Published in final edited form as:

Inorg Chem. 2007 November 26; 46(24): 10087–10092. doi:10.1021/ic700383q.

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

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Abstract

The interaction of Cr^{II} with taurine/ α -ketoglutarate (α KG) dioxygenase (TauD) was examined. Cr^{II} replaces Fe^{II} and binds stoichiometrically with α KG to the Fe^{II}/ α KG-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 pre-formed 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 NaBH₄-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 (α KG) dioxygenase (TauD) is an Fe^{II}/ α KG-dependent hydroxylase that couples the oxidative decarboxylation of α KG 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 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 α KG, which chelates the metal to produce a MLCT transition (λ_{max} 530 nm, ϵ_{530} 140–240 M⁻¹ cm⁻¹).⁶ The remaining water is displaced upon binding enter, 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 α KG, leading to its decomposition and formation of an Fe^{IV}-oxo intermediate that has been characterized by stopped-flow UV-visible, 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 formation of a dihydroxyphenyl-alanine (DOPA) side chain replacing Tyr-73^{13–15} or the hydroxylation 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 α KG binding, while 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

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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 α KG to the Fe^{II}/ α KG binding site of 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 NaBH₄-reduced TauD, and from several variant TauD proteins, and we show that the chromophore arises from 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 pME4141¹ 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 by using Ellman's reagent to quantify sulfite,¹ with one unit (U) of enzyme activity defined as the amount of enzyme that releases 1 μ mol of sulfite per min at 30 °C in assay buffer containing 25 mM Tris (pH 8.0), 50 μ M Fe^{II}, 100 μ M ascorbate, 500 μ M α KG, and 1 mM taurine. Several other Fe^{II}/ α KG 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 *E. 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 Bradford²⁷

Spectroscopy

Samples of TauD (500 or 250 μ M 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}, α KG, or other additives. UV-visible 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 D₂O.

HPLC organic acids analysis

Cr^{II}- and α KG-treated enzyme aliquots (200 μ L) were precipitated by addition of 50 μ L 6 M HCl, filtered through a 0.2 μ m 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 254

nm and by use of refractive index detector. Standard samples included α KG, succinate, and hydroxyglutarate.

Chromium content

TauD (550 μ M subunit) was mixed with 2 mM α KG and 500 μ M 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 ($30 \text{ M}^{-1} \text{ cm}^{-1}$) and another feature at 426 nm ($23 \text{ M}^{-1} \text{ cm}^{-1}$) as shown in Figure 1A. These transitions do not arise from Cr^{II} , which exhibits a broad and very weak ($5 \text{ M}^{-1} \text{ cm}^{-1}$) absorption at 714 nm in water; rather, the observed features are likely to correspond to low concentrations of Cr^{III} generated during the spontaneous reduction of protons.²⁸ Upon addition of α KG, an intense spectrum is instantaneously generated with a broad feature centered at 434 nm ($1000 \text{ M}^{-1} \text{ cm}^{-1}$), a small feature at 630 nm ($100 \text{ M}^{-1} \text{ cm}^{-1}$), and a sharper transition at around 690 nm ($350 \text{ M}^{-1} \text{ cm}^{-1}$) 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 disappearance of the intense spectrum, with the final species displaying weak transitions at 525 nm ($85 \text{ M}^{-1} \text{ cm}^{-1}$), ~426 nm (shoulder, $\sim 123 \text{ M}^{-1} \text{ cm}^{-1}$), and 380 nm ($200 \text{ M}^{-1} \text{ cm}^{-1}$).

The intense spectrum generated for anaerobic TauD plus Cr^{II} and α KG exhibits a remarkable similarity to that of $[\text{Cr}^{\text{III}}(\text{tren})(3,6\text{-di-}i\text{-tert-butylorthosemiquinone})](\text{PF}_6)_2$ ²⁹ and $\text{Cr}^{\text{III}}(\text{tren})(\text{semi-quinato})(^n\text{Bu}_4\text{N})\text{BF}_4$,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, α KG-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 dyad²⁹ 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 $S = \frac{1}{2}$ 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 $100 \text{ M}^{-1} \text{ cm}^{-1}$.²⁹ The assignment is less clear for the intense feature found at 460 nm in this model complex ($5600 \text{ M}^{-1} \text{ cm}^{-1}$), with suggestions including a ${}^3\text{MLCT} \leftarrow {}^3B_1$ 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 $13600 \text{ M}^{-1} \text{ cm}^{-1}$.^{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 α KG-dependent process forms an Fe^{IV} -oxo intermediate

that adds an oxygen atom derived from solvent.¹³ In the second mechanism, succinate and O₂ are used to create a putative Fe^{III}-OOH intermediate that generates DOPA with the added hydroxyl group derived from gaseous O₂.¹⁵ One might expect that aerobically grown cultures, which contain α KG 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 Cr-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 four equivalents of NaBH₄ to reduce any endogenous quinone species. This reduced protein was separated from excess reagent by gel filtrations using a Sephadex G25 spin column, and subsequently shown to be incapable of forming the Cr^{II}/ α KG-dependent chromophore (data not shown). Similarly, TauD isolated from anaerobically grown cells failed to generate the presumed Cr^{III}-semiquinone spectrum (Figure 2A); whereas it does form the MLCT transitions associated with α KG-Fe^{II}- and taurine- α KG-Fe^{II}-bound enzyme (530 nm and 520 nm, respectively), with the latter species producing an Fe^{III}-catecholate spectrum (~700 nm) when exposed to oxygen (Figure 2B). Finally, the Y73F variant of TauD was ineffective at generating the novel chromophore (Figure S2, panel A).

On the basis of the above studies, we conclude that electron transfer from 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). Subsequent reaction of this chromophoric sample with O₂ is suggested to yield the nearly colorless Cr^{III}-semiquinone that dissociates to yield Cr^{III} and the DOPA quinone sidechain. 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 α KG. 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% 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 α KG (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 spectra²¹ nor the Cr^{III}-semiquinone species (Figure S2, panels B-G). Surprisingly, the essentially inactive D101C variant which does not generate the Fe^{III}-catecholate spectra²¹ exhibits significant levels of the Cr^{III}-semiquinone (Figure S2, panel H). Also of interest is the R270K variant of TauD that is inactive because it cannot bind taurine. This protein does bind Fe^{II} and α KG (as shown by 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 (Figure 3D). 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 α KG for Chromophore Generation

The Cr^{III}-semiquinone chromophore reached maximal intensity with the addition of approximately one equivalent of Cr^{II} per α KG-bound enzyme (Figure 4). Cr^{II} in excess of 1.5 equivalents 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 one equivalent of Cr^{II} binds along with α KG at the typical Fe^{II}/ α KG-binding site of TauD to yield an essentially featureless species while 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; i.e., for sample incubated with 0.91 equivalents of Cr^{II}, 0.86 equivalents of metal were detected after multiple cycles of buffer exchange.

For Cr^{II} concentrations that were less than stoichiometric compared to protein and α KG, 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 α KG (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 generated at higher concentrations of Cr^{II} (Figure 1). The apparent first-order rate constants of chromophore formation ($3.3 \pm 1.2 \text{ s}^{-1}$) and decay ($0.43 \pm 0.29 \text{ s}^{-1}$) 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 α KG (P- α KG), 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- α KG).

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

In a similar manner, the α KG 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 α KG added to each sample. A similar stoichiometry was observed when a single sample was titrated with increasing levels of α KG; 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 α KG 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 α KG-Cr^{II}-TauD species is inherently unstable; e.g., the absorption at 434 nm decayed with an apparent rate constant of 0.0015 absorption unit per min for 0.5 mM TauD sample provided with 0.5 mM Cr^{II} plus 0.5 mM α KG.

Role of α KG 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 α KG 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 (Figure S3, panel A, 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 (Figure S3, panels B and C). ¹H-NMR experiments reveal that α KG 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 α KG 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}/ α KG dioxygenases were examined for their ability to generate a Cr^{III}-semiquinone species. Such a spectrum was observed (Figure S4) 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 α KG; 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 addition of Cr^{II}.

Conclusions

These studies demonstrate that stoichiometric levels of Cr^{II} and α KG bind to the Fe^{II}/ α KG-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 these species and Cr^{II} itself are oxygen sensitive and would not accumulate under aerobic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Kermit Johnson for assistance with the NMR studies, Denis Proshlyakov for use of his spectrophotometer, Dave Dooley and Doreen Brown for pea seedling amine oxidase, Matthew Ryle for TauD from anaerobically grown cells and providing the Y73F construct, Tina Müller for SdpA, Meng Li for *B. pertussis* TfdA-like protein and XanA, Thalia Kalliri for CsiD, and Melody Campbell for assistance with some of the experiments. Finally, we thank Dong Guo, Joel Schrauben, and Jim McCusker for helpful comments. This work was supported by NIH grant GM065384.

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Figure 1.

Electronic absorption difference spectra of Cr^{II} -treated TauD. (A) 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 addition of gaseous oxygen (○). The difference spectra are versus protein alone, with ϵ based on the subunit concentration.

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} ($\blacklozenge$) 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} ($\blacklozenge$), 2 mM αKG (solid line), and 2 mM taurine ($\circ$), and then exposed to gaseous O_2 for 12 h ($\times$).

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 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 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. α KG 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 α KG. The absorbance difference at 434 nm is depicted for each sample.

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.