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Direct Detection of Oxygen Intermediates in the Non-Heme Fe Enzyme Taurine/α-Ketoglutarate Dioxygenase

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Reactive oxygen species play a central role in catalysis by many Fe oxygenases. Several oxygen intermediates have been extensively characterized for heme and non-heme enzymes;[1−3] however, no O_2 intermediates were directly observed for the α-ketoglutarate (αKG)-dependent dioxygenases.[3,4] *Escherichia coli* TauD is a typical αKG-dependent hydroxylase that transforms taurine (2-aminoethanesulfonate) to aminoacetaldehyde and sulfite while decomposing αKG to succinate and CO_2.[5] Spectroscopic and structural studies have shown that αKG coordinates to the iron by the C-1 carboxylate and C-2 carbonyl groups.[6,7] Taurine does not bind directly to iron upon formation of a taurine/αKG-Fe(II)TauD complex, but creates an open metal coordination site for oxygen which reacts readily with this complex.[6] Proposed intermediates in the O_2 reaction include Fe(III)-superoxo or Fe(IV)-peroxo species, an Fe(IV) peroxyhemiketal bicyclic complex, and Fe(IV)-oxo. Stopped-flow UV−visible and freeze-quench Mössbauer and EPR spectroscopies reveal an Fe(IV) intermediate exhibiting an absorption maximum near 318 nm.[8] The identity of the intermediate remains unknown, but this species is responsible for abstracting a hydrogen atom from taurine.[9] Here, we combine oxygen isotope difference resonance Raman (rR) spectroscopy with cryogenic continuous flow to provide evidence that the hydrogen atom abstraction in TauD is accomplished by an Fe(IV)═O species.

The method of continuous flow, whereby two reactants are continuously mixed and passed through the excitation laser beam, is an inclusive approach to achieve time-resolution in biological rR experiments. The Fe(IV) species of TauD was reported to be maximized at ∼20 ms in the presence of O_2 at 5 °C.[8] In a continuous-flow system with 2 μL dead volume, a 20-ms delay would be achieved at a flow rate of 6 mL/min. Given the extended periods of spectral accumulation of rR scattering required for weak chromophores (ϵ_{318} 1.5 mM^{-1} cm^{-1}),[8] such a flow rate would require prohibitive amounts of protein sample.

To reduce the sample consumption to a manageable level, we developed a modified version of our original mixer[10] that allowed us to achieve fast and complete mixing of liquid reactants entirely at cryogenic temperatures.[11] By addition of 50% ethylene glycol to the reaction medium, we were able to carry out continuous-flow rR and UV−vis absorption measurements at −38 °C while maintaining fluidity and optical transparency of the sample.

We recorded transient absorption spectra during the anticipated decay of the Fe(IV) species,[12] as shown in Figure 1A. The spectrum at the shortest delay time exhibited a weak visible absorption centered at 520−530 nm and previously assigned to the charge-transfer transition of the Fe(II)-α-keto acid bidentate complex (ϵ_{530} 180 M^{-1} cm^{-1}).[6] The observed molar absorptivity of this band is in reasonable agreement with earlier reports.[6,8]

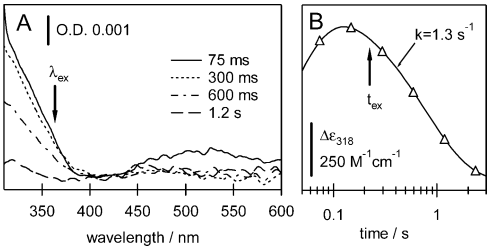

Figure 1. UV−vis absorption changes in the reaction of taurine/αKG-Fe(II)TauD with O_2 at −38 °C. (A) Transient difference absorption spectra are shown versus that obtained at 4.8 s. An empirical baseline at 425−700 nm was used for all data, and spectra were smoothed. (B) Temporal profile at 318 nm averaged over the 313−323-nm window. The optical path length was 0.025 cm. The delay from the reaction start was achieved by varying flow rates at a fixed dead volume of ∼2 μL. Sample composition: Anaerobic solution containing 0.55 mM TauD (purified as the apoprotein), 0.5 mM ferrous ammonium sulfate, 1.2 mM taurine, 1.2 mM αKG, and 0.1 mM ascorbate in 25 mM Tris, pH 8.0, 50% v/v ethylene glycol mixed with an equal volume of O_2-saturated buffer of the same composition. Arrows indicate temporal and spectral positions probed by rR.

At longer delay times, the ∼525-nm band disappears, leaving the spectrum dominated by a UV absorption below 400 nm. Figure 1B shows that temporal changes at 318 nm follow an exponential decay with a molar extinction change of 1 mM^{-1} cm^{-1}. The decay rate is in good agreement with an extrapolation of reaction rates observed at 4 °C and 25 °C (not shown). The amplitude of the absorption change is in good agreement with the value of 0.7 mM^{-1} cm^{-1} estimated from the recently published transient spectra.[8] Taken together, these observations show that the pathway for reaction of TauD with O_2 is not significantly affected by either temperature or ethylene glycol. Consequently, the phase of the reaction observed at ∼150 ms for the sample at −38 °C corresponds to that probed recently by Mössbauer spectroscopy at ∼20 ms at 5 °C.[8]

Figure 2 shows the transient rR spectrum of taurine/αKG-Fe(II)TauD in its reaction with O_2 at the delay time and excitation indicated in Figure 1. The spectrum is shown as a difference of the data obtained with $^{16}O_2$ versus that obtained with $^{18}O_2$, where all the vibrations not involving oxygen bound to a chromophore are canceled. An absolute rR spectrum of ^{16}O isotope used in the subtraction is shown for comparison, reduced by a factor of 1000. The most prominent vibration can be seen at 821 cm^{-1} for ^{16}O and 787 cm^{-1} for ^{18}O. A second vibration was observed at 583/555 cm^{-1} for ^{16}O and ^{18}O, respectively.

The presence of intense vibrations of ethylene glycol at 865 and 1089 cm^{-1} made it difficult to achieve reliable subtraction in these regions. Nevertheless, using several independent preparations of the enzyme, we found that it was impossible to achieve complete cancellation of both bands simultaneously. The spectrum in Figure

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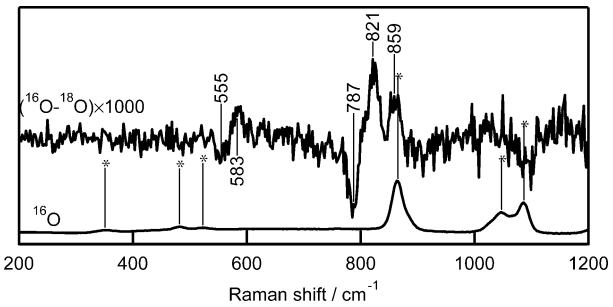
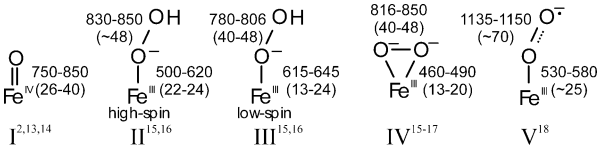


Figure 2. Cryogenic $^{16}O_2/^{18}O_2$ isotope difference spectrum of taurine/αKG-Fe(II)TauD during its reaction with O_2. The spectra were acquired at 0.22 s delay time, −38 °C; excitation, 363.7 nm at 70 mW, path length 0.75 mm; scattered light was collected at 90° geometry; spectral slit width 17 cm^{-1}, resolution 2.1 cm^{-1}/pixel at 800 cm^{-1}; calibration using acetone and acetonitrile; accumulation, 3 h per isotope in 35 min increments in alternating order; fluorescence background was corrected using a polynomial function. Asterisks indicate positions of solvent bands. Sample composition was the same as in Figure 1.

Scheme 1. Structures and Characteristic Vibrations ($^{16}O/^{18}O$ Shifts) of Potential Oxygen Species



2 represents the subtraction factor that provides the most neutral balance between the two regions. The frequencies of the residual bands, most clearly the positive band at 859 cm^{-1}, appear to be shifted from those of ethylene glycol. We therefore conclude that at least one additional $^{16}O/^{18}O$ sensitive mode is likely to be present.

Structures and characteristic frequencies of oxygen intermediates that could give rise to the observed vibrations are shown in Scheme 1. Vibrations in the 800 cm^{-1} region could originate from either the $\nu_{Fe=O}$ of **I**[2,13,14] or ν_{O-O} of **II**, **III**, or **IV**.[15−17] These compounds are, however, distinguished by the magnitude of the downshift upon $^{16}O/^{18}O$ substitution. The downshift of 34 cm^{-1} seen in Figure 2 is a good match to typical values for ferryloxo species (**I**) calculated by normal coordinate analysis and extensively documented for heme proteins and model compounds.[2,13] The O−O vibrations of peroxy species (**II**-**IV**) typically exhibit a larger shift of ∼45 cm^{-1}. While the frequency of the $\nu_{Fe=O}$ mode may be affected by the spin state of the iron, the amplitude of the $^{16}O/^{18}O$ shift is likely to be less sensitive as illustrated by species **II**−**IV**.[15−17] We therefore assign the 821/787 cm^{-1} mode to structure **I**.

Since Fe(IV)═O does not exhibit known vibrations in the 600 cm^{-1} region, the 583/555 cm^{-1} band most likely originates from a precursor in the reaction sequence. Of particular interest is structure **V**. Well-characterized in heme systems, it exhibits a ν_{Fe-O} that is close to the vibration reported here in both its frequency and amplitude of the $^{16}O/^{18}O$ shift.[18] Prior studies[8] did not detect such an intermediate, perhaps due to its low population under corresponding conditions compounded by spectral overlap. The second mode of structure **V** (ν_{O-O}) can be expected around 1100 cm^{-1} with a $^{16}O/^{18}O$ shift of ∼70 cm^{-1}.[15,16,18] A weak derivative pattern is present at 1157/1095 cm^{-1} in Figure 2, but interference from the solvent band at 1089 cm^{-1} does not allow us to conclusively identify this vibration.

A less likely origin of the 583 cm^{-1} band is a ν_{Fe-O} mode of a protonated end-on peroxy (**II** or **III**) or alkylperoxy species. Reduction of O_2 to the peroxy level requires an additional electron, which could come from iron, αKG, or an external source. Oxidation of Fe would lead to the formation of Fe(IV)−O−O(H), which may occur but has never been observed nor is likely to be stable enough to achieve a detectable population. Lack of evidence for additional electron donors and an apparent absence of Fe(III) during turnover[8] do not support external reduction. Oxidation of αKG by a ferric superoxo complex is an intriguing possibility that would lead to a formation of a peroxyhemiketal bicyclic complex.[8] It should be noted, however, that the superoxo (**V**), all end-on (**II**, **III**), and some side-on (**IV**) peroxy species are expected to exhibit an intense ($\epsilon \geq 1000\ M^{-1}$) absorption in the near UV and/or visible region,[15−17] which was not detected. While the six-coordinated side-on peroxy species (**IV**), which is characterized by absorption below 300 nm, is a rare exception,[17] the frequency of its ν_{Fe-O} mode appears to be too low to be responsible for the observed 583/555 cm^{-1} mode. Ongoing studies seek to discriminate between these possibilities.

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(11) The reduced flow rates at lower temperature greatly conserved the sample; however, low velocity and the viscosity increase due to the presence of ethylene glycol and low temperature caused a rapid drop in Reynold's number and led to a strictly laminar flow, preventing spontaneous mixing of two reactants. An externally driven rotor in our setup induced complete mixing.

(12) Optical measurements were carried out by using an Ocean Optics SD2000 spectrophotometer equipped with 300 μm solarization-resistant fibers and DT1000 light source. Sample point was 0.7 mm in diameter centered at the excitation laser beam used for rR measurements; path length was 0.025 cm. Approximately (2−4) × 10^3 10-ms spectra were averaged.

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