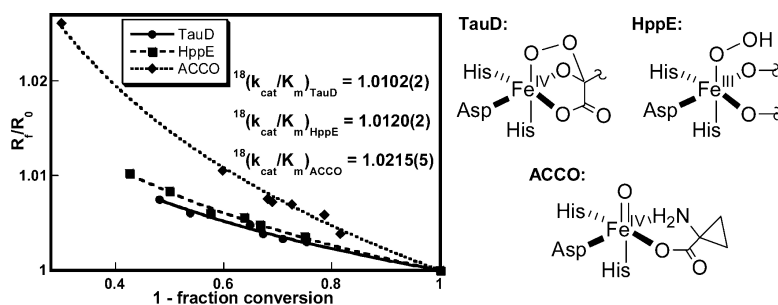## Communication

O Kinetic Isotope Effects in Non-Heme Iron Enzymes: Probing the Nature of Fe/O Intermediates

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^{18}O Kinetic Isotope Effects in Non-Heme Iron Enzymes: Probing the Nature of Fe/O$_2$ Intermediates

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The O$_2$-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.[1] 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$_2$-activation processes for these enzymes may provide key insights into the basis of their divergent reactivities despite similarly coordinated active-site metal centers.[1]

Several recent studies have employed the measurement of competitive $^{16}O/^{18}O$ kinetic isotope effects (^{18}O KIEs) on $k_{cat}/K_m(O_2)$ for O$_2$-activating enzymes in order to probe the early steps involved in O$_2$ activation up to and including the rate-determining step (RDS) of $k_{cat}/K_m(O_2)$.[2,3] Contrasted herein are the ^{18}O KIEs for three non-heme iron enzymes that activate O$_2$ 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}O KIE measurements allow, for the first time, a direct comparison of the O$_2$-activation processes by non-heme iron enzymes employing different substrates and co-reductants.[1]

TauD is an α-ketoglutarate (αKG)-dependent non-heme iron enzyme that catalyzes the hydroxylation of taurine in bacteria.[4] Its mechanism has been extensively investigated, and several Fe/O$_2$ intermediates have been characterized, including a high-valent Fe^{IV}=O species.[5–7] The RDS of $k_{cat}/K_m(O_2)$ in TauD is proposed to be either the binding of O$_2$ to the iron center, the attack of the formed Fe^{III}–OO$^{•-}$ species on αKG to form a cyclic peroxohemiketal intermediate, or the subsequent oxidative decarboxylation to form the Fe^{IV}=O species.[6] The measured ^{18}O KIE for TauD is 1.0102 ± 0.0002 at 30 °C (Figure 1).[8]

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.[9] The mechanism of HppE is not as well-known as for TauD, with formation of an Fe^{III}–OOH species being proposed to involve either a hydrogen atom transfer (HAT) from S-HPP or proton-coupled electron transfer (PCET) from the reductant.[10] The measured ^{18}O KIE for HppE is 1.0120 ± 0.0002 at 25 °C, using FMN in the presence of NADH as the reductant (Figure 1).[8]

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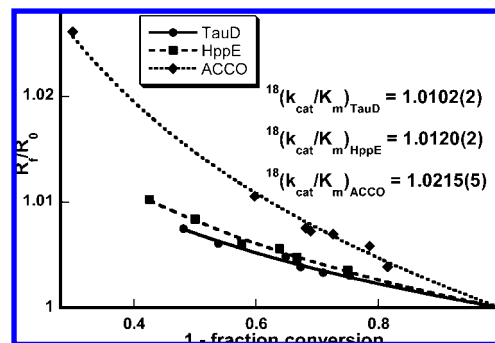

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Figure 1. Isotope fractionation plots for TauD (●), HppE (■), and ACCO (◆). The fits for obtaining ^{18}O KIEs are shown in solid, dashed, and dotted lines, respectively.[8] Conditions: TauD: 0.2 μM TauD, 0.4–0.6 mM O$_2$, 2 mM taurine, 2 mM αKG, 0.2 mM ascorbate, 50 mM bis-Tris pH 6.2, 30 °C; HppE: 10 μM HppE, 0.4–0.6 mM O$_2$, 1 mM S-HPP, 11 μM FMN, 1.5 mM NADH, 20 mM Tris-HCl pH 7.5, 25 °C; ACCO: 5 μM ACCO, 0.3–0.5 mM O$_2$, 3 mM ACC, 20 mM ascorbate, 20 mM NaHCO$_3$, 100 mM NaCl, 100 mM MOPS pH 7.2, 25 °C.

that substrate oxidation occurs after the RDS of O$_2$ activation, which most probably involves the formation of an Fe^{IV}=O species.[12] The ^{18}O KIE for ACCO is 1.0215 ± 0.0005 at 25 °C (Figure 1), one of the largest values measured for O$_2$-activating metalloenzymes.[13]

Competitive ^{18}O KIEs on $k_{cat}/K_m(O_2)$ reflect changes in the oxygen bond order that occur in all steps from initial O$_2$ binding up to and including the first irreversible step.[19] To help interpret the measured ^{18}O KIEs, calculated ^{18}O equilibrium isotope effects (^{18}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,[14] following the formalism developed by Bigeleisen and Mayer.[20] The calculated ^{18}O EIEs for reactions involving no O–O bond cleavage can be used as upper limits for the measured ^{18}O KIEs (entries 1–4, Table 1), assuming a negligible isotope effect contribution from the reaction coordinate frequency for such reactions.[21] Using known frequencies for Fe^{III}–OO$^{•-}$,[15] Fe^{III}–OOH,[1] and Fe^{IV}=O species,[7] the relevant Fe/O$_2$ intermediates for the studied enzymes,[1,22] we calculated ^{18}O EIEs of 1.0080, 1.0172, and 1.0287, respectively (Table 1).[8] In addition, ^{18}O EIEs of 1.0187 and 1.0129 were calculated for an Fe–alkylperoxo species, Fe^{III}–OO^{t}Bu,[16,17] and an Fe–peroxocarbonate species,[18] respectively, the latter being similar to a proposed intermediate in TauD.[6]

Interpretation of the measured ^{18}O KIEs using the calculated ^{18}O EIEs allows us to obtain information on the first irreversible step of O$_2$ activation. On the basis of stopped-flow data, Bollinger, Krebs, et al. have proposed a kinetic mechanism in which O$_2$ binding may be either irreversible or reversible, the latter model providing a better fit to the data.[6] In the latter model, the obtained

Table 1. Vibrational Frequencies (cm⁻¹) of Fe/O₂ Species, the Corresponding Calculated ^{18}O EIEs,[a] and Experimental ^{18}O EIEs and KIEs

Entry $Fe^{II} + O_2 \rightleftharpoons$	mode	Frequency (cm⁻¹) ν_{16-16}	ν_{18-18}	^{18}O EIE (calc)[a]	^{18}O EIE (expt)[b]	^{18}O KIE (expt)
1 Fe^{III}-OO$^{•-}$	Fe-O[c]	555	526	1.0080[d]	1.0054	ND[e]
	O-O[c]	1136	1100[f]	1.0093[g]	(Mb)	
2 Fe^{III}-OOH	Fe-O[h]	621	599	1.0172[d]	1.0113	1.0120[i]
	O-O[h]	844	820[f]	1.0137[g]	(Hr)	(HppE)
	O-H[j]	3539	3527			
	O-O-H[j]	1205	1199			
3 Fe^{III}-OO^{t}Bu	Fe-O[k]	637	612	1.0187[d]	ND	ND
	O-O[l]	860	829[f]			
	O-^{t}Bu[l]	746	738			
4 (O–O / Fe^{III}=O / O peroxocarbonate)	Fe-O[m]	547	524	1.0129[d]	ND	1.0102[i]
	O-O[m]	884	862[f]			(TauD)
	O-C[m]	965	946			
	O-C[m]	728	710			
5 Fe^{IV}=O	Fe-O[n]	821	787	1.0287[d]	ND	1.0215[o] (ACCO)

[a] The ^{18}O EIEs correspond to the formation of the shown species in equilibrium with Fe^{II} and O_2. [b] Measured ^{18}O EIEs for O_2-binding proteins myoglobin (Mb) and hemerythrin (Hr) (ref 14). [c] Ref 15. [d] ^{18}O EIEs calculated using known frequencies and the Bigeleisen-Mayer equation (ref 8). [e] Not determined. [f] $\nu_{18-16} = (\nu_{16-16} \, \nu_{18-18})^{1/2}$ (ref 8). [g] ^{18}O EIEs obtained using DFT-calculated frequencies (ref 8). [h] Ref 1. [i] This work. [j] Ref 14. [k] Ref 16. [l] Ref 17. [m] Ref 18. [n] Ref 7. [o] Ref 13.

Scheme 1. Proposed Mechanisms of O_2 Activation for TauD, HppE, and ACCO (RDS = Proposed Rate-Determining Step of O_2 Activation, R–H = Taurine, Asc = Ascorbate): The First Step Represents Several Reversible Substrate Binding Events
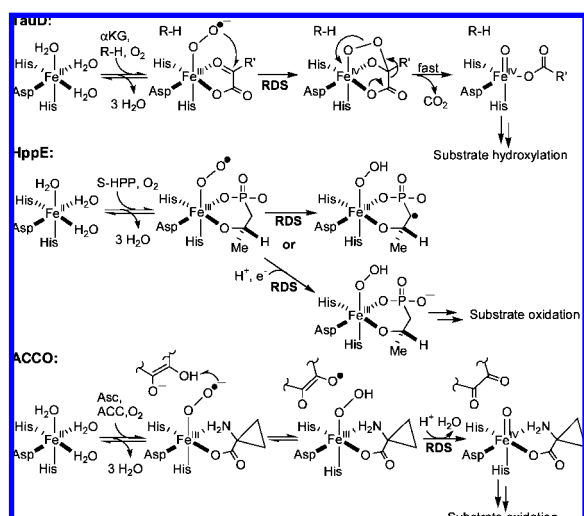
kinetic parameters support a small enough forward commitment such that the measured ^{18}O KIEs approximate intrinsic KIEs.[8] When compared to the calculated ^{18}O EIEs (Table 1), the TauD ^{18}O KIE (1.0102 ± 0.0002) is greater than the calculated ^{18}O EIE for an Fe^{III}–OO$^{•-}$ species formation (entry 1, Table 1) and the measured ^{18}O EIE for Mb (1.0054 ± 0.0006, Table 1),[14] but less than the calculated ^{18}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).[8]

The ^{18}O KIE of 1.0120 ± 0.0002 for HppE is similar to the measured ^{18}O EIE of 1.0113 ± 0.0005 for Hr[14] and less than the calculated ^{18}O EIEs for an Fe^{III}–OOH species (entry 3, Table 1), pointing toward the formation of an Fe^{III}–OOH species in the first irreversible step of O_2 activation. A partially rate-limiting O_2-binding step in HppE may diminish, to a small extent, the measured ^{18}O KIE from its intrinsic value, as suggested by preliminary data.[23]

For ACCO, the large ^{18}O KIE of 1.0215 ± 0.0005 (less than the calculated ^{18}O EIE of 1.0287, Table 1, entry 5) implies a significant change in the oxygen bond order and points toward Fe^{IV}=O species formation as the RDS of O_2 activation (Scheme 1).[13] In ACCO, the reduction of the Fe^{III}–O$_2^{•-}$ species to Fe^{III}–OOH by ascorbate is proposed to be reversible, similar to the reversible O_2 binding observed in hemerythrin.[14] Interestingly, the initial inner-sphere activaton of O_2 does not appear to be rate-limiting for any of these enzymes, in accordance with a recently proposed reversible O_2 binding as a requisite for the reactivity of non-heme iron enzymes.[24] This is in contrast to a rate-limiting and irreversible outer-sphere O_2 activation by glucose oxidase.[2,3,25]

In conclusion, measured ^{18}O KIEs for three O_2-activating non-heme iron enzymes that use different reductants have been directly related to each enzyme's distinct chemical mechanism. For TauD, the ^{18}O KIE measurement provides direct evidence for the first irreversible step of O_2 activation, information that was not available from pre-steady-state studies. Similarly, a rate-limiting formation of an Fe^{III}–OOH species is suggested for HppE. By contrast, in ACCO, the conclusion of formation of an Fe^{IV}=O species in the first irreversible step provides a frame of reference for processes involving such an intermediate.[13] Overall, the calculated ^{18}O EIEs and measured ^{18}O KIEs reported herein provide unique insights into the inner-sphere mechanism of O_2 activation at a metal center.

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

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