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Cr^{II} Reactivity of Taurine/α-Ketoglutarate Dioxygenase

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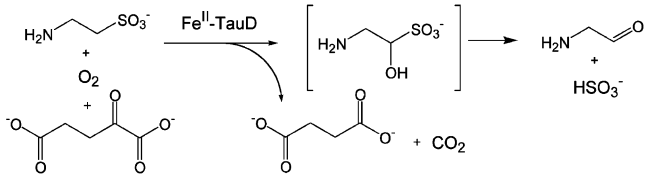
The interaction of Cr^{II} with taurine/α-ketoglutarate (αKG) dioxygenase (TauD) was examined. Cr^{II} replaces Fe^{II} and binds stoichiometrically with αKG to the Fe^{II}/αKG binding site of the protein, with additional Cr^{II} used to generate a chromophore attributed to a Cr^{III}–semiquinone in a small percentage of the sample. Formation of the latter oxygen-sensitive species requires the dihydroxyphenylalanine (DOPA) quinone form of Tyr-73. This preformed side chain is generated by intracellular self-hydroxylation of Tyr-73 to form DOPA, which is subsequently oxidized to the quinone. No chromophore is generated when using $NaBH_4$-treated sample, protein isolated from anaerobically grown cells, inactive TauD variants that are incapable of self-hydroxylation, or the Y73F active mutant of TauD. A Cr^{III}–DOPA semiquinone also was observed in the herbicide hydroxylase SdpA.

Introduction

Taurine/α-ketoglutarate (αKG) dioxygenase (TauD) is an Fe^{II}/αKG-dependent hydroxylase that couples the oxidative decarboxylation of αKG to the hydroxylation of taurine (2-aminoethanesulfonate) as depicted in Scheme 1.[1] This extensively characterized enzyme serves as the prototype of a large group of related enzymes with diverse functions ranging from DNA repair to hypoxic signaling to the synthesis or degradation of a plethora of biologically significant small molecules.[2−5] TauD apoprotein binds Fe^{II} via its His-99, Asp-101, and His-255 side chains, with three water molecules completing the hexacoordinate geometry. Two waters are displaced by αKG, which chelates the metal to produce an MLCT transition (λ_{max} 530 nm, ϵ_{530} 140−240 M^{-1} cm^{-1}).[6] The remaining water is displaced upon the binding of the substrate near the metal center, leading to a perturbation of the MLCT band (λ_{max} 520 nm, ϵ_{520} 180−270 M^{-1} cm^{-1}) and creating a site where oxygen binds. The resulting Fe^{III}−superoxo species attacks the keto group of the bound αKG, leading to its decomposition and the formation of an Fe^{IV}−oxo intermediate that has been characterized by stopped-flow UV−vis, freeze-quench Mössbauer, X-ray absorbance, and continuous-flow resonance Raman spectroscopies.[7−12] In the absence of its primary substrate taurine, TauD carries out aberrant side reactions that result in the formation of a dihydroxyphenylalanine (DOPA) side chain replacing Tyr-73[13−15] or the hydroxyla-

Scheme 1



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tion of Trp-128, Trp-240, and Trp-248 (unpublished data summarized earlier).[16]

Other divalent metal ions cannot substitute for Fe^{II} in generating TauD activity; however, several metals bind to the TauD active site and inhibit the enzyme. Bound Co^{II} exhibits a broad absorption (λ_{max} 530 nm, 70 M^{-1} cm^{-1}) that changes little upon αKG binding, whereas the subsequent addition of taurine generates three distinct transitions (λ = 500, 552, and 565 nm, with ϵ = 127, 204, and 200 M^{-1} cm^{-1}, respectively) consistent with a shift from 6- to 5-coordinate geometry.[17] Ni^{II} also binds to the TauD active site, but does not afford any chromophore. Both of these metal ions are slow-binding inhibitors of this and several related enzymes.[17] Of special interest, these metals are hypoxia mimics due to their ability to bind to and inhibit specific prolyl hydroxylases that modify the hypoxia inducible factor involved in the response to varied oxygen concentrations.[18] Cu^{II} also substitutes for Fe^{II} in members of this class of enzyme and provides a useful paramagnetic probe, as illustrated by studies with the herbicide-degrading TfdA.[19] For example, the TfdA metal ligands were identified by spectroscopic analysis of Cu^{II}-substituted site-directed variants.[20]

Here, we characterize the surprisingly complex interaction of TauD with Cr^{II}. We report that stoichiometric amounts of Cr^{II} bind with αKG to the Fe^{II}/αKG binding site of the anaerobic apoprotein and that additional Cr^{II} participates in the formation of an intense spectrum consistent with a Cr^{III}–semiquinone in a portion of the sample. We compare the extent of chromophore generation in protein isolated from anaerobically grown cells, from $NaBH_4$-reduced TauD, and from several variant TauD proteins, and we show that the chromophore arises from the metal interaction with a preformed protein-bound quinone at Tyr-73 that is generated during cell culture and enzyme purification under aerobic conditions. Finally, we demonstrate the formation of a similar chromophore in another member of this enzyme family.

Experimental Section

Enzyme Purification and Assays. Wild-type TauD and its variants were purified as apoproteins, as previously described.[6,13,21] The Y73F variant of TauD was newly constructed by mutagenesis of tauD in pME4141[1] by using the Stratagene Quickchange System (Stratagene, La Jolla, CA) as detailed previously.[13] A sample of wild-type TauD was purified from cells grown anaerobically. TauD activity was measured using Ellman's reagent to quantify the sulfite,[1] with one unit (U) of enzyme activity defined as the amount of the enzyme that releases 1 μmol of sulfite per min at 30 °C in assay buffer containing 25 mM Tris (pH 8.0), 50 μM Fe^{II}, 100 μM ascorbate, 500 μM αKG, and 1 mM taurine. Several other Fe^{II}/αKG dioxygenases were purified as previously described, including SdpA,[22] a TfdA-like protein from *Bordetella pertussis* (fused to the maltose binding protein),[23] *Aspergillus nidulans* xanthine hydroxylase (XanA) purified from *Escherichia coli*,[24] and a full-length version (unpublished studies) of the Gab protein (CsiD) from *E. coli*.[25] Pea seedling amine oxidase,[26] provided by David Dooley and Doreen Brown, was examined as an authentic trihydroxyphenylalanyl quinone-containing protein. Protein concentrations were determined on the basis of the method of Bradford.[27]

Spectroscopy. Samples of TauD (500 or 250 μM subunit in 25 mM Tris buffer, pH 8.0) were subjected to several cycles of vacuum and argon purging in an anaerobic cuvette and adjusted to the indicated concentrations of Cr^{II}, αKG, or other additives. UV–vis difference spectra (using a Shimadzu 2401PC) were obtained for the metal-loaded wild-type and variant enzymes (with the spectrum of TauD apoprotein subtracted) as previously described.[6,21] Kinetic measurements utilized a Hewlett-Packard 8453 diode array spectrophotometer fitted with a thermostatted cuvette holder and a magnetic stirrer. ^1H NMR spectroscopy made use of a 300 MHz Varian spectrometer and used sample prepared in D_2O.

HPLC Organic Acids Analysis. Cr^{II}- and αKG-treated enzyme aliquots (200 μL) were precipitated by the addition of 50 μL 6 M HCl, filtered through a 0.2 μm spin column, and chromatographed on a Waters Breeze Chromatography system using a Bio-Rad HPX-87H organic acids column (Bio-Rad Laboratories; Richmond, CA) with 13 mM sulfuric acid as solvent, at a flow rate of 0.5 mL/min at room temperature. Detection was accomplished by means of a UV detector set at 210 nm and by the use of a refractive index detector. Standard samples included αKG, succinate, and hydroxyglutarate.

Chromium Content. TauD (550 μM subunit) was mixed with 2 mM αKG and 500 μM Cr^{II} in 25 mM Tris buffer (pH 8) under anaerobic conditions, exchanged into deionized water by repeated concentration/dilution using an Amicon Ultra 15, and the metal content was assessed by inductively coupled plasma emission spectrometry at the University of Georgia Chemical Analysis Laboratory.

Results and Discussion

Electronic Absorption Spectra of TauD plus Cr^{II}. Anaerobic addition of Cr^{II} to TauD apoprotein in Tris buffer, pH 8.0, yields a weak and broad absorption at approximately 600 nm (30 M^{-1} cm^{-1}) and another feature at 426 nm (23 M^{-1} cm^{-1}) as shown in Figure 1. These transitions do not arise from Cr^{II}, which exhibits a broad and very weak (5 M^{-1} cm^{-1}) absorption at 714 nm in water; rather, the

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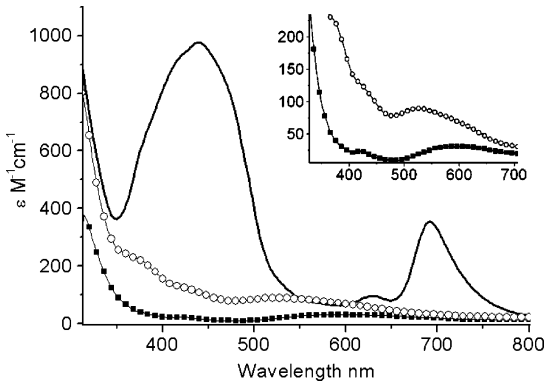

Figure 1. Electronic absorption difference spectra of Cr^{II}-treated TauD. Wild-type TauD (0.5 mM subunit in 25 mM Tris buffer, pH 8.0) was made anaerobic and adjusted to contain 0.5 mM Cr^{II} (♦) and 2 mM αKG (solid line), followed by the addition of gaseous oxygen (○). The difference spectra are versus protein alone, with ϵ based on the subunit concentration.

observed features are likely to correspond to low concentrations of Cr^{III} generated during the spontaneous reduction of protons.[28] Upon the addition of αKG, an intense spectrum is instantaneously generated with a broad feature centered at 434 nm (1000 M^{-1} cm^{-1}), a small feature at 630 nm (100 M^{-1} cm^{-1}), and a sharper transition at around 690 nm (350 M^{-1} cm^{-1}), with the resulting sample exhibiting a distinct yellow color. Subsequent addition of buffer saturated with 100% oxygen or sparging with gaseous oxygen results in the immediate loss of the yellow color and the disappearance of the intense spectrum, with the final species displaying weak transitions at 525 nm (85 M^{-1} cm^{-1}), ∼426 nm (shoulder, ∼123 M^{-1} cm^{-1}), and 380 nm (200 M^{-1} cm^{-1}) as shown in the inset.

The intense spectrum generated for anaerobic TauD plus Cr^{II} and αKG exhibits a remarkable similarity to that of [Cr^{III}(tren)(3,6-di-*tert*-butylorthosemiquinone)]$(PF_6)_2$[29] and Cr^{III}(tren)(semiquinato)]($^nBu_4N$)BF_4,[30] both illustrated in Figure S1 (Supporting Information), and other related Cr^{III}−semiquinones.[31−36] In contrast to the yellow color of the anaerobic Cr^{II}-treated, αKG-bound protein sample, the model complexes are typically referred to as green or yellow-green in color. Ligand-field theory analysis of the spectrum of the Cr^{III}-quinone dyad[29] indicates symmetry lowering to C_{2v} with consequent splitting, followed by further splitting as the unpaired spins of the metal ion antiferromagnetically couple to the semiquinone $S = {}^1/_2$ spin of b_1 symmetry. The 665 nm doublet feature associated with the first model complex is assigned to spin-allowed $^3B_1 \leftarrow {}^3B_1$ absorptions; however, the observed extinction coefficient is an order of magnitude greater than the expected value of approximately 100 M^{-1} cm^{-1}.[29] The assignment is less clear for the intense feature found at 460 nm in this model complex (5600 M^{-1} cm^{-1}), with suggestions including a $^3MLCT \leftarrow {}^3B_1$ transition and another exchange-enhanced ligand-field band.[29] Related model compounds display variations in the positions and intensities of these features, with the ∼450 nm band ranging from 2860 to 13 600 M^{-1} cm^{-1}.[30−36] As might be expected for a protein metallocenter, the corresponding features in the TauD spectrum are broadened compared to most, but not all, of the model compounds. The intensities of these features varied for different TauD preparations, with the absorption at 434 nm typically ranging from 7 to 35% of those reported in the model systems. Thus, the chromophore likely is present at much less than stoichiometric levels in the protein.

Source of Cr^{III}−Semiquinone Chromophore in TauD. To account for the formation of a putative Cr^{III}−semiquinone chromophore in TauD, we propose that the added Cr^{II} binds and transfers an electron to a pre-existing protein-bound quinone. A potential source of a quinone is the DOPA side chain created by self-hydroxylation of Tyr-73.[13−15] Two distinct chemical mechanisms have been shown to generate this protein modification. In one case, an O_2- and αKG-dependent process forms an Fe^{IV}−oxo intermediate that adds an oxygen atom derived from solvent.[13] In the second mechanism, succinate and O_2 are used to create a putative Fe^{III}−OOH intermediate that generates DOPA, with the added hydroxyl group derived from gaseous O_2.[15] One might expect that aerobically grown cultures, which contain αKG and succinate, would generate small amounts of DOPA in TauD. The DOPA could further oxidize spontaneously to DOPA quinone during protein purification, and we have found increased production of the chromium-related chromophore in enzyme samples where the isolation had taken more time. As further evidence for DOPA quinone in the protein, the addition of Fe^{II} to anaerobic TauD samples results in the development of an apparent Fe^{III}−catecholate chromophore at fractional abundance.[21] Furthermore, a redox-cycling nitroblue tetrazolium gel blot stain[37,38] confirms the presence of a catechol/quinone side chain in TauD isolated from aerobic cultures.[21]

Three experiments were carried out to test the hypothesis that the DOPA quinone state of Tyr-73 reacts with Cr^{II} to generate a Cr^{III}−semiquinone chromophore in TauD. First, TauD from aerobically grown cells was treated with 4 equiv of $NaBH_4$ to reduce any endogenous quinone species. This reduced protein was separated from excess reagent by gel filtration using a Sephadex G25 spin column, and subsequently shown to be incapable of forming the Cr^{II}/αKG-dependent chromophore (data not shown). Similarly, TauD isolated from anaerobically grown cells failed to generate the presumed Cr^{III}−semiquinone spectrum (part A of Figure 2); whereas it does form the MLCT transitions associated

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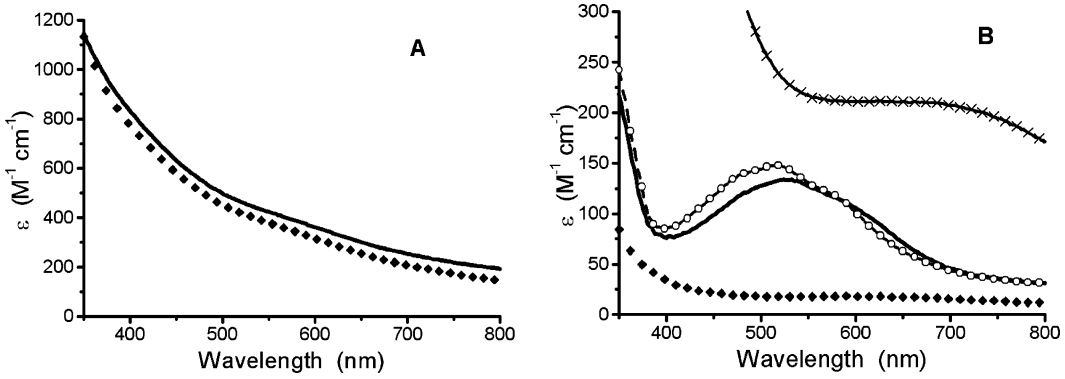
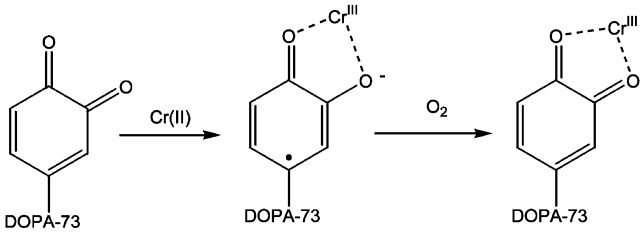


Figure 2. Electronic absorption difference spectra of Cr^{II}- and Fe^{II}-treated TauD purified from anaerobically grown cultures. (A) TauD was isolated from cells grown in the absence of oxygen (0.5 mM subunit in 25 mM Tris buffer, pH 8.0), made anaerobic, and adjusted to 0.5 mM Cr^{II} (♦) plus 2 mM αKG (solid line), and difference spectra were obtained versus protein alone. (B) Anaerobic TauD (0.5 mM) was adjusted to contain 0.5 mM Fe^{II} (♦), 2 mM αKG (solid line), and 2 mM taurine (○), and then exposed to gaseous O_2 for 12 h (×).

Scheme 2



with αKG-Fe^{II}- and taurine-αKG-Fe^{II}-bound enzyme (530 and 520 nm, respectively), with the latter species producing an Fe^{III}−catecholate spectrum (∼700 nm) when exposed to oxygen (part B of Figure 2). Finally, the Y73F variant of TauD was ineffective at generating the novel chromophore (panel A in Figure S2, Supporting Information).

On the basis of the above studies, we conclude that electron transfer from the Cr^{II} to the quinone form of DOPA-73 found in a small proportion of TauD leads to the formation of a Cr^{III}−semiquinone (Scheme 2). A subsequent reaction of this chromophoric sample with O_2 is suggested to yield the nearly colorless Cr^{III}−semiquinone that dissociates to yield Cr^{III} and the DOPA quinone side chain. The oxygen reactivity of the chromophore-containing protein sample differs from some Cr^{III}−semiquinone model complexes that are stable in the presence of this oxidant.

Characterization of Cr^{II} Interaction with Additional TauD Variants. A suite of TauD variants was examined for their reactivity with Cr^{II} and αKG. Prior studies had reported on the steady-state kinetics of these mutant proteins and examined their abilities to generate an Fe^{III}−catecholate chromophore, presumably formed by quinone interaction with one Fe^{II} and electron donation by a second metal ion.[21] The highly active variants (H255Q, H255E, and D101E) exhibit 81, 33, and 22% of the k_{cat} of wild-type enzyme and generate Fe^{III}−catecholate spectra;[21] these samples all form spectra consistent with the formation of significant levels of Cr^{III}−semiquinone when amended with Cr^{II} and αKG (Figure 3, panels A, B, and C). In contrast, the essentially inactive H99A, H99E, H99Q, D101Q, H255D, and H255N variants form neither the Fe^{III}−catecholate spectra[21] nor the Cr^{III}−semiquinone species (panels B−G in Figure S2, Supporting Information). Surprisingly, the essentially inactive D101C variant, which does not generate the Fe^{III}−catecholate spectra[21] exhibits significant levels of the Cr^{III}−semiquinone (panel H in Figure S2, Supporting Information). Also of interest is the R270K variant of TauD that is inactive because it cannot bind taurine. This protein does bind Fe^{II} and αKG (as shown by the formation of the resulting chromophore)[12] and should be able to catalyze the uncoupled reaction to hydroxylate Tyr-73; as expected, this protein forms the Cr^{III}−semiquinone spectrum (part D of Figure 3). In general, the ability to form a Cr^{III}−semiquinone chromophore in TauD requires that the enzyme be capable of using iron-based chemistry to first hydroxylate its Tyr-73.

Concentration Dependence of Cr^{II} and αKG for Chromophore Generation. The Cr^{III}−semiquinone chromophore reached maximal intensity with the addition of approximately 1 equiv of Cr^{II} per αKG-bound enzyme (Figure 4). Cr^{II} in excess of 1.5 equiv led to protein precipitation. Given the low intensity of the chromophore formed in TauD compared to that of model compounds,[29−36] we interpret the stoichiometry data to indicate that 1 equiv of Cr^{II} binds along with αKG at the typical Fe^{II}/αKG binding site of TauD to yield an essentially featureless species, whereas additional Cr^{II} binds to the quinone present in only a small proportion of the protein. Nearly stoichiometric binding of metal was confirmed by ICP analysis; that is, for sample incubated with 0.91 equiv of Cr^{II}, 0.86 equiv of metal were detected after multiple cycles of buffer exchange.

For Cr^{II} concentrations that were less than stoichiometric compared to protein and αKG, the samples exhibited a flash of yellow color that decayed to the indicated absorbance values. Kinetic studies were performed to determine the effects of varied Cr^{II} concentrations on the apparent first-order rate constants of chromophore formation and decay at 22 °C. Because of the inherent instability of solutions containing low concentrations of Cr^{II}, stopped-flow spectrophotometric studies were precluded. Rather, the kinetics were examined by injecting small volumes of concentrated Cr^{II} solutions (30 mM) into anaerobic buffered solutions of protein (0.5 mM) and αKG (2 mM) with rapid mixing, while monitoring changes in the chromophore intensity. The transitions associated with the transient chromophore were indistinguishable from those observed in the species gener-

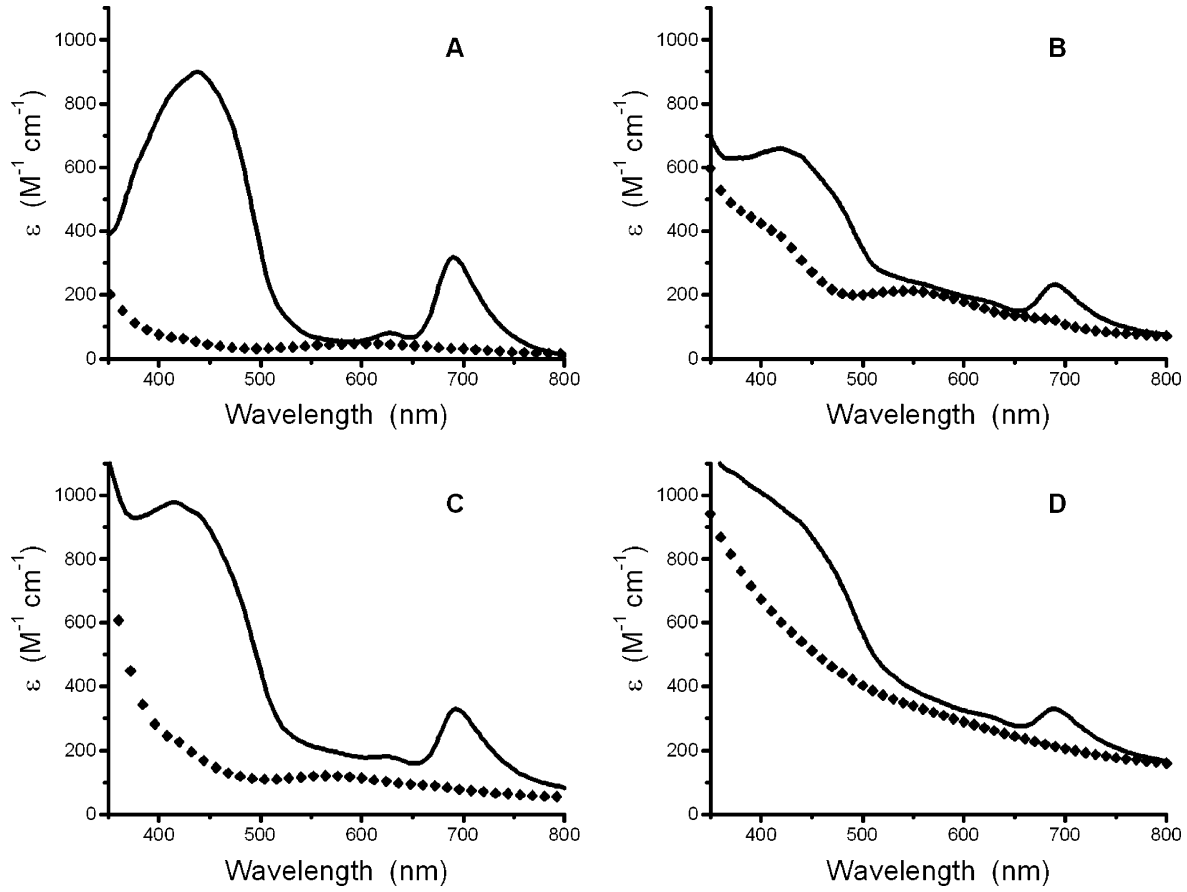


Figure 3. Electronic absorption difference spectra of Cr^{II}- and αKG-treated TauD mutants. Variant forms of TauD (0.5 mM subunit in 25 mM Tris buffer, pH 8.0) were made anaerobic and adjusted to contain 0.5 mM Cr^{II} (closed diamonds) followed by the addition of 2 mM αKG (solid line), and the difference spectra were obtained versus protein alone. (A) H255Q, (B) H255E, (C) D101E, and (D) R270K TauD. Spectra of additional mutants are provided in Figure S2 in the Supporting Information.

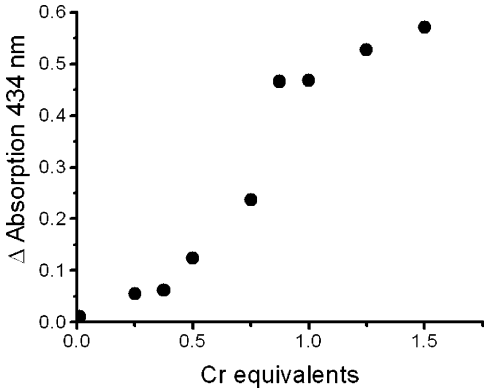


Figure 4. Cr^{II} concentration dependence of Cr^{III}−semiquinone formation. A series of difference absorption spectra (versus protein alone) were collected for 0.5 mM TauD samples in 25 mM Tris (pH 8.0) buffer after making them anaerobic and adjusting to the indicated concentration of Cr^{II} and 0.5 mM αKG. The absorbance difference at 434 nm is depicted for each sample.

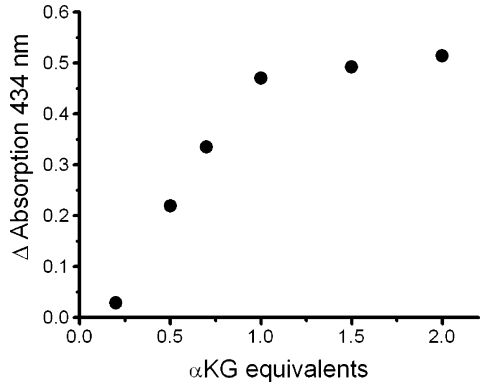
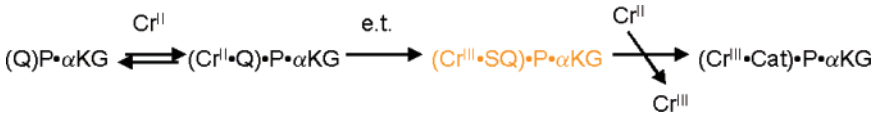


Figure 5. αKG concentration dependence of Cr^{III}−semiquinone formation. A series of difference absorption spectra (versus protein alone) were collected for 0.5 mM TauD samples in 25 mM Tris (pH 8.0) buffer that were made anaerobic and adjusted to contain 0.5 mM Cr^{II} and various concentrations of αKG. The absorbance difference at 434 nm is depicted for each sample.

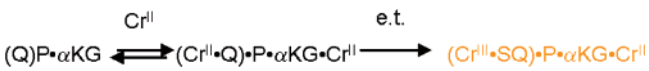
ated at higher concentrations of Cr^{II} (Figure 1). The apparent first-order rate constants of chromophore formation (3.3 ± 1.2 s^{-1}) and decay (0.43 ± 0.29 s^{-1}) were essentially independent of Cr^{II} concentration from 50 μM to 250 μM Cr^{II}, indicating that these color changes are associated with steps that follow the binding of Cr^{II}. We interpret these results in terms of Scheme 3. Thus, when substoichiometric levels of Cr^{II} are added to TauD plus αKG (P·αKG), a portion of the metal reacts with the endogenous quinone (Q), found in a small proportion of the enzyme, to generate the chromophoric Cr^{III}−semiquinone species (Cr^{III}·SQ). The Cr^{II} remaining in solution can access the Cr^{III}−semiquinone, and reduces it to yield the Cr^{III}−catecholate species (Cr^{III}·Cat·P·αKG).

As illustrated in Scheme 4, rapid decay of the chromophore does not occur when using stoichiometric levels of Cr^{II} because the metal is available to bind to the Fe^{II} binding site. We suggest that the Cr^{III}−semiquinone species is

Scheme 3



Scheme 4



inaccessible to excess Cr^{II} when both αKG and Cr^{II} are bound at the TauD active site.

In a similar manner, the αKG concentration dependence was examined for anaerobic protein in the presence of Cr^{II} (Figure 5). These data represent a series of separate Cr^{II}–TauD samples with the indicated concentrations of αKG added to each sample. A similar stoichiometry was observed when a single sample was titrated with increasing levels of αKG; however, the intensity of the final response was somewhat lower in that experiment. This discrepancy likely arises from three processes. First, excess Cr^{II} may reduce some of the Cr^{III}–semiquinone (analogous to Scheme 3) because αKG is not available to sequester this species from metal ions in the solvent. Second, the repeated injections may lead to the inadvertent introduction of oxygen during the experiment. Finally, the chromophore associated with the αKG-Cr^{II}-TauD species is inherently unstable; for example, the absorption at 434 nm decayed with an apparent rate constant of 0.0015 absorption units per min for the 0.5 mM TauD sample provided with 0.5 mM Cr^{II} plus 0.5 mM αKG.

Role of αKG in Formation of the Cr^{III}–Semiquinone Chromophore. The spectroscopic species of interest does not form when TauD is treated with just Cr^{II}, raising questions about whether αKG undergoes some type of chemical reactivity in generating the chromophore. Pyruvate, an alternative α-ketoacid, is not utilized as a cosubstrate by the enzyme, but it yields the chromophore with low efficiency (panel A in Figure S3, Supporting Information). In contrast, neither *N*-oxalylglycine, a cosubstrate analogue and competitive inhibitor,[17] nor the reduced analogue 2-hydroxyglutarate support formation of the Cr^{III}–semiquinone species (panels B and C in Figure S3, Supporting Information). ^{1}H NMR experiments reveal that αKG is not consumed during chromophore formation, a result confirmed by HPLC studies, which detect no new species associated with this reaction. These results are consistent with αKG acting in a structural role to prevent excess Cr^{II} from accessing and reducing the protein-bound Cr^{III}–semiquinone (Scheme 4).

Characterization of Cr^{II} Interaction with Other Proteins. A variety of other Fe^{II}/αKG dioxygenases were examined for their ability to generate a Cr^{III}–semiquinone species. Such a spectrum was observed (Figure S4, Supporting Information) in the herbicide hydroxylase SdpA; thus, the chromophore species is not unique to TauD. SdpA is 30% identical to TauD and is predicted to have Tyr-107 positioned near its active site.[22] In contrast, neither XanA, a xanthine hydroxylase of *A. nidulans*,[24] a TfdA-like protein from *B. pertussis*,[23] nor CsiD from *E. coli*[25] exhibit this feature when incubated with Cr^{II} and αKG; thus, suggesting that quinones are not produced to a detectable extent in these proteins or, if present, the Cr^{III}–semiquinone species are immediately reduced by excess Cr^{II} (as in Scheme 3). Similarly, bovine serum albumin failed to generate a Cr^{III}–semiquinone spectrum (data not shown). Pea seedling amine oxidase, an authentic quinone-containing protein,[26] precipitated upon the addition of Cr^{II}.

Conclusions

These studies demonstrate that stoichiometric levels of Cr^{II} and αKG bind to the Fe^{II}/αKG binding site of TauD and that additional Cr^{II} binds to previously formed Tyr-73 DOPA quinone (present at substoichiometric levels in catalytically active forms of TauD) forming, after electron transfer, a Cr^{III}–semiquinone. This work shows for the first time that fundamental investigations of model Cr^{III}–semiquinones[29–36,39,40] are relevant to biological samples. Cr^{III}-containing compounds can enter cells and, depending on the ligand identity (which controls the metal redox potential), can be reduced to Cr^{II} by biological reductants such as ascorbate.[41] It is not unreasonable to speculate that Cr^{II} in the cell could bind to protein-associated DOPA quinones to generate Cr^{III}–semiquinones; however, both of these species and Cr^{II} itself are oxygen sensitive and would not accumulate under aerobic conditions.

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Supporting Information Available: Spectra for two Cr^{III}–semiquinone model compounds and several Cr^{II}-treated samples as described in the text. Ordering information is given on any current masthead page. This material is available free of charge via the Internet at http://pubs.acs.org.

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