The nature of O\textsubscript{2} activation by the ethylene-forming enzyme 1-aminocyclopropane-1-carboxylic acid oxidase

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Ethylene is a plant hormone important in many aspects of plant growth and development such as germination, fruit ripening, and senescence. 1-Aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACCO), an O\textsubscript{2}-activating ascorbate-dependent nonheme iron enzyme, catalyzes the last step in ethylene biosynthesis. The O\textsubscript{2} activation process by ACCO was investigated using steady-state kinetics, solvent isotope effects (SIEs), and competitive oxygen kinetic isotope effects (\textsuperscript{18}O KIEs) to provide insights into the nature of the activated oxygen species formed at the active-site iron center and its dependence on ascorbic acid. The observed large \textsuperscript{18}O KIE of 1.0215 ± 0.0005 strongly supports a rate-determining step formation of an Fe\textsuperscript{V}=O species, which acts as the reactive intermediate in substrate oxidation. The large SIE on $k_{cat}/K_m(O_2)$ of 5.0 ± 0.9 suggests that formation of this Fe\textsuperscript{V}=O species is linked to a rate-limiting proton or hydrogen atom transfer step. Based on the observed decrease in SIE and \textsuperscript{18}O KIE values for ACCO at limiting ascorbate concentrations, ascorbate is proposed to bind in a random manner, depending on its concentration. We conclude that ascorbate is not essential for initial O\textsubscript{2} binding and activation but is required for rapid Fe\textsuperscript{V}=O formation under catalytic turnover. Similar studies can be performed for other nonheme iron enzymes, with the \textsuperscript{18}O KIEs providing a kinetic probe into the chemical nature of Fe/O\textsubscript{2} intermediates formed in the first irreversible step of the O\textsubscript{2} activation.

2 His, 1-Asp proteins | high-valent iron oxo species | oxygen-18 kinetic isotope effects | nonheme iron enzymes

Ethylene is a plant hormone important in many aspects of plant growth and development such as germination, fruit ripening, and senescence (1). The ability to control ethylene formation in a time-dependent manner would have far-reaching economic, agricultural, and environmental implications. The last step in the biosynthesis of ethylene, the two-electron oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, CO\textsubscript{2}, and HCN, is catalyzed by ACC oxidase (ACCO) (2–4). This reaction also requires the concomitant reduction of O\textsubscript{2} to water and the oxidation of ascorbate to dehydroascorbate (Fig. 1), whereas CO\textsubscript{2} (or bicarbonate) has been shown to act as an activator for ethylene formation (5).

ACCO belongs to the family of O\textsubscript{2}-activating nonheme iron enzymes. Although the sequence homology among these enzymes is not high, all their active sites contain a single ferrous ion bound in a tridentate ligand arrangement referred to as a “2-His-1-carboxylate facial triad” (4). A crystal structure of ACCO has been reported (6), revealing a solvent-exposed active site and confirming the ligation of the iron center (His-177, Asp-179, and His-234) inferred from mutagenesis studies (7), and the general jellyroll motif found in other nonheme iron enzymes (8, 9).

What distinguishes ACCO from the other enzymes of this family is the two-electron donating cosubstrate needed for reducing O\textsubscript{2} to water: ascorbate in the case of ACCO (10–13) and α-ketoglutarate (αKG) in almost all other known examples (4). The mechanism of αKG-dependent enzymes has been studied extensively, and it generally is accepted that O\textsubscript{2} activation at the iron center is linked to oxidative decarboxylation of the αKG, ultimately forming an Fe\textsuperscript{V}=O species as the reactive intermediate (14–17). This high-valent Fe/O\textsubscript{2} species functions as a “generic” oxidant for a wide range of oxidation/oxygenation reactions.

In contrast to the αKG-dependent enzymes, where αKG rather than substrate binds to the metal center, spectroscopic studies of ACCO have shown that ACC coordinates to the iron center via both its amino and carboxylate groups (10, 18). This finding implies a distinctly different mode for reductant interaction with ACCO than for most other nonheme iron enzymes. Magnetic circular dichroism (MCD) studies indicate that the active-site iron center is six-coordinate in the resting state Fe(II)-ACCO (19). On addition of ACC and ascorbate, the iron center becomes five-coordinate, allowing for O\textsubscript{2} binding and activation. These results are in line with steady-state kinetic analyses that suggest an ordered process where ACC binding to ACCO must precede O\textsubscript{2} binding, although it could not be distinguished whether ascorbate binds before ACC or after O\textsubscript{2} (12).

Single-turnover experiments showed that a substoichiometric amount of ethylene was formed in the absence of ascorbate, with the presence of either CO\textsubscript{2} or bicarbonate being essential for enzyme turnover (13). Although ascorbate was proposed to act mainly as a reductant for restoring the iron to the ferrous state, the rate of ethylene formation under single-turnover conditions was significantly lower than under steady-state conditions, suggesting that ascorbate is needed for O\textsubscript{2} activation under catalytic turnover (Fig. 2).

From these accumulated results, it is clear that the complexity of the ACCO chemistry presents a challenge for mechanistic enzymologists and requires further examination. In this study, steady-state kinetics, solvent isotope effects (SIEs), and competitive oxygen kinetic isotope effects (\textsuperscript{18}O KIEs) are used to provide insights into the rate-limiting steps contributing to the interaction of O\textsubscript{2} with ACCO, the nature of the Fe/O\textsubscript{2} intermediates, and the role of ascorbate in O\textsubscript{2} activation. Of particular interest is the investigation of the Fe\textsuperscript{V}=O species formation and its proposed role in substrate oxidation (20).

Results

SIEs on Steady-State Parameters. The kinetic parameters for ACCO have been measured by monitoring the amount of O\textsubscript{2} consumed by using a Clark electrode. The obtained kinetic...
parameters $k_{\text{cat}} = 36.7 \pm 1.1 \text{ min}^{-1}$, $K_m(\text{ACC}) = 92 \pm 10 \mu \text{M}$, $K_m(\text{Asc}) = 5.5 \pm 0.7 \text{ mM}$, $K_m(O_2) = 67 \pm 6 \mu \text{M}$ are similar to our previous results (12). Because both ACC and ascorbate have solvent-exchangeable protons, the only deuterium isotope effects that can be measured are kinetic SIEs. Comparison of the kinetic parameters measured in both H2O and D2O revealed the presence of SIEs (Table 1). The measured values for $D^{2}O k_{\text{cat}}, D^{2}O K_m(\text{ACC})$, and $D^{2}O k_{\text{cat}}/K_m(\text{Asc})$ are $\sim 2.3$, identical within experimental error, suggesting changes in hydrogen bonding between the reactants and the transition state. None of the kinetic parameters has been found to vary between pH 6 and 8 (data not shown), indicating the lack of a pK$_a$ effect as the origin of the SIEs. Interestingly, the $D^{2}O k_{\text{cat}}/K_m(O_2)$ value is $5.0 \pm 0.9$ (Table 1 and Fig. 3), an uncommonly large value for a SIE. Such a value suggests that the rate-determining step in O2 activation involves either a proton-coupled electron transfer (PCET) or hydrogen atom transfer (HAT), as discussed below.

18O KIE. The $18O$ KIE on $k_{\text{cat}}/K_m(O_2)$, $18O K_m(O_2)$, was measured for ACCO to determine the extent of change in oxygen bond order in the steps leading up to the first irreversible step in O2 activation. Competitive 18O KIEs were determined from the fractionation of oxygen isotopes, i.e., the change in the $18O/16O$ ratio during the consumption of O2 catalyzed by ACCO (21). The fractional conversions used for these measurements were between 15% and 70%. The isotope fractionation plot $18O$ isotopic ratios of ratios versus fractional conversion) for ACCO is shown in Fig. 4. The 18O KIEs were obtained by fitting the data to Eq. 1, where $R_f$ is the $18O/16O$ isotopic ratio at $f$ fractional conversion and $R_0$ is the isotopic ratio before the enzymatic reaction.

$$R_f = \frac{R_0}{(1-f)^{18O\text{ KIE} - 1}}$$

The data are well fitted by Eq. 1 to give an 18O KIE value of $1.0215 \pm 0.0005$. This observed 18O KIE is among the largest for O2-activating metalloenzymes (22), suggesting a significant change in the oxygen bond order, attributable to formation of a high-valent Fe$^{IV}=O$ species in the first irreversible step (see below). The measured 18O KIE for the much slower background O2 consumption caused by ascorbate and iron in the absence of enzyme is $1.0111 \pm 0.0002$ (data not shown), excluding an artificial inflation of the ACCO 18O KIE value from the non-enzymatic reactions.

SIE on 18O KIE. To test the role of protons during O2 activation, the 18O KIE also was measured in D2O. Because of lower activity of ACCO in D2O, data points for the ACCO reaction were collected only for fractional conversions between 10% and 30%. The measured 18O KIE for ACCO in D2O is $1.0209 \pm 0.0004$ (Fig. 4A). This value is within experimental error of the 18O KIE in H2O. The lack of a SIE on the 18O KIE of ACCO indicates that solvent deuteration does not impact the relative activation barriers of the steps leading up to and including the first irreversible step of $k_{\text{cat}}/K_m(O_2)$.

Ascorbate Effect on SIE and 18O KIE. Ascorbate is an essential substrate for ACCO and acts as a reductant to fully reduce O2 to water. Its role in ACCO activity was tested by measuring the kinetic parameters at lower ascorbate concentration (2 mM, 35% of its $K_m$ value). Because of lower activity of ACCO at this ascorbate concentration, data points were collected only for fractional conversions between 10% and 30%. Measurement of the 18O KIE for ACCO under these conditions reveals a value of 1.0157 $\pm 0.0004$ (Fig. 4B), lower than the value obtained under saturating ascorbate concentration (1.0215 $\pm 0.0005$).

In addition, the SIEs measured at 2 mM ascorbate show that the apparent $D^{2}O k_{\text{cat}}/K_m(O_2)$ value decreases to 2.7 $\pm 0.7$, within experimental error of the SIEs of $\sim 2.3$ for the other kinetic parameters that do not change with ascorbate concentration (Table 1). The decrease in both 18O KIE and $D^{2}O k_{\text{cat}}/K_m(O_2)$ values at lower ascorbate concentrations is not expected for an ordered terterreactant enzymatic reaction, suggesting a possible change in the order of ascorbate binding to ACCO that has an effect on the O2 activation steps (see below).

Discussion

The gene for the tomato ACCO has been expressed recently in E. coli and purified to homogeneity to yield a protein with approximately 10 times greater activity than previously reported (12). Some of the initial studies undertaken with this enzyme have included the kinetic order for binding of all substrates to enzyme, the relationship of O2 activation to substrate activation, and the chemical steps that lead from ACC to its products. Steady-state kinetic studies indicated a sequential mechanism involving a quaternary complex (ACCO, ACC, O2, and ascorbate) and an equilibrium-ordered pattern with ACC binding before O2 but were unable to distinguish whether ascorbate binds before ACC or after O2 (12).

Activation of ACC by ACCO is likely to occur by one-electron oxidation or hydrogen atom abstraction from the amine group to form an amine radical cation or aminyl radical, respectively (23). Rapid radical rearrangement results in cleavage of the cyclopropane ring and eventually formation of ethylene, HCN, and CO2. In a recent study, the behavior of three cyclic and three acyclic substrate analogs was analyzed in regard to turnover rates, product distribution, and O2 uncoupling (20). Although these analogs have different structures, the turnover rates ($k_{\text{cat}}$) are within a factor of four of ACC, suggesting that the rate-determining step occurs before substrate oxidation (20). It also was proposed that the first committed step involved the formation of an Fe$^{IV}=O$ species, which acts as the oxidant in substrate activation. An Fe$^{IV}=O$ species had been implicated previously as the oxidant that activates the substrate in the αKG-dependent enzymes (14–16). However, as noted ACCO uses ascorbate as the reductant, which can serve as a one- or two-electron donor;
Lipscomb, Que, and colleagues suggested that ACC oxidation may involve either direct HAT to an FeIII—OOH or prior O—O bond heterolysis to generate FeV=O as the oxidizing species (13). In their examples, an FeIV=O species would be formed concomitant with substrate activation. Although multiple species (FeIII=OOH, FeV=O, or FeV=O) have been discussed as possible oxidants in the context of the ACCO mechanism (13, 20), direct evidence for the catalytic intermediate has been lacking.

SIE. The kinetic parameters for ACCO measured in both H2O and D2O show the presence of SIEs. D2O/kcat, D2O/kcat/Km(ACC), and D2O/kcat/Km(Asc) are identical within experimental error, with values ≈2.3 (Table 1). These SIEs are suggestive of proton transfers occurring along hydrogen bonds involved in general acid/base catalytic stabilization of the transition state (24). However, given the large number of protons in the active site that can exchange with solvent (water molecules coordinated to the iron center, amine protons of ACC, acidic protons of ascorbate, bicarbonate), these SIEs may represent the product of multiple effects and are referred to as background SIEs. A pKa origin of the SIE can be eliminated because all kinetic parameters are found to be invariant between pH 6 and 8. Interestingly, D2O/kcat/Km(O2) is 5.0 ± 0.9 (Table 1 and Fig. 3). Such a large SIE indicates that either a PCET or HAT from a solvent-exchangeable site occurs in the rate-limiting step of kcat/Km(O2) (24). Possible proton sources for a PCET process may include acidic side-chain residues, ascorbate, or bicarbonate, whereas a hydrogen atom may be removed from ascorbate or the ACC amine group.

18O KIE. In recent years, a number of studies have addressed the early steps in O2 activation for a series of enzymes, with measurement of competitive 18O KIEs on kcat/Km(O2) being an important factor in determining the nature of the first commit-

Table 1. Kinetic parameters for ACCO in H2O and D2O

| Parameter                      | H2O          | D2O          | SIE          | 2 mM ascorbate† |
|--------------------------------|--------------|--------------|--------------|----------------|
| kcat, min−1                 | 3.67 ± 1.1   | 17.5 ± 1.2   | 2.1 ± 0.2    | 13.4 ± 0.9§    |
| kcat/Km(O2), μM−1 min−1     | 0.547 ± 0.06 | 0.109 ± 0.017| 5.0 ± 0.9   | 0.114 ± 0.018§ |
| kcat/Km(Asc), μM−1 min−1    | 0.280 ± 0.045| 0.117 ± 0.015| 2.4 ± 0.5   | 0.069 ± 0.011§ |
| kcat/Km(Asc), mM−1 min−1    | 5.68 ± 0.60  | 2.32 ± 0.49  | 2.4 ± 0.6   | N/A            |

* Saturating conditions: 1 mM ACC, 258 μM O2, 20 mM ascorbate, and 20 mM NaHCO3, 25°C, pH 7.2. †Varied ACC concentration and 258 μM O2, 20 mM ascorbate, and 20 mM NaHCO3. ‡Varied O2 concentration and 1 mM ACC, 258 μM ascorbate, and 20 mM NaHCO3. §Apparent kcat, kcat/Km(O2), and kcat/Km(Asc).

Fig. 3. Michaelis–Menten plot for ACCO with O2 as varied substrate, in H2O (●) and D2O (●). Conditions: 0.5 μM ACCO, 1 mM ACC, 20 mM ascorbate, and 20 mM NaHCO3, 25°C, pH 7.2. The solid and dashed lines are fits to the Michaelis–Menten equation.
The Role of Ascorbate in O₂ Activation. Given the ambiguous role of ascorbate during O₂ activation, the impact of ascorbate was investigated by measuring the ¹⁸O KIE at a lower ascorbate concentration (2 mM), below its Kₘ value. Assuming an ordered terreactant mechanism, if ascorbate binds first, followed by ACC, and O₂ binds last, then k_cat/Kₘ(O₂) and consequently the ¹⁸O KIE will be independent of ascorbate concentration (33). If ascorbate binds last, after O₂, then at higher ascorbate concentration, the reaction will be fully committed, because high ascorbate concentration prevents the release of O₂ and its equilibration with excess O₂. A large forward commitment factor translates into an observed ¹⁸O KIE of 1.0 (33), and lowering the ascorbate concentration will increase the measured ¹⁸O KIE.

In contrast to the predicted effect, the measurements reveal that the ¹⁸O KIE decreases from 1.0215 ± 0.0005 at saturating ascorbate concentration (20 mM) to 1.0157 ± 0.0004 at low ascorbate (2 mM, Fig. 4B), which suggests that another step becomes rate-limiting such that a smaller change in the oxygen bond order has been measured. Additionally, D₂O shows that ¹⁸O KIE (27) decreases from 5.0 ± 0.9 (at 20 mM ascorbate) to 2 ± 0.7 (at 2 mM ascorbate), a value within experimental error of the background SIEs for the other kinetic parameters. The reduction of both ¹⁸O KIE and D₂O k_cat/Kₘ(O₂) is attributed to a partially rate-determining binding of ascorbate at the reduced ascorbate concentrations. In an earlier study (13), the possibility of an effector site for ascorbate was postulated. In light of the proposal of very tight binding of ascorbate to such a site (13), this explanation does not appear relevant to the conditions of the present experiments (2 and 20 mM ascorbate). Further, since the kinetic properties of ACCO can accommodate the available data, there is no need to invoke the presence of an additional ascorbate binding site.

Proposed Mechanism for O₂ Activation. Considering all experimental observations, a mechanism for O₂ activation by ACCO is proposed (Fig. 5). Our working model is that, although an ordered mechanism is still in place for substrate and O₂, ascorbate interaction with enzyme occurs in a random manner, depending on its concentration. At high, saturating concentrations, ascorbate is proposed to bind first, followed by ACC and O₂. For such a kinetic mechanism, the fully rate-limiting step for k_cat/Kₘ(O₂) is proposed to be the formation of an Fe^{IV}=O species in a process that involves a PCET, consistent with the large ¹⁸O KIE and D₂O k_cat/Kₘ(O₂). The oxidation of the Fe^{III}=O₂ species to Fe^{IV}=OOH by ascorbate is proposed to be reversible, similar to the reversible O₂ binding observed in hemerythrin (21). When the ascorbate concentration is below its Kₘ value, ascorbate is proposed to bind last, subsequent to O₂, with the actual binding being partially rate-limiting. Because ascorbate binding most probably does not involve a change in the oxygen bond order or a direct proton transfer, a reduction in both ¹⁸O KIE and SIE values is expected, as observed experimentally. These findings suggest
that, unlike substrate, which directly coordinates to the iron center, binding of ascorbate at the active site of ACCO is not an essential step for the conversion of the metal center from six- to five-coordinate and initial reduction of O₂ to form the Fe^{III}–O₂ species. Although it is possible that product release or the reduction of Fe(III) to Fe(II) may be rate-limiting under certain conditions (13), the rate-limiting step on $k_{cat}/K_m (O_2)$ is unlikely to depend on these steps. Because the ascorbate concentration has a pronounced effect on $^{18}$O KIE and on $D_{O2} k_{cat}/K_m (O_2)$, it becomes clear that under catalytic conditions high ascorbate concentrations facilitate the rapid formation of the active oxidant.

In summary, several insights have been obtained about the mechanism of O₂ activation by the nonheme iron enzyme ACCO. SIEs and competitive $^{18}$O KIEs reveal a detailed picture of the Fe/O₂ intermediates formed during O₂ activation. The presence of a large $D_{O2} k_{cat}/K_m (O_2)$ suggests that O₂ activation involves a PCET (or HAT), although the source of proton cannot be unambiguously determined. The elevated $^{18}$O KIE value for ACCO, which is independent of solvent deuteration, strongly supports the formation of an Fe^{IV}═O intermediate in the rate-determining step of O₂ activation; this species thus is implicated as the oxidant responsible for subsequent substrate activation. Based on the data herein, the slow single-turnover reaction reported for ACCO in the absence of ascorbate (13) is attributed to an alternate, non-catalytic pathway involving electron transfer from another molecule of ACCO. These studies make ACCO a unique system, providing kinetic as opposed to the more conventional spectroscopic evidence (14–17) for the presence of an Fe^{IV}═O species. It is important to mention that, to our knowledge, $^{18}$O KIE measurements have not been performed before for an O₂-activating, nonheme iron enzyme. Although $^{18}$O KIEs values were reported for the pterin-dependent tyrosine hydroxylase, in that case, the initial O₂ activation is proposed to occur at the pterin cofactor and not at the iron center (34). Additionally, the role of ascorbate on O₂ activation has been investigated, leading to a kinetic model in which ascorbate binds to enzyme in a random manner. Thus, although ascorbate is required for rapid Fe^{IV}═O formation under catalytic turnover, the presence of this reductant is not required for initial O₂ binding (13).

Several recent studies have used $^{18}$O KIEs to probe the steps involved in O₂ reduction and to reveal the nature of the metal/O₂ intermediate formed in the first irreversible step of enzymatic reactions (22, 25). This technique emerges as a valuable companion to pre-steady-state kinetic analyses, which investigate the nature of the intermediate immediately preceding the rate-determining step. We are presently employing stopped-flow methods to monitor the intermediate that precedes the formation of the postulated Fe^{IV}═O species in ACCO. From this study, the observed $^{18}$O KIE is available as a standard for other enzymatic or model systems proposed to involve similar Fe/O₂ intermediates. The results reported herein open up the opportunity for similar measurements on a range of αKG-dependent and other nonheme iron enzymes.

Materials and Methods

General. All reagents were purchased from commercial sources and used without further purification unless otherwise indicated.

Overexpression and Purification of ACCO. ACCO from Lycopersicon esculentum (ACO1) was produced in Escherichia coli strain BL21 (DE3) pLysS and purified by a two-column purification procedure as previously described (12).

Steady-State Kinetics. Initial velocities were measured by the rate of oxygen consumption at 25°C, pH 7.2, using a Yellow Springs Instrument biological oxygen monitor (model 5300) as previously described (12). 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, 20 mM NaHCO₃, 100 mM NaCl, and various amounts of ascorbate and ACC. When ACC and ascorbate were kept constant, concentrations were maintained at 1 and 20 mM, respectively. When the oxygen concentration was varied, the reaction mixture was equilibrated by stirring for at least 10 min with the appropriate premixed O₂/N₂ gas mixture to obtain the desired oxygen concentration. Starting oxygen concentrations were determined by using the oxygen monitor that was calibrated with air-saturated water (258 μM oxygen at 25°C). Reactions were initiated with 2 μl of ACCO reconstituted with equimolar Fe(NH₄)₂(SO₄)₂. Because of the loss of activity on prolonged exposure to Fe(II) in the presence of oxygen (35, 36), ACCO was reconstituted in small aliquots and used within 30 min. Concentration of ACCO is as indicated in figure legends. All initial rates were measured under conditions where <5% of any given substrate was consumed. All rates were calculated subtracting background oxygen consumption attributable 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 by using the program KaleidaGraph. The kinetic parameters are reported with errors of ± 1σ.

SIEs on Steady-State Parameters. Initial rates were measured as described above. The standard buffer was prepared in D₂O (99.9%) by dissolving MOPS, NaHCO₃, and NaCl in D₂O and then titrating to pH 7.2 with a KOH solution. A value of 0.4 was added to the reading on the pH meter to correct to pH 7.2. To avoid H₂O contamination in the D₂O reactions, the electrode tip was soaked in D₂O before each reaction. In parallel, a buffer was prepared in H₂O.
for direct comparison. SIEs are reported with errors obtained by using appropriate error propagation methods.

18O KIEs. 18O KIEs were measured competitively as described in refs. 21 and 26. Reactions were carried out in 100 mM MOPS, pH 7.2, 20 mM NaHCO3, 100 mM NaCl, and 2 mM or 20 mM sodium ascorbate at 25°C with 0.3–0.5 mM O2 and 3 mM ACC. The enzymatic reactions were initiated with a preincubated mixture of ACCO:Fe(NH4)2(SO4)2 in a 1:7.5:1 ratio to minimize free iron in solution. Final concentrations typically were 3.5–8 μM ACCO and 2–4 μM Fe. Reactions were carried out in D2O under similar conditions, the buffer being prepared as described above. The amount of O2 consumed was corrected for the background O2 consumption attributed to ascorbate and Fe in absence of enzyme. In all experiments, the O2 consumed in nonenzymatic reactions accounted for <10% of the total O2 consumed. The 18O KIE also was measured for the background oxygen consumption (at 1 μM Fe), for comparison with the enzymatic reaction. The 18O/16O ratios were measured by using isotopic ratio mass spectrometry (Laboratory for Environmental and Sedimentary Geochemistry, Department of Earth and Planetary Science, University of California, Berkeley, CA). The 18O KIEs were 21.9±0.1 (21). For direct comparison. SIEs are reported with errors obtained by using appropriate error propagation methods. 18O KIEs were measured competitively as described in refs. 21 and 26. Reactions were carried out in 100 mM MOPS, pH 7.2, 20 mM NaHCO3, 100 mM NaCl, and 2 mM or 20 mM sodium ascorbate at 25°C with 0.3–0.5 mM O2 and 3 mM ACC. The enzymatic reactions were initiated with a preincubated mixture of ACCO:Fe(NH4)2(SO4)2 in a 1:7.5:1 ratio to minimize free iron in solution. Final concentrations typically were 3.5–8 μM ACCO and 2–4 μM Fe. Reactions were carried out in D2O under similar conditions, the buffer being prepared as described above. The amount of O2 consumed was corrected for the background O2 consumption attributed to ascorbate and Fe in absence of enzyme. In all experiments, the O2 consumed in nonenzymatic reactions accounted for <10% of the total O2 consumed. The 18O KIE also was measured for the background oxygen consumption (at 1 μM Fe), for comparison with the enzymatic reaction. The 18O/16O ratios were measured by using isotopic ratio mass spectrometry (Laboratory for Environmental and Sedimentary Geochemistry, Department of Earth and Planetary Science, University of California, Berkeley, CA). The 18O KIEs were 21.9±0.1 (21).

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The calculation of the 18O EIEs. The EIEs 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): EIE = ZPE × EXC × MMI (28). All three terms are related to vibrational frequencies of 18O- and 16O-containing reactants and products, as described in ref. 21. The measured frequency of 82 cm−1 (18O = 34–34 cm−1) for the Fe(II)O intermediate in Taud was used for calculation of the 18O EIE (17). For the model reaction (Table 2, entry 3), the 18O label can end up in either Fe(IV)O or H2O. The populations of the two isotopic distributions are expected to be close to each other; hence, the 18O EIE was calculated by using the formula: 18O EIE = 2[^16O:18O + 16,18O (1)^2] (21).