O [d]	555	526	526	555
	O–O [d]	1136	1066	1100 [e]	1100 [e]
Fe^{III}-OOH	Fe–O [f]	621	599	599	621
	O–O [f]	844	796	820 [e]	820 [e]
	O–H [b]	3539	-	3527	3539
	O-O-H [b]	1205	1199	1199	1204
Fe^{III}-$OO^{t}Bu$	Fe–O [g]	637	612	612	637
	O–O [g]	860	797	829 [e]	829 [e]
	O–^{t}Bu [h]	746	-	746	738
Fe^{IV}=O	Fe–O [i]	821	-	787	-

[a] Frequencies $\nu_{x\text{-}x}$ represent the modes for the species derived from $^{x,x}O_2$, the first label corresponding to the O atom closest to the metal center. [b] Ref 10. [c] Not applicable. [d] Ref 14. [e] $\nu_{18\text{-}16}$ was calculated as follows: $\nu_{18\text{-}16} = (\nu_{16\text{-}16}\,\nu_{18\text{-}18})^{½}$. [f] Ref 15. [g] Ref 16. [h] Ref 17. [i] Ref 18.

Table S2. Calculated ^{18}O EIEs using vibrational frequencies.

Reaction	ZPE	EXC	MMI	^{18}O EIE_{calc}	^{18}O EIE_{net} [a]
$Fe^{II} + O_2 \rightleftharpoons Fe^{III}\text{-}O_2^{\bullet-}$					
$Fe\text{-}^{18}O\text{-}^{16}O^{\bullet-}$	0.9493	0.9887	1.0590	0.9939	
$Fe\text{-}^{16}O\text{-}^{18}O^{\bullet-}$	1.0193	0.9994	1.0037	1.0224	**1.0080**
$Fe^{II} + O_2 \overset{e^-,\,H^+}{\rightleftharpoons} Fe^{III}\text{-}OOH$					
$Fe^{III}\text{-}^{18}O^{16}OH$	0.9947	0.9921	1.0370	1.0234	
$Fe^{III}\text{-}^{16}O^{18}OH$	1.0044	0.9979	1.0087	1.0110	**1.0172**
$Fe^{II} + O_2 \overset{e^-,\,{}^{t}Bu^+}{\rightleftharpoons} Fe^{III}\text{-}OO^{t}Bu$					
$Fe^{III}\text{-}^{18}O^{16}O^{t}Bu$	0.9710	0.9914	1.0494	1.0101	
$Fe^{III}\text{-}^{16}O^{18}O^{t}Bu$	1.0117	0.9964	1.0191	1.0273	**1.0187**
$Fe^{II} + O_2 \overset{2e^-,\,2H^+}{\rightleftharpoons} Fe^{IV}{=}O + H_2O$					
$Fe^{IV}{=}{}^{18}O,\ H_2{}^{16}O$	1.0239	0.9967	1.0138	1.0347	
$Fe^{IV}{=}{}^{16}O,\ H_2{}^{18}O$	1.0414	1.0001	0.9820	1.0228	**1.0287**

[a] The net ^{18}O EIE was calculated using the formula: $^{18}EIE_{net} = 2/(^{18,16}EIE^{-1} + {}^{16,18}EIE^{-1})$ (Ref 10).

References

(1) Liu, P. H.; Murakami, K.; Seki, T.; He, X. M.; Yeung, S. M.; Kuzuyama, T.; Seto, H.; Liu, H. W. *J. Am. Chem. Soc.* **2001**, *123*, 4619.

(2) Liu, P. H.; Liu, A. M.; Yan, F.; Wolfe, M. D.; Lipscomb, J. D.; Liu, H. W. *Biochemistry* **2003**, *42*, 11577.

(3) Munos, J.W.; Hong, L.; Mansoorabadi, S. O.; Yan, F.; Liu, A.; Liu, H.w., manuscript in preparation.

(4) Thrower, J. S.; Blalock, R.; Klinman, J. P. *Biochemistry* **2001**, *40*, 9717.

(5) Barlow, J. N.; Zhang, Z. H.; John, P.; Baldwin, J. E.; Schofield, C. J. *Biochemistry* **1997**, *36*, 3563.

(6) Zhang, Z. H.; Barlow, J. N.; Baldwin, J. E.; Schofield, C. J. *Biochemistry* **1997**, *36*, 15999.

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

(8) Eichhorn, E.; vanderPloeg, J. R.; Kertesz, M. A.; Leisinger, T. *J. Biol. Chem.* **1997**, *272*, 23031.

(9) Grzyska, P. K.; Ryle, M. J.; Monterosso, G. R.; Liu, J.; Ballou, D. P.; Hausinger, R. P. *Biochemistry* **2005**, *44*, 3845.

(10) Tian, G. C.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 8891.

(11) Tian, G. C.; Berry, J. A.; Klinman, J. P. *Biochemistry* **1994**, *33*, 226.

(12) Bigeleisen, J.; Mayer, M. G. *J. Chem. Phys.* **1947**, *15*, 261.

(13) Smirnov, V. V.; Brinkley, D. W.; Lanci, M. P.; Karlin, K. D.; Roth, J. P. *J. Mol. Catal. A: Chem.* **2006**, *251*, 100.

(14) Das, T. K.; Couture, M.; Ouellet, Y.; Guertin, M.; Rousseau, D. L. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 479.

(15) Costas, M.; Mehn, M. P.; Jensen, M. P.; Que, L., Jr. *Chem. Rev.* **2004**, *104*, 939.

(16) Lehnert, N.; Ho, R. Y. N.; Que, L.; Solomon, E. I. *J. Am. Chem. Soc.* **2001**, *123*, 12802.

(17) Lehnert, N.; Fujisawa, K.; Solomon, E. I. *Inorg. Chem.* **2003**, *42*, 469.

(18) Proshlyakov, D. A.; Henshaw, T. F.; Monterosso, G. R.; Ryle, M. J.; Hausinger, R. P. *J. Am. Chem. Soc.* **2004**, *126*, 1022.