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Spectroscopic Analyses of 2-Oxoglutarate-Dependent Oxygenases: TauD as a Case Study

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Abstract

A wide range of spectroscopic approaches have been used to interrogate the mononuclear iron metallocenter in 2-oxoglutarate (2OG)-dependent oxygenases. The results from these spectroscopic studies have provided valuable insights into the structural changes at the active site during substrate binding and catalysis, thus providing critical information that complements investigations of these enzymes by x-ray crystallography, biochemical, and computational approaches. This mini-review highlights taurine hydroxylase (taurine:2OG dioxygenase, TauD) as a case study to illustrate the wealth of knowledge that can be generated by applying a diverse array of spectroscopic investigations to a single enzyme. In particular, electronic absorption, circular dichroism, magnetic circular dichroism, conventional and pulse electron paramagnetic, Mössbauer, X-ray absorption, and resonance Raman methods have been exploited to uncover the properties of the metal site in TauD.

Keywords

2-Oxoglutarate; Nonheme iron; Oxygenase; Spectroscopy

Introduction

The 2-oxoglutarate (2OG)-dependent oxygenases (2OG oxygenases) belong to a large family of enzymes that catalyze hydroxylation, chlorination, desaturation, ring formation, ring expansion/contraction, epoxidation, endoperoxidation, and epimerization reactions using a wide variety of substrates [1, 2]. This remarkable catalytic diversity is united by chemical mechanisms that include Fe(IV)-oxo (ferryl) intermediates [3]. The mononuclear iron active sites of these enzymes are located at the opening of a double-stranded β -helix scaffold, with the metal generally bound by three protein side chains in a His-X-Asp(Glu)-X_n-His motif [4]. A simplified depiction of the hydroxylation reaction cycle (Fig. 1) shows the major spectroscopically characterized species. The resting enzyme (Fig. 1a) contains

Fe(II) in octahedral coordination where three water molecules occupy one face of the metal ion. Two waters are displaced by 2OG which binds in bidentate coordination to Fe(II) via its C1 carboxylate and C2 keto group (Fig. 1b). The primary substrate (R-H) binds near the metallocenter leading to displacement of the remaining water and resulting in square pyramidal geometry of Fe(II) (Fig. 1c). O₂ binds to the newly opened coordination site and presumably forms an Fe(III)-superoxo species that has not been spectroscopically detected. Subsequent C-C and O-O cleavage reactions result in release of the 2OG C1 group as CO₂ and formation of succinate and the Fe(IV)-oxo intermediate (Fig. 1d), both containing O atoms derived from O₂. Oxygen insertion into the primary substrate C-H bond returns the metallocenter to the Fe(II) state (Fig. 1e), and the cycle is completed by dissociation of products and association of three water molecules.

Taurine hydroxylase (also known as taurine:2OG dioxygenase or TauD) is the best-characterized representative of this enzyme family [5]. The microbial enzyme catalyzes the hydroxylation of taurine (aminoethanesulfonate) forming an the unstable intermediate that spontaneously decomposes to aminoacetaldehyde and sulfite, which is utilized as a sulfur source [6]. Here, we use TauD as a case study to illustrate how various spectroscopic approaches have provided powerful insights into the structure and properties of the enzyme metallocenter during substrate binding and catalysis.

Electronic absorption spectroscopy of TauD

Like other proteins, TauD exhibits electronic transitions in the ultraviolet (UV) associated with its Trp and Tyr residues, and the spectrum is nearly identical when Fe(II) is added to apoprotein to provide the holoenzyme (Fig. 2a) [7]. Close inspection of the difference spectrum for these samples (Fig. 2b), however, reveals modulations associated with conformational changes affecting the environment of the aromatic residues. Similar perturbations were observed upon binding of other metal ions or taurine, and titration studies were used to estimate binding affinities [7]. Because aromatic residues are commonly observed at the active sites of 2OG oxygenases, such UV absorption difference spectra may be useful for monitoring metal and ligand interactions in favorable cases with other members of this enzyme family.

In contrast to the lack of a visible absorption spectrum in TauD·Fe(II) (Fig. 2a), TauD·Fe(II)·2OG and TauD·Fe(II)·2OG·taurine possess visible (pink/purple-colored) chromophores (Fig. 2c; $\epsilon_{530} = 140\text{--}240 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{520} = 180\text{--}270 \text{ M}^{-1} \text{ cm}^{-1}$) [8, 10–12]. Analogous features in clavaminic acid synthase (another 2OG oxygenase) were identified as metal-to-ligand charge-transfer (MLCT) transitions associated with Fe(II) d_{yz} , $d_x^2 - y^2$, and $d_z^2 \rightarrow 2OG \pi^*$ energy levels with 2OG in bidentate coordination to the metal ion [13]. Stopped-flow (SF) spectroscopy was used to show the Fe(II)/2OG chromophore forms at a rate that is independent of substrate or enzyme concentration, consistent with initial binding of 2OG to the enzyme followed by a conformational change to create the bidentate state that generated the color. The Fe(II)/2OG MLCT transitions have been used as a diagnostic probe to demonstrate correct protein folding and proper active site formation in other proteins, even when the primary substrate is unknown (e.g. [14]).

Visible spectroscopic changes are sometimes observed during non-productive reactions of 2OG oxygenases. For example, when TauD·Fe(II)·2OG (lacking the substrate taurine) is exposed to O₂ (Fig. 2d) the protein forms a transient tyrosyl radical ($\epsilon_{408} = 1,600 \text{ M}^{-1} \text{ cm}^{-1}$) followed by the slow development of a greenish brown color ($\epsilon_{550} = 460 \text{ M}^{-1} \text{ cm}^{-1}$) [9]. This chromophore very slowly shifts to a bright green species ($\epsilon_{720} \sim 300 \text{ M}^{-1} \text{ cm}^{-1}$) upon standing for several days [15]. Similarly, a green species ($\epsilon_{720} = 380 \text{ M}^{-1} \text{ cm}^{-1}$) is formed when TauD·Fe(II)·succinate is exposed to O₂ (or H₂O₂), and the spectrum of this species is shifted to one with a 550 nm maximum when bicarbonate is added [15]. Mutagenesis, resonance Raman, and mass spectrometric evidence were used to show these chromophores derive from the conversion of Tyr73 into a dihydroxyphenylalanine (DOPA) residue that chelates Fe(III) [9, 15, 16]. The ligand-to-metal charge-transfer (LMCT) transitions of this catecholate-like species are modulated by the binding of product bicarbonate. As purified from aerobically grown cells, a portion of TauD has the oxidized form of DOPA, DOPA quinone, as shown by the addition of Fe(II) leading to the generation of a DOPA-Fe(III) LMCT transition or by the addition of Cr(II) to an anaerobic protein resulting in a Cr(III) semiquinone spectrum ($\epsilon_{434} = 1,000 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{690} = 350 \text{ M}^{-1} \text{ cm}^{-1}$) [17, 18]. Self-hydroxylation reactions of active site Tyr residues (resulting in DOPA-Fe(III) species) or Trp residues (yielding hydroxyindole-Fe(III) species) give rise to green or blue LMCT transitions in several other family members (e.g. [19–22]).

SF-UV-visible spectroscopy has proven to be a powerful tool to monitor the productive reaction of 2OG oxygenases with O₂. When an anaerobic sample of TauD·Fe(II)·2OG·taurine ($\epsilon_{520} \sim 200 \text{ M}^{-1} \text{ cm}^{-1}$) is mixed with O₂ at 5 °C, an intermediate identified as the ferryl species (Fig. 1D, $\epsilon_{318} = 1,550 \text{ M}^{-1} \text{ cm}^{-1}$) forms within 25 ms and decays by 600 ms (Fig. 2e and 2f) [10]. Return of the 520 nm absorbing species is delayed compared to loss of the 318 nm intermediate, providing evidence for a second intermediate in the reaction. The apparent first-order rate constant for ferryl formation is linearly dependent on the O₂ concentration [11, 23], and it decays with a first-order rate constant that is diminished by substrate deuteration demonstrating that it is responsible for hydrogen atom abstraction ($k_{\text{H}}/k_{\text{D}} = 28\text{--}50$) [24]. Modifications of the kinetics of the ferryl intermediate have been examined using this technique for a suite of TauD variants and with alternative sulfonic acid substrates [11, 25, 26]. SF-UV-visible spectroscopy has been used to detect this intermediate in TauD from *Pseudomonas putida* [12] and in several other family members (e.g. [27–29]).

CD and MCD spectroscopy of TauD

The study of mononuclear $S = 2$ Fe(II) sites in proteins has been greatly aided by recent developments in circular dichroism (CD) and especially magnetic circular dichroism (MCD) spectroscopies [30–32]. The TauD holoenzyme at 298 K exhibits positive CD transitions at 8,850 and 10,600 cm^{-1} which offer little insight into the metalcenter geometry. By contrast, the positive MCD transitions at 8,900 and 10,700 cm^{-1} at 1.8 K (Fig. 3a and 3b, green traces) [33] are consistent with $^5\text{E}_g \rightarrow d_{x^2-y^2}$ and d_{z^2} ligand field transitions centered at $\sim 10,000 \text{ cm}^{-1}$ and split by $\sim 2,000 \text{ cm}^{-1}$ as seen in distorted six-coordinate Fe(II) sites (Fig. 3c). Also compatible with distorted 6-coordinate geometry, TauD·Fe(II)·2OG has a positive transition at 7,400 cm^{-1} and a negative transition at 11,150 cm^{-1} while the MCD spectra reveals positive features at 8,190 and 11,450 cm^{-1} (Fig. 3a and 3b, blue traces) [33].

Furthermore, TauD·Fe(II)·2OG-aurine provides a negative band at 5,000 cm^{-1} and a positive band at 10,150 cm^{-1} in the CD spectrum and an intense positive transition at 5,500 cm^{-1} along with weak positive features at 8,550 and 10,400 cm^{-1} in the MCD spectrum (Fig. 3a and 3b, red traces) [33]. By comparison to spectra of species with known coordination geometry (Fig. 3c), this complex was identified as a mixture of 6-coordinate and square pyramidal 5-coordinate sites. This finding is consistent with the hypothesis that the binding of taurine results in loss of a water ligand (Fig. 1, b conversion to c) to create a site for O_2 addition. Substrate-triggered creation of an O_2 -binding site has been noted in clavaminic acid synthase [34], a 2OG-dependent halogenase [33], and other mononuclear iron oxygenases [35, 36].

EPR spectroscopy of NO-bound TauD

Electron paramagnetic resonance (EPR) studies of the Fe(II) catalytic site of TauD have required the addition of NO as a substitute for substrate O_2 to form an $S=3/2$, $\{\text{FeNO}\}^7$ paramagnetic center [37]. This approach for the EPR study of non-heme Fe enzymes was pioneered by Lipscomb and Münck [38–40], and its utility for structural studies using Q-band ENDOR spectroscopy to measure ligand hyperfine couplings has been demonstrated by Hoffman and coworkers [41–43]. In addition to creating an EPR-active catalytic site, NO coordination serves to approximate O_2 binding and the Fe-NO bond provides a reference for the interpretation of ligand hyperfine couplings in terms of structure [44, 45]. We have used the pulse EPR methods of electron spin echo envelope modulation (ESEEM) and hyperfine sublevel correlation (HYSCORE) at conventional X-band frequency to characterize Fe ligation for enzyme samples prepared with different combinations of substrate and cofactor. ESEEM is especially useful for this task as it is sensitive to both the moderate hyperfine couplings typical for Fe(II)-bound ligands and the weak hyperfine couplings expected for second coordination sphere ligands like substrate taurine [46].

Figure 4a shows the X-band continuous wave (cw) EPR spectrum of the quaternary complex of TauD with 2OG, taurine, and NO (TauD· $\{\text{FeNO}\}^7$ ·2OG-aurine). The spectrum stems from the lower energy Kramer's doublet of an $S = 3/2$ $\{\text{FeNO}\}^7$ center characterized by a large, positive zero-field interaction ($D > 1 \text{ cm}^{-1}$). The spectrum is nearly axial with $|E|/D$ 0.003 and an EPR absorbance that covers a 170 mT range of magnetic field strengths from $g = 4$ to $g = 2$. The top two traces of Figure 4b show normalized 3-pulse ESEEM data that were collected for this sample at 330 mT. The top trace is for a sample prepared with 1,1- ^2H -taurine while the middle trace was prepared with unlabeled taurine. These data sets are nearly identical with either sample showing overall modulation depths of about 20% that consist of contributions from the nitrogen atoms of coordinated NO and histidine, and from the protons of primary and secondary coordination sphere ligands. The bottom trace of Figure 4b, obtained by dividing the ^2H -taurine trace (top) by the taurine trace (center), represents the contribution of a single coupled ^2H [47]. The ^2H -ESEEM spectrum corresponding to these data is shown in Figure 4c in the box marked 330 mT (black trace).

Figure 4c shows ^2H -ESEEM spectra collected at six magnetic field positions across the cw-EPR spectrum of the TauD· $\{\text{FeNO}\}^7$ ·2OG-aurine complex (Fig. 4a). These data contain detailed information on the location of the coupled taurine deuteron with respect to the Fe-

NO bond that is manifest in the frequency, lineshape, and amplitude of the ^2H -ESEEM response as a function of magnetic field strength. Analysis of these data was accomplished using EasySpin, a public-domain EPR simulation program that runs in the MATLAB environment, to simultaneously fit all six of the spectra shown in Figure 4c [49–51]. The results, shown as red traces in Figure 4c, arise from a single coupled ^2H characterized by a dipolar coupling of 0.19 ± 0.03 MHz, and an orientation that places the coupled ^2H on the base of a cone that makes a $26 \pm 6^\circ$ angle with the Fe-NO bond axis [48]. The drawing shown in Figure 4d was produced from a crystal structure obtained for TauD (PDB: 1GY9) where the crystallization was done in the presence of 2OG and taurine [52]. If one assumes that NO binds directly opposite to the axial histidyl imidazole ligand, along the black arrow, then the crystal structure indicates the closest C_1 - ^2H would be $3.1 \text{ \AA}$ from the metal ion with the coupled nucleus lying on the base of a cone that makes a 29° angle with the Fe-NO bond. Using the point dipole-dipole approximation, and an Fe spin density weighting of 0.9, our measured deuterium dipolar coupling translates into a $3.9 \pm 0.2 \text{ \AA}$ distance between the coupled deuteron and Fe [48]. While there is good agreement between our ^2H -ESEEM results and the crystal structure for the orientation of the coupled deuteron with respect to the Fe-NO bond, there is a substantial difference in its distance from the metal ion. In addition, our ^2H -ESEEM analysis also provided information on the orientation of the principle axis of the ^2H -nuclear quadrupole interaction, coincident with the taurine C_1 - ^2H bond that was at odds with the 1GY9 structure. Specifically, we found a $52 \pm 17^\circ$ orientation for the C_1 - ^2H bond axis with respect to the Fe-NO bond, while the 1GY9 structure shows this angle to be close to 30° . To explore differences in the 1GY9 structure that may result from the binding of NO to Fe, we used the Avogadro software package to re-optimize the position of taurine using the unified force field option. This exercise yielded a taurine orientation at the active site that shows good agreement with both the dipolar distance and the C_1 - ^2H bond orientation derived from ESEEM measurements [48].

The study of primary coordination sphere ligands using ESEEM involves separating the modulations from ^{14}N and ^1H nuclei that are substantially overlapped in frequency. This process is readily accomplished for nonheme $\{\text{FeNO}\}^7$ centers using 4-pulse HYSCORE spectroscopy, a 2-dimensional ESEEM experiment. Figure 5a shows the 3-pulse ESEEM spectrum collected for a ternary complex of TauD- $\{\text{FeNO}\}^7$ -taurine at 280 mT (see inset for the cw-EPR spectrum). This 1-dimensional spectrum shows approximately 20 different modulation components arising from strong- and weak-coupled ^{14}N and ^1H nuclei. The 4-pulse HYSCORE spectrum that corresponds to these data is shown as the upper contour plot of Figure 5b. In HYSCORE spectroscopy, hyperfine couplings that are large compared to the Larmor frequency of the coupled nucleus are sorted by the 2-dimensional fast Fourier transform (FT) into the quadrant where one of the correlated frequencies has a negative phase, the $(-,+)$ quadrant [53]. As a result, the directly coordinated ^{14}N nuclei of the coordinated histidine ligands and NO contribute to the cross-peaks resolved in the $(-,+)$, or left-hand quadrant. Cross-peaks arising from the coupled protons of the histidyl imidazole side chains and bound water molecules are resolved in the $(+,+)$ quadrant. The protons of bound water ligands are further characterized by strong dipolar couplings and easily distinguished by HYSCORE spectroscopy [54, 55]. The cross-peaks due to these protons are circled in red on the HYSCORE spectrum shown (Fig. 5b, top). The HYSCORE spectrum

collected at 280 mT for a sample of the ternary complex of TauD·{FeNO}⁷·2OG also is shown (Fig. 5b, bottom). These data show the displacement of bound water that occurs upon 2OG chelation to the {FeNO}⁷ paramagnetic center and also show that the electronic structure of the center has been altered in a fashion that affects the ¹⁴N hyperfine coupling from NO and the histidines.

Mössbauer and XAS analysis of TauD

The initial TauD reaction intermediate detected by SF-UV-visible spectroscopy (Fig. 2e) was identified as a ferryl species by the combined use of freeze-quench (FQ) Mössbauer analysis, FQ X-ray absorption spectroscopy (XAS), and continuous-flow resonance Raman spectroscopy. The first two approaches are described here and the third is summarized in the following section.

The Mössbauer spectrum of ⁵⁷Fe-containing TauD·Fe(II) (Fig. 6a, top trace) reveals a quadrupole doublet (with an isomer shift, δ , of 1.27 mm s⁻¹ and a quadrupole splitting, E_Q , of 3.06 mm s⁻¹), indicating high-spin Fe(II) [10]. This spectrum shifts to one with altered parameters (δ = 1.16 mm s⁻¹ and E_Q = 2.76 mm s⁻¹) in the presence of 2OG and taurine (Fig. 6a, second trace), consistent with the transition from 6-coordinate to 5-coordinate geometry [10]. When TauD·Fe(II)·2OG·taurine is mixed with O₂ and frozen after selected intervals, the FQ Mössbauer samples exhibit a new spectrum (δ = 0.31 mm s⁻¹ and E_Q = 0.88 mm s⁻¹; Fig. 6a, bottom traces) that forms and decays with the same kinetic constants (Fig. 6b) as the reaction intermediate detected by electronic absorption spectroscopy. The Mössbauer spectrum of the reaction intermediate varies with the external applied magnetic field (Fig. 6c) in a manner consistent with an integer spin species possessing $S = 2$ [10, 56, 57]. This reaction intermediate exhibits no EPR features whereas cryoreduction of the sample leads to a high-spin Fe(III) signal (data not shown), leading to assignment of the intermediate as a high-spin Fe(IV) species [10]. FQ Mössbauer analysis has been used to detect similar Fe(IV) intermediate species in several other family representatives (e.g. [27–29, 58, 59]).

FQ trapping of the TauD reaction intermediate (at 79% abundance when using ²H-taurine as the substrate) also allowed for its analysis by XAS [60]. The Fe K-edge of the intermediate species (7,123 eV) and the intensity of the 1s → 3d transition were both significantly greater than for these features of the anaerobic sample, consistent with the accumulation of an Fe(IV) species containing a short Fe-O bond in the reaction sample. The FT of the extended X-ray absorption fine structure (EXAFS) data (Fig. 6d) did not resolve a separate peak at short radius, but the Fourier filtered EXAFS results were only well fit by including a scattering shell at 1.62 Å, compatible with Fe-O bonding, in addition to scattering shells at 2.05 and probably 2.42 Å (Fig. 6e) [60]. XAS studies have been carried out with other family members (e.g. [61–63]), including the FQ trapping of reaction intermediates containing short Fe-O bonds for the CytC3 and SyrB2 halogenases (along with evidence for an Fe-Br bond in CytC3 that was reacted in the presence of bromide) [28, 64].

One other Mössbauer study of TauD is worthy of mention due to its novelty [65]. TauD·Fe(II)·2OG·taurine was treated with NO, similar to the EPR studies described in the

earlier section, and the resulting $\text{TauD} \cdot \{\text{FeNO}\}^7 \cdot 2\text{OG} \cdot \text{taurine}$ species was examined by Mössbauer analysis at several fields. As expected, the sample possesses a $S = 3/2$ ground state, $\delta = 0.69 \text{ mm s}^{-1}$, and $E_Q = -1.70 \text{ mm s}^{-1}$ (not shown), similar to other $\{\text{FeNO}\}^7$ complexes. In addition, the sample was subjected to cryoreduction to afford $\text{TauD} \cdot \{\text{FeNO}\}^8 \cdot 2\text{OG} \cdot \text{taurine}$; this was the first paramagnetic $\{\text{FeNO}\}^8$ complex observed and is important because it is isoelectronic with $\{\text{FeO}_2\}^8$, the initial O_2 adduct. This new species exhibited $\delta = 1.07 \text{ mm s}^{-1}$ and $E_Q = 2.39 \text{ mm s}^{-1}$ (not shown), consistent with reduction of the high-spin Fe(III) to high-spin Fe(II) with the NO-ligand remaining NO^- [65].

RR spectroscopy of TauD

Resonance Raman (RR) spectroscopy has been used to examine both anaerobic TauD containing the $\text{Fe(II)} \cdot 2\text{OG}$ chromophore and the catalytic intermediates formed after oxygen addition to $\text{TauD} \cdot \text{Fe(II)} \cdot 2\text{OG} \cdot \text{taurine}$.

An anaerobic spinning cell was used to probe the RR vibrations associated with $\text{TauD} \cdot \text{Fe(II)} \cdot 2\text{OG}$ and $\text{TauD} \cdot \text{Fe(II)} \cdot 2\text{OG} \cdot \text{taurine}$ (Fig. 7) [66]. On the basis of comparison to corresponding vibrations of model compounds, the RR shifts at 460 cm^{-1} and $1,686 \text{ cm}^{-1}$ in $\text{TauD} \cdot \text{Fe(II)} \cdot 2\text{OG}$ are assigned to the $\text{Fe(II)} \cdot 2\text{OG}$ chelate mode and the $\nu(\text{C}=\text{O})$ of the keto carbonyl group, respectively, with both features displaying a downshift in H_2^{18}O . The addition of taurine to the sample results in vibrations at 470 cm^{-1} and $1,688 \text{ cm}^{-1}$, which also are downshifted in H_2^{18}O . The magnitude of the $^{16}\text{O}/^{18}\text{O}$ isotopic shift of the $1,686 \text{ cm}^{-1}$ mode is in agreement with the value obtained for model complexes and, therefore, this mode involves only the keto carbonyl oxygen. By contrast, the small magnitude of the isotopic shift observed for the 460 cm^{-1} mode (-9 cm^{-1}) upon $^{16}\text{O}/^{18}\text{O}$ substitution indicates that it involves motion of multiple atoms. This vibration in model compounds is assigned to an Fe(II) chelate mode and involves both carboxyl and keto oxygen atoms, but only the latter atom undergoes exchange with the bulk water in $\text{TauD} \cdot \text{Fe(II)} \cdot \text{taurine}$ (Fig. 7). The terminal water ligand of Fe(II) is excluded as a possible origin of the 460 cm^{-1} mode because the observed isotopic shift is much smaller than expected for an isolated diatomic Fe-O oscillator. Furthermore, a similar chelate mode is observed upon binding of taurine, where the terminal water is lost [52]. The concomitant 10 cm^{-1} upshift in Raman frequency is interpreted as arising from the conversion of 6-coordinate to 5-coordinate geometry [66]. No other vibrations attributable to the $\text{Fe(II)}\text{-OH}_{(2)}$ are detected in $\text{TauD} \cdot \text{Fe(II)} \cdot 2\text{OG}$.

Transient oxygen-derived species that form during TauD catalysis were characterized by use of isotope-difference continuous-flow (CF) RR spectroscopy [67, 68]. Anaerobic samples of $\text{TauD} \cdot \text{Fe(II)} \cdot 2\text{OG} \cdot \text{taurine}$ were actively mixed with $^{16}\text{O}_2$, $^{18}\text{O}_2$, or $^{16}\text{O}/^{18}\text{O}$ mixed isotope-enriched oxygenated buffer and passed through a flow cell while collecting RR spectra (Fig. 8a). Spectra of each isotopomer were integrated across multiple alternating measurements and isotope-difference spectra were generated by subtracting the spectra obtained with $^{18}\text{O}_2$ from those obtained with $^{16}\text{O}_2$. Such subtraction cancels out all vibrations except for those involving atoms originating from O_2 and oxygen isotope-sensitive species appear as derivative shifts (Fig. 8a). Time resolution was achieved by varying the flow rate and the physical volume between the mixing point and the probe laser beam. To accumulate sufficient Raman intensity using practically achievable amounts of protein sample, the rate

of the reaction was decreased by using low temperature ($-36\text{ }^{\circ}\text{C}$) in the presence of cryoprotectant in the sample buffer. To verify that the transient species with characteristic optical absorption at 320 nm was populated at these conditions in agreement with the conventional stopped-flow studies [10], in situ absorption spectra were acquired. Close similarity of the observed absorption spectra suggested that the reaction proceeds via the same transient species.

The initial transient isotope-difference RR spectrum of TauD reveals the vibration at $821/787\text{ cm}^{-1}$. Both the frequency and the magnitude of the $^{16}\text{O}/^{18}\text{O}$ isotopic shift support the formation of a transient ferryl species during TauD catalysis [67]. Follow-up studies confirm the presence of this intermediate ($825/788\text{ cm}^{-1}$ labeled F4, Fig. 8b left) and directly probe its structure using $^{16}\text{O}^{18}\text{O}$ mixed oxygen isotope (Fig. 8c). The result conclusively shows that all detectable species in TauD contain a single oxygen atom, finalizing the assignment of the 825 cm^{-1} species as a ferryl intermediate and the observed frequency to its $\nu_{\text{Fe}=\text{O}}$ mode. This assignment is further corroborated by the observed kinetic isotope effect of substrate deuteration. Careful internal calibration using solvent bands allowed us to detect an upshift in this mode to $827/790\text{ cm}^{-1}$, Fig. 8b, right) in the presence of ^2H -taurine. By analogy with well-characterized heme enzymes, this upshift is attributed to direct interaction between the oxo group and the C1 hydrogen of the primary substrate [68].

Two new transient species are resolved in the TauD reaction at $-36\text{ }^{\circ}\text{C}$. The most prominent species is observed at $578/555\text{ cm}^{-1}$ (labeled F3 in Fig. 8). It shows time-dependence, trailing that of F4, and is assigned to the catalytic successor of F4, although formation of this species is not yet resolved. The second and weaker species is detected at the exhaustion of F4 using global spectral regression analysis. It is characterized by an unusually small magnitude of the isotopic shift at $815/787\text{ cm}^{-1}$ and termed FX in Fig. 8. To understand the vibrational origin of this species, several isotopically-labeled models of possible products of the reaction were synthesized, of which only one – the metal-alkoxo complex – shows isotopic sensitivity comparable to FX in its frequency and magnitude of the $^{16}\text{O}/^{18}\text{O}$ downshift. Based on this similarity, the FX species is assigned to a putative Fe(II)-alkoxo species following oxygen rebinding to substrate radical; however, the mechanism for Raman enhancement of such a species requires further investigation.

The vibrational signature of the F3 species is the most intriguing among the transient Raman intermediates of TauD. First, the time-resolved Raman study shows that both F3 and FX exhibit lifetimes longer than that of F4 and that the kinetics of all three species are sensitive to substrate deuteration. In contrast, the time-dependencies of the Fe(IV) and Fe(II) Mössbauer species at room temperature do not suggest the existence of any additional steps between the two intermediates [10]. Because the time resolution of the rapid FQ method used in the Mössbauer study is at least as high as that in the CF Raman studies, it is unlikely that the F3 and F4 species are not detected by Mössbauer for this reason. Instead, these results suggest that the populations of F3 and FX species are temperature dependent and either are increased at low temperature due to the relatively high activation energies of $\text{F4} \rightarrow \text{F3}$ and $\text{F3} \rightarrow \text{FX}$ steps or, in contrast, the activation energies are very low which may lead to their decay during FQ Mössbauer studies.

The second peculiarity of the F3 species is the lack of vibrational signature from the associated proton. The kinetic isotope effect of the reaction of Fe(IV)=O with taurine clearly supports a large tunneling component of hydrogen atom transfer over proton-coupled electron transfer [69–72]. Hydrogen atom transfer from C1 of taurine to Fe(IV)=O which should yield Fe(III)-OH. Yet, careful vibrational analysis shows no detectable sensitivity of the $\nu_{\text{Fe-O}}$ of F3 to either bulk water or substrate deuteration. Three alternative scenarios can explain this disparity. First, the Fe(III)-oxo species could reasonably arise by deprotonation of an Fe(III)-OH, following hydrogen atom abstraction from substrate. The main contradiction to this mechanism is the consensus expectation of significant basic character of the Fe(III)-OH, although this expectation is based on only a few model complexes characterized under very different conditions [5]. Preliminary efforts to detect the Fe(III)-O(H) Raman mode directly have been hindered by a very small scattering cross-section due to the low molar absorptivity of the mono-nuclear non-heme iron center. This problem may be circumvented by redox-difference infrared (IR) spectroscopy, which allows one to detect redox-linked pK_a s from pH dependencies of experimentally measured redox potentials [73–75].

An alternative possibility involves an unusual geometry that renders the Fe(III)-OH proton Raman-invisible. Such a scenario is plausible if the Fe-O-H is highly distorted from a relaxed geometry. When this ligand bending angle approaches 90°, the vibrational contribution of the proton into the $\nu_{\text{Fe-O}}$ stretching mode is minimized and the effect of $^1\text{H}/^2\text{H}$ substitution on the observed frequency becomes negligible or is reversed in the presence of a strong hydrogen bond [76–79]. Considering the direct interactions of Fe(IV)=O with the substrate proton and the unremarkably “normal” frequency of $\nu_{\text{Fe(IV)=O}}$ in TauD, such steric hindrance appears to be unlikely [5]. The detectable population of the putative alkoxo species FX also argues against the oxo ligand in F3 being protonated. Alkoxides are highly sensitive to hydrolysis and the formation of spectroscopically detectable Fe-O-C species is plausible only if the alkoxo oxygen is not protonated. The hydroxy ligand in the precursor species (presumably F3) would yield a hydro-alkoxy bridge, followed by a rapid dissociation from the metal and the loss of an isotope-sensitive vibration. Lastly, the observed F3 species may belong to a different spin pathway, which is populated to detectable levels at reduced temperature. This possibility appears to be the least likely [5], yet it cannot be completely discounted until direct kinetic resolution between F3 and F4 species is achieved. Further studies are necessary to discern the identity of these species, but their observation by RR methods demonstrates the power of this approach.

Conclusions and Perspective

As demonstrated by this case study of TauD, the application of an array of spectroscopic methods can provide detailed insights into the properties of a 2OG oxygenase metallocenter during substrate binding and catalysis. Additional spectroscopic methods to probe the metallocenter, such as nuclear resonance vibrational spectroscopy [80, 81], have not yet been applied to this enzyme, but have been used for other family members [82]. Non-spectroscopic techniques further extend the significant information available on this enzyme. For example, structural information was obtained for TauD in various states by crystallographic analyses [52, 83]. Metal binding was examined by isothermal titration

calorimetry [84]. ^{18}O -kinetic isotope effect measurements were used to identify the first irreversible reaction of O_2 activation, the step following Fe(III)-superoxo formation [85]. It also has proven useful to supplement these experimental results with computational approaches, such as quantum mechanics/molecular mechanics and density functional theory calculations [57, 86–93], and by comparison to biomimetic models [94, 95]. The combination of these diverse approaches has greatly enhanced our fundamental understanding of the TauD reaction, and such a range of methods applied to other enzymes is certain to provide keen insights into their reaction mechanisms.

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Abbreviations

CD	Circular dichroism
CF	Continuous flow
cw	Continuous wave
DOPA	Dihydroxyphenylalanine
EPR	Electron paramagnetic resonance
ESEEM	Electron spin echo envelope modulation
EXAFS	Extended X-ray absorption fine structure
FQ	Freeze quench
FT	Fourier transform
HYSCORE	Hyperfine sublevel correlation
IR	Infrared
LMCT	Ligand-to-metal charge-transfer
MCD	Magnetic circular dichroism
MLCT	Metal-to-ligand charge-transfer
2OG	2-Oxoglutarate
RR	Resonance Raman
SF	Stopped-flow
TauD	Taurine:2OG dioxygenase
UV	Ultraviolet

XAS X-ray absorption spectroscopy**References**

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Fig. 1. Simplified depiction of the 2OG-dependent hydroxylase reaction cycle illustrating the major spectroscopically characterized intermediates

Fig. 2.

Electronic absorption spectroscopic investigations of TauD. **a** Absolute spectra of TauD apoprotein and holoenzyme (25 μ M, pH 8.0) and **b** difference spectrum associated with Fe(II) binding to apoprotein. **c** Difference spectra of TauD-Fe(II)-2OG-taurine, TauD-Fe(II)-2OG, and TauD-Fe(II)-taurine minus the spectrum of TauD-Fe(II); 0.5 mM protein final concentration. **d** Repetitive spectra obtained for TauD-Fe(II)-2OG (red) mixed with an equal volume of O₂-saturated buffer at 13 s (yellow) to 3 h (blue); 0.28 mM protein final concentration. **e** Stopped-flow difference spectra of TauD-Fe(II)-2OG-taurine mixed with O₂-saturated buffer for 20 ms (solid trace), 68 ms (dot-dash trace), 210 ms (dotted trace), and 10 s (dashed trace) minus the spectrum of TauD-Fe(II); 1.2 mM protein final concentration. **f** Time-dependent changes in absorbance at 318, 520, and 700 nm for the sample shown in panel **e**. Reprinted with permission from [7] (**a** and **b**), [8] (**c**), [9] (**d**), and [10] (**e** and **f**)

Fig. 3.

CD and MCD spectra of TauD. **a** CD spectra of TauD apoprotein (black), TauD·Fe(II) (green), TauD·Fe(II)·2OG (blue), and TauD·Fe(II)·2OG·taurine (red) at 298 K. **b** MCD spectra of TauD·Fe(II) (green), TauD·Fe(II)·2OG (blue), and TauD·Fe(II)·2OG·taurine (red) at 1.8 K in the presence of a 7 tesla (T) magnetic field. **c** Representative MCD spectra of Fe(II) species with octahedral, square pyramidal, trigonal bipyramidal, and tetrahedral ligand geometries. Reprinted with permission from [33] (**a** and **b**) and [31] (**c**)

Fig. 4.

EPR and ESEEM spectra of TauD. **a** Continuous wave (cw)-EPR spectrum of the quaternary complex of TauD·{FeNO}⁷·2OG·taurine collected at 9.682 GHz and at 4.0 K. **b** The top two traces are normalized 3-pulse ESEEM data collected for TauD·{FeNO}⁷·2OG·1,1-²H-taurine (top) and TauD·{FeNO}⁷·2OG·taurine (middle). These data were collected under the following conditions: microwave frequency, 9.682 GHz; magnetic field, 330 mT; τ -value, 144 ns; and sample temperature, 4.0 K. ESEEM data were normalized by division of a bi-exponential decay function. The lowest trace is the ²H-ESEEM component obtained by dividing the top trace by the middle trace. **c** ²H-ESEEM spectra (black traces) obtained by Fourier transformation of the ²H/¹H time domain data. The red traces are fits to a Hamiltonian model for a single coupled ²H nucleus with $A_{\text{iso}} = 0$; $T = 0.191$ MHz; $A_{\text{Frame}} = (0, 26^\circ, 0)$; $e2qQ/h = 0.22$ MHz; and $Q_{\text{Frame}} = (-21^\circ, 52^\circ, 0)$. **d** Stick drawing made from the TauD active site crystal structure (PDB: 1GY9) using the Pymol visualization program. Adapted with permission from [46] (**a** and **b**) and [48] (**c**)

Fig. 5. HYSCORE spectroscopy of TauD. **a** 3-pulse ESEEM spectrum of the ternary complex of TauD• {FeNO}⁷•taurine collected under the following conditions: microwave frequency, 9.68 GHz; magnetic field, 280 mT; τ -value, 84 ns; and sample temperature, 4.0 K. **b** 4-pulse HYSCORE spectra collected under the same conditions as **a**, for the ternary complexes of TauD• {FeNO}⁷•taurine (top) and TauD• {FeNO}⁷•2OG (bottom). Pi pulses for the HYSCORE were 16 ns FWHM and approximately 800 W microwave power, 90° pulse were 16 ns FWHM with a peak power of 200W

Fig. 6.

Mössbauer and XAS analyses of TauD. **a** Mössbauer spectra (4.2 K, 40 mT magnetic field) of TauD·Fe(II) or TauD·Fe(II)·2OG-taurine exposed to O₂ for the times indicated. The separate line just above the 20 ms trace is a simulated spectrum of the reaction intermediate species. **b** Kinetics of formation and decay for the reaction intermediate from panel **a**. **c** Field-dependent changes to the Mössbauer spectrum of the reaction intermediate species at 50 mT or 1, 4, and 8 T. **d** FT of the Fe K-edge EXAFS data for the reaction intermediate (thick line) or anaerobic control sample (thin line). **e** Fourier-filtered EXAFS spectra of the reaction intermediate (thick lines) superimposed with selected fits (thin lines). Reprinted with permission from [10] (**a** and **b**), [56] (**c**), and [60] (**d** and **e**)

Fig. 7.

Static resonance Raman spectra of TauD-Fe(II). The Fe(II)-2OG chromophore was excited using the 568.2 nm laser line and scattering was collected using 90° geometry in an anaerobic spinning cell at room temperature: A, TauD apoprotein; B, TauD-Fe(II)-2OG; C, TauD-Fe(II)-2OG in H_2^{18}O ; D, TauD-Fe(II)-2OG-aurine; and E, TauD-Fe(II)-2OG-aurine in H_2^{18}O . Vibrational assignments for TauD-Fe(II)-2OG (top) and TauD-Fe(II)-2OG-aurine (bottom) show the keto carbonyl stretching and chelate ring vibration modes, the site of isotopic labeling, and the loss of coordinated water upon taurine binding. The spectra were reprinted with permission from [66]

Fig. 8.

Time-resolved RR spectra of TauD. **a** Schematic diagram of the isotope-difference cryogenic CF system with its active mixing, including an in situ optical probe. Processing of the alternating isotope substitution to absolute and isotope-difference Raman spectra is illustrated underneath. **b** Time-resolved, isotope difference ($^{16}\text{O}_2$ - $^{18}\text{O}_2$) spectra of oxygen-containing species of TauD obtained with ^1H - and ^2H -taurine at -36°C (gray traces) and their quantitative analysis using global spectral regression (black traces). The ▲ and ◆ symbols indicate a laser plasma line and a major ethylene glycol peak, respectively. **c** Illustration of the use of $^{16}\text{O}^{18}\text{O}$ mixed oxygen isotope to distinguish between oxo and peroxo structures of reactive intermediates, whose $^{16}\text{O}_2$ / $^{18}\text{O}_2$ differences are similar. **d** Vibrational assignments of two major transient intermediates detected in TauD at -36°C . Two alternative structures are shown for F3. Panel **b** was reprinted with permission from [68]