

## $^{18}\text{O}$ Kinetic Isotope Effects in Non-Heme Iron Enzymes: Probing the Nature of Fe/O<sub>2</sub> Intermediates

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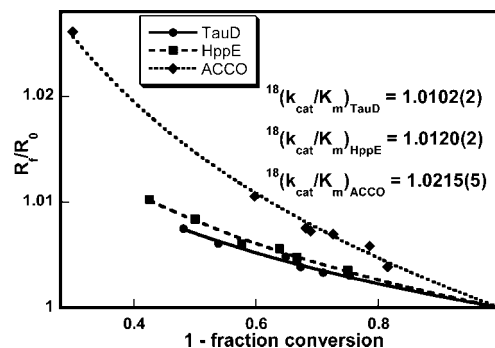
The O<sub>2</sub>-activating, non-heme iron enzymes catalyze a wide range of oxygenation and oxidation reactions with important biological implications, such as DNA repair, hypoxic response, collagen biosynthesis, and histone demethylation.<sup>1</sup> Most of these enzymes contain a single iron center coordinated by two His and one Asp/Glu residues in a tridentate binding motif referred to as “2-His-1-carboxylate facial triad”. Understanding the O<sub>2</sub>-activation processes for these enzymes may provide key insights into the basis of their divergent reactivities despite similarly coordinated active-site metal centers.<sup>1</sup>

Several recent studies have employed the measurement of competitive  $^{16}\text{O}/^{18}\text{O}$  kinetic isotope effects ( $^{18}\text{O}$  KIEs) on  $k_{\text{cat}}/K_{\text{m}}(\text{O}_2)$  for O<sub>2</sub>-activating enzymes in order to probe the early steps involved in O<sub>2</sub> activation up to and including the rate-determining step (RDS) of  $k_{\text{cat}}/K_{\text{m}}(\text{O}_2)$ .<sup>2,3</sup> Contrasted herein are the  $^{18}\text{O}$  KIEs for three non-heme iron enzymes that activate O<sub>2</sub> at an iron center coordinated by a 2-His-1-carboxylate facial triad: taurine dioxygenase (TauD), (*S*)-(2)-hydroxypropylphosphonic acid (S-HPP) epoxidase (HppE), and 1-aminocyclopropyl-1-carboxylic acid oxidase (ACCO). These  $^{18}\text{O}$  KIE measurements allow, for the first time, a direct comparison of the O<sub>2</sub>-activation processes by non-heme iron enzymes employing different substrates and co-reductants.<sup>1</sup>

TauD is an  $\alpha$ -ketoglutarate ( $\alpha$ KG)-dependent non-heme iron enzyme that catalyzes the hydroxylation of taurine in bacteria.<sup>4</sup> Its mechanism has been extensively investigated, and several Fe/O<sub>2</sub> intermediates have been characterized, including a high-valent Fe<sup>IV</sup>=O species.<sup>5–7</sup> The RDS of  $k_{\text{cat}}/K_{\text{m}}(\text{O}_2)$  in TauD is proposed to be either the binding of O<sub>2</sub> to the iron center, the attack of the formed Fe<sup>III</sup>-OO<sup>•-</sup> species on  $\alpha$ KG to form a cyclic peroxo-hemiketal intermediate, or the subsequent oxidative decarboxylation to form the Fe<sup>IV</sup>=O species.<sup>6</sup> The measured  $^{18}\text{O}$  KIE for TauD is  $1.0102 \pm 0.0002$  at 30 °C (Figure 1).<sup>8</sup>

HppE is a reductase-dependent non-heme iron enzyme that catalyzes the epoxidation of S-HPP, the last step in the biosynthesis of the antibiotic fosfomycin.<sup>9</sup> The mechanism of HppE is not as well-known as for TauD, with formation of an Fe<sup>III</sup>-OOH species being proposed to involve either a hydrogen atom transfer (HAT) from S-HPP or proton-coupled electron transfer (PCET) from the reductant.<sup>10</sup> The measured  $^{18}\text{O}$  KIE for HppE is  $1.0120 \pm 0.0002$  at 25 °C, using FMN in the presence of NADH as the reductant (Figure 1).<sup>8</sup>

ACCO is an ascorbate-dependent non-heme iron enzyme that catalyzes the last step in ethylene biosynthesis, an important plant hormone.<sup>11</sup> Recent steady-state kinetic studies of ACCO suggest



**Figure 1.** Isotope fractionation plots for TauD (●), HppE (■), and ACCO (◆). The fits for obtaining  $^{18}\text{O}$  KIEs are shown in solid, dashed, and dotted lines, respectively.<sup>8</sup> Conditions: TauD: 0.2  $\mu\text{M}$  TauD, 0.4–0.6 mM O<sub>2</sub>, 2 mM taurine, 2 mM  $\alpha$ KG, 0.2 mM ascorbate, 50 mM bis-Tris pH 6.2, 30 °C; HppE: 10  $\mu\text{M}$  HppE, 0.4–0.6 mM O<sub>2</sub>, 1 mM S-HPP, 11  $\mu\text{M}$  FMN, 1.5 mM NADH, 20 mM Tris-HCl pH 7.5, 25 °C; ACCO: 5  $\mu\text{M}$  ACCO, 0.3–0.5 mM O<sub>2</sub>, 3 mM ACC, 20 mM ascorbate, 20 mM NaHCO<sub>3</sub>, 100 mM NaCl, 100 mM MOPS pH 7.2, 25 °C.

that substrate oxidation occurs after the RDS of O<sub>2</sub> activation, which most probably involves the formation of an Fe<sup>IV</sup>=O species.<sup>12</sup> The  $^{18}\text{O}$  KIE for ACCO is  $1.0215 \pm 0.0005$  at 25 °C (Figure 1), one of the largest values measured for O<sub>2</sub>-activating metalloenzymes.<sup>13</sup>

Competitive  $^{18}\text{O}$  KIEs on  $k_{\text{cat}}/K_{\text{m}}(\text{O}_2)$  reflect changes in the oxygen bond order that occur in all steps from initial O<sub>2</sub> binding up to and including the first irreversible step.<sup>19</sup> To help interpret the measured  $^{18}\text{O}$  KIEs, calculated  $^{18}\text{O}$  equilibrium isotope effects ( $^{18}\text{O}$  EIEs, the product of the EIEs for all preequilibrium steps and the EIE corresponding to the first irreversible step) can be obtained from vibrational frequencies of reactants and products,<sup>14</sup> following the formalism developed by Bigeleisen and Mayer.<sup>20</sup> The calculated  $^{18}\text{O}$  EIEs for reactions involving no O–O bond cleavage can be used as upper limits for the measured  $^{18}\text{O}$  KIEs (entries 1–4, Table 1), assuming a negligible isotope effect contribution from the reaction coordinate frequency for such reactions.<sup>21</sup> Using known frequencies for Fe<sup>III</sup>-OO<sup>•-</sup>,<sup>15</sup> Fe<sup>III</sup>-OOH,<sup>1</sup> and Fe<sup>IV</sup>=O species,<sup>7</sup> the relevant Fe/O<sub>2</sub> intermediates for the studied enzymes,<sup>1,22</sup> we calculated  $^{18}\text{O}$  EIEs of 1.0080, 1.0172, and 1.0287, respectively (Table 1).<sup>8</sup> In addition,  $^{18}\text{O}$  EIEs of 1.0187 and 1.0129 were calculated for an Fe-alkylperoxy species, Fe<sup>III</sup>-OO'Bu,<sup>16,17</sup> and an Fe-peroxocarbonate species,<sup>18</sup> respectively, the latter being similar to a proposed intermediate in TauD.<sup>6</sup>

Interpretation of the measured  $^{18}\text{O}$  KIEs using the calculated  $^{18}\text{O}$  EIEs allows us to obtain information on the first irreversible step of O<sub>2</sub> activation. On the basis of stopped-flow data, Bollinger, Krebs, et al. have proposed a kinetic mechanism in which O<sub>2</sub> binding may be either irreversible or reversible, the latter model providing a better fit to the data.<sup>6</sup> In the latter model, the obtained

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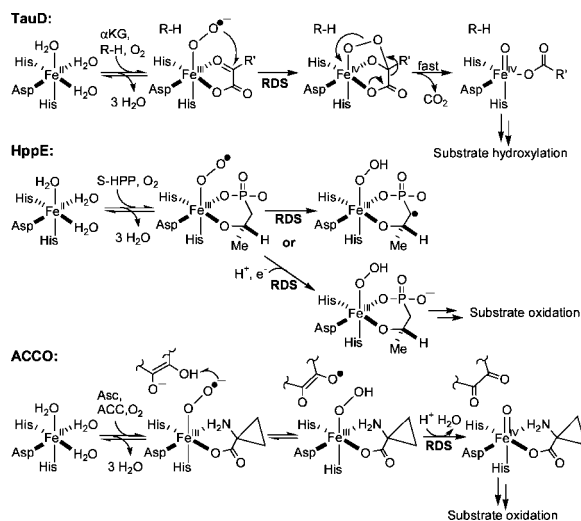
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**Table 1.** Vibrational Frequencies ( $\text{cm}^{-1}$ ) of Fe/O<sub>2</sub> Species, the Corresponding Calculated <sup>18</sup>O EIEs,<sup>a</sup> and Experimental <sup>18</sup>O EIEs and KIEs

Entry	Fe <sup>II</sup> + O <sub>2</sub> ⇌	Frequency ( $\text{cm}^{-1}$ )		<sup>18</sup> O EIE	<sup>18</sup> O EIE	<sup>18</sup> O KIE	
		mode	$\nu_{16-16}$	$\nu_{18-16}$	(calc) <sup>a</sup>	(expt) <sup>b</sup>	(expt)
1	Fe <sup>III</sup> -OO <sup>-</sup>	Fe-O <sup>c</sup>	555	526	1.0080 <sup>d</sup>	1.0054	ND <sup>e</sup>
		O-O <sup>c</sup>	1136	1100 <sup>f</sup>	1.0093 <sup>g</sup>	(Mb)	
2	Fe <sup>III</sup> -OOH	Fe-O <sup>h</sup>	621	599	1.0172 <sup>d</sup>	1.0113	1.0120 <sup>i</sup>
		O-O <sup>h</sup>	844	820 <sup>f</sup>	1.0137 <sup>k</sup>	(Hr)	(HppE)
		O-H <sup>j</sup>	3539	3527			
		O-O-H <sup>j</sup>	1205	1199			
3	Fe <sup>III</sup> -OO <sup>l</sup> Bu	Fe-O <sup>k</sup>	637	612	1.0187 <sup>d</sup>	ND	ND
		O-O <sup>k</sup>	860	829 <sup>f</sup>			
		O <sup>l</sup> Bu <sup>l</sup>	746	738			
4		Fe-O <sup>m</sup>	547	524	1.0129 <sup>d</sup>	ND	1.0102 <sup>i</sup>
		O-O <sup>m</sup>	884	862 <sup>f</sup>			(TauD)
		O-C <sup>m</sup>	965	946			
		O-C-O <sup>m</sup>	728	710			
5	Fe <sup>IV</sup> =O	Fe-O <sup>n</sup>	821	787	1.0287 <sup>d</sup>	ND	1.0215 <sup>o</sup>
						(ACCO)	

<sup>a</sup> The <sup>18</sup>O EIEs correspond to the formation of the shown species in equilibrium with Fe<sup>II</sup> and O<sub>2</sub>. <sup>b</sup> Measured <sup>18</sup>O EIEs for O<sub>2</sub>-binding proteins myoglobin (Mb) and hemerythrin (Hr) (ref 14). <sup>c</sup> Ref 15. <sup>d</sup> <sup>18</sup>O EIEs calculated using known frequencies and the Bigeleisen-Mayer equation (ref 8). <sup>e</sup> Not determined. <sup>f</sup>  $\nu_{18-16} = (\nu_{16-16} \nu_{18-18})^{1/2}$  (ref 8). <sup>g</sup> <sup>18</sup>O EIEs obtained using DFT-calculated frequencies (ref 8). <sup>h</sup> Ref 1. <sup>i</sup> This work. <sup>j</sup> Ref 14. <sup>k</sup> Ref 16. <sup>l</sup> Ref 17. <sup>m</sup> Ref 18. <sup>n</sup> Ref 7. <sup>o</sup> Ref 13.

**Scheme 1.** Proposed Mechanisms of O<sub>2</sub> Activation for TauD, HppE, and ACCO (RDS = Proposed Rate-Determining Step of O<sub>2</sub> Activation, R-H = Taurine, Asc = Ascorbate): The First Step Represents Several Reversible Substrate Binding Events



kinetic parameters support a small enough forward commitment such that the measured <sup>18</sup>O KIEs approximate intrinsic KIEs.<sup>8</sup> When compared to the calculated <sup>18</sup>O EIEs (Table 1), the TauD <sup>18</sup>O KIE (1.0102 ± 0.0002) is greater than the calculated <sup>18</sup>O EIE for an Fe<sup>III</sup>-OO<sup>-</sup> species formation (entry 1, Table 1) and the measured <sup>18</sup>O EIE for Mb (1.0054 ± 0.0006, Table 1),<sup>14</sup> but less than the calculated <sup>18</sup>O EIE for an Fe-peroxocarbonate species (1.0129). This observation, combined with the stopped-flow data, strongly suggests a rate-limiting formation of the peroxohemiketal intermediate (Scheme 1).<sup>8</sup>

The <sup>18</sup>O KIE of 1.0120 ± 0.0002 for HppE is similar to the measured <sup>18</sup>O EIE of 1.0113 ± 0.0005 for Hr<sup>14</sup> and less than the calculated <sup>18</sup>O EIEs for an Fe<sup>III</sup>-OOH species (entry 3, Table 1), pointing toward the formation of an Fe<sup>III</sup>-OOH species in the first irreversible step of O<sub>2</sub> activation. A partially rate-limiting O<sub>2</sub>-binding step in HppE may diminish, to a small extent, the measured <sup>18</sup>O KIE from its intrinsic value, as suggested by preliminary data.<sup>23</sup>

For ACCO, the large <sup>18</sup>O KIE of 1.0215 ± 0.0005 (less than the calculated <sup>18</sup>O EIE of 1.0287, Table 1, entry 5) implies a significant change in the oxygen bond order and points toward Fe<sup>IV</sup>=O species formation as the RDS of O<sub>2</sub> activation (Scheme 1).<sup>13</sup> In ACCO, the reduction of the Fe<sup>III</sup>-O<sub>2</sub><sup>-</sup> species to Fe<sup>III</sup>-OOH by ascorbate is proposed to be reversible, similar to the reversible O<sub>2</sub> binding observed in hemerythrin.<sup>14</sup> Interestingly, the initial inner-sphere activation of O<sub>2</sub> does not appear to be rate-limiting for any of these enzymes, in accordance with a recently proposed reversible O<sub>2</sub> binding as a requisite for the reactivity of non-heme iron enzymes.<sup>24</sup> This is in contrast to a rate-limiting and irreversible outer-sphere O<sub>2</sub> activation by glucose oxidase.<sup>2,3,25</sup>

In conclusion, measured <sup>18</sup>O KIEs for three O<sub>2</sub>-activating non-heme iron enzymes that use different reductants have been directly related to each enzyme's distinct chemical mechanism. For TauD, the <sup>18</sup>O KIE measurement provides direct evidence for the first irreversible step of O<sub>2</sub> activation, information that was not available from pre-steady-state studies. Similarly, a rate-limiting formation of an Fe<sup>III</sup>-OOH species is suggested for HppE. By contrast, in ACCO, the conclusion of formation of an Fe<sup>IV</sup>=O species in the first irreversible step provides a frame of reference for processes involving such an intermediate.<sup>13</sup> Overall, the calculated <sup>18</sup>O EIEs and measured <sup>18</sup>O KIEs reported herein provide unique insights into the inner-sphere mechanism of O<sub>2</sub> activation at a metal center.

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**Supporting Information Available:** Protein expression and purification procedures, <sup>18</sup>O KIE experimental details, <sup>18</sup>O EIE calculations, and mechanistic interpretation for TauD. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (1) Costas, M.; Mehn, M. P.; Jensen, M. P.; Que, L., Jr. *Chem. Rev.* **2004**, *104*, 939.
- (2) Roth, J. P.; Klinman, J. P. In *Isotope Effects in Chemistry and Biology*; Limbach, H.-H., Ed.; Marcel Dekker: New York, 2004; p 645.
- (3) Roth, J. P. *Curr. Opin. Chem. Biol.* **2007**, *11*, 142.
- (4) Eichhorn, E.; van der Ploeg, J. R.; Kertesz, M. A.; Leisinger, T. *J. Biol. Chem.* **1997**, *272*, 23031.
- (5) Price, J. C.; Barr, E. W.; Tirupati, B.; Bollinger, J. M.; Krebs, C. *Biochemistry* **2003**, *42*, 7497.
- (6) Price, J. C.; Barr, E. W.; Hoffart, L. M.; Krebs, C.; Bollinger, J. M. *Biochemistry* **2005**, *44*, 8138.
- (7) Proshlyakov, D. A.; Henshaw, T. F.; Monterosso, G. R.; Ryle, M. J.; Hausinger, R. P. *J. Am. Chem. Soc.* **2004**, *126*, 1022.
- (8) See Supporting Information.
- (9) 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.
- (10) Yan, F.; Munos, J. W.; Liu, P. H.; Liu, H. W. *Biochemistry* **2006**, *45*, 11473.
- (11) John, P. *Physiol. Plant.* **1997**, *100*, 583.
- (12) Thrower, J. S.; Mirica, L. M.; McCusker, K. P.; Klinman, J. P. *Biochemistry* **2006**, *45*, 13108.
- (13) Mirica, L. M.; Klinman, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 1814.
- (14) Tian, G. C.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 8891.
- (15) Das, T. K.; Couture, M.; Ouellet, Y.; Guertin, M.; Rousseau, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 479.
- (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) Hashimoto, K.; Nagatomo, S.; Fujinami, S.; Furutachi, H.; Ogo, S.; Suzuki, M.; Uehara, A.; Maeda, Y.; Watanabe, Y.; Kitagawa, T. *Angew. Chem., Int. Ed.* **2002**, *41*, 1202.
- (19) Tian, G. C.; Berry, J. A.; Klinman, J. P. *Biochemistry* **1994**, *33*, 14650.
- (20) Bigeleisen, J.; Mayer, M. G. *J. Chem. Phys.* **1947**, *15*, 261.
- (21) Lanci, M. P.; Brinkley, D. W.; Stone, K. L.; Smirnov, V. V.; Roth, J. P. *Angew. Chem., Int. Ed.* **2005**, *44*, 7273.
- (22) Burger, R. M.; Tian, G. C.; Drlica, K. *J. Am. Chem. Soc.* **1995**, *117*, 1167.
- (23) Detailed KIE mechanistic studies of HppE are underway.
- (24) Bollinger, J. M.; Krebs, C. *Curr. Opin. Chem. Biol.* **2007**, *11*, 151.
- (25) Klinman, J. P. *Acc. Chem. Res.* **2007**, *40*, 325.

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