Published in final edited form as:

Anal Biochem. 2010 April 1; 399(1): 64–71. doi:10.1016/j.ab.2009.11.022.

Metal and substrate binding to Fe(II) dioxygenase resolved by UV spectroscopy with global regression analysis

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Abstract

The addition of divalent metal ions or substrate taurine to TauD, an α -ketoglutarate dependent dioxygenase, alters its UV absorption, as clearly observed by monitoring the protein's difference spectra. Binding of metal ions leads to a decrease in absorption at ~ 297 nm and modulation of other features. A separate signature with enhanced absorption at ~ 295 nm is identified for binding of taurine. These narrow (~ 700 cm⁻¹) and intense (~ 0.5 mM⁻¹ cm⁻¹) spectral changes are attributed to ligand-induced protein conformational changes affecting the environment of aromatic residues. The changes in the UV difference spectra were exploited to assess directly the thermodynamics and kinetics of ligand interactions in wild-type TauD and selected variants. This approach holds promise as a new tool to probe ligand-induced conformational changes in a wide range of other proteins. Experimental and quantification approaches for a reliable analysis of protein absorption below 320 nm are presented.

Keywords

Ultraviolet absorption; Ligand binding; Thermodynamics; Conformational changes; Dioxygenase; Mutagenesis

Spectrophotometric approaches to examine protein-ligand interactions generally are limited to circumstances where the protein or ligand possesses a distinct visible or fluorescent chromophore that is perturbed in the complex. Such a situation is observed in many heme, flavin, and other cofactor-containing proteins, or in favorable cases where the fluorescence of endogenous aromatic residues undergoes changes upon ligand binding. The basic properties of many other complexes remain unexplored for the lack of visible spectral signatures. Here, we describe a novel, accessible, and quantitative approach that exploits changes in the ultraviolet (UV)¹ spectrum of a model enzyme, taurine/ α -ketoglutarate (α KG) dioxygenase (TauD) from *Escherichia coli*, to assess the thermodynamics and kinetics of its interactions with metal ions and substrate. The α KG-dependent dioxygenases are mononuclear, non-heme Fe(II) enzymes that couple the oxidative decarboxylation of an α -ketoacid to the oxidation of a target

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¹Abbreviations used: α KG, α -ketoglutarate; AU, absorbance units; MLCT, metal-to-ligand charge-transfer; TauD, taurine/ α KG dioxygenase; UV, ultraviolet

substrate. Reactions catalyzed by members of this enzyme superfamily are used to repair alkylation damage to DNA and RNA, modify structural proteins, regulate hypoxia-induced transcription factors, synthesize antibiotics, and metabolize many other cellular components [1;2;3]. TauD decomposes taurine (2-aminoethanesulfonate) as a sulfur source to produce sulfite and aminoacetaldehyde [4], as illustrated in Scheme 1.

Crystal structures were reported for anaerobic TauD with bound Fe(II), α KG, and taurine as well as for apoprotein with bound taurine [5;6], providing insight into the critical active site residues and the conformational changes that accompany active site assembly. As depicted in Figure 1, the Fe(II) center is formed by two imidazoles (His99 and His255) and a carboxylate (Asp101). The co-substrate α KG chelates the metal ion with additional stabilization provided by a salt bridge with Arg266 and a hydrogen bond with Thr128 (not depicted). Taurine binds near the active site and primes the five-coordinate Fe(II) to react with oxygen. The protein interactions with the taurine sulfonate (involving His70 plus the non-depicted backbone amide of Val102 and the side chain of Arg270) are similar in the absence of Fe(II) and α KG [6], but the amino group interactions are slightly altered. Numerous subtle shifts of active site residues are noted when comparing the holoprotein and apoprotein structures. Of significance to the present work, several aromatic residues (including Tyr73, Trp98, Trp128, Trp240, Trp248, and Tyr256) are located in the vicinity of the active site and interact directly or indirectly with its components.

Among the divalent metals, only Fe(II) is capable of supporting TauD activity; however, several other metal ions are known to bind to the TauD active site and inhibit the enzyme. For example Ni(II), a common contaminant of proteins purified by using metal interaction chromatography, is a slow-binding inhibitor of TauD [7]. Co(II) also inhibits TauD, and its TauD complex with bound α KG exhibits a characteristic visible absorption chromophore [7]. These metals can mimic the effects of hypoxia 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 [8]. Cu(II) can be substituted 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 [9;10]. Finally, the complex interplay of Cr(II) with anaerobic TauD with a partially oxidized ortho-quinone side chain has been examined [11].

It was shown earlier that UV absorption spectroscopy could be used to quantitatively monitor the oxidation of a single aromatic amino acid residue in a protein complex as large as mammalian cytochrome *c* oxidase (210 kDa) and that UV difference absorption spectra could identify the nature of the protein radical and follow its migration between two residues [12]. The studies described here utilize a similar approach to characterize more subtle perturbations in the UV spectrum of TauD that occur upon binding of metal ions and the substrate. These specific effects are further characterized by using selected TauD mutants that alter substrate or cofactor binding. This general approach provides an inclusive spectroscopic tool for direct analysis of kinetic and thermodynamic parameters associated with metal or other ligand binding in proteins.

Materials and methods

Enzyme purification

Wild-type, H70A, H99A, D101E, D101Q, H255A, and H255Q variants of TauD were purified in their apoprotein forms, as previously described [13;14;15]. Inductively-coupled plasma-emission mass spectrometry (Chemical Analysis Facility, University of Georgia) was used to confirm the wild-type TauD apoprotein contained no metals at the end of the purification procedure. TauD concentrations were determined by using the theoretical extinction coefficient at 280 nm of $46.4 \text{ mM}^{-1}\text{cm}^{-1}$.

Anaerobic sample preparation

Apoprotein solutions (2 mM) were made anaerobic by cycling 4–5 times between mild vacuum and 1 atm argon with gentle agitation in vials sealed with stoppers. Argon gas (Linde, Murray Hill, NJ) was scrubbed of residual oxygen to < 5 ppm and moistened prior to introduction into the sample. Thoroughly degassed stock buffer solutions were transferred into a dry, anaerobic cuvette sealed with a rubber septum and equipped with a magnetic stirring bar. The gently degassed protein stock solutions were added to yield a final concentration of 25 μ M subunit and the samples were subjected to another gas exchange to remove traces of oxygen introduced during sample transfers. Aliquots of Fe(II) stock solutions were introduced while stirring by using gas-tight microsyringes flushed of oxygen. Such treatment consistently produced anaerobic solutions which exhibit no detectable oxygen binding to deoxymyoglobin (i.e., $P_{50} \leq 3$ torr or 4 μ M). For uniformity, studies involving addition of other metal ions, taurine, and α KG (all from Sigma-Aldrich, St. Louis, MO) were conducted in the same manner.

Spectroscopy

Visible and preliminary UV absorption spectra were recorded by using a Hewlett-Packard photodiode array spectrophotometer (HP 8453; Agilent Technologies, Inc., Santa Clara, CA) at 22 °C. The UV difference absorption spectra were recorded by using a custom, open-bench UV spectrophotometer, also at 22 °C. The probe beam (5 mm diameter) from the combination deep-UV/visible light source (model DH2000-DUV, Ocean Optics, Inc., Dunedin, FL) was formed and routed by using far UV enhanced ($\lambda > 180$ nm) achromatic reflective optics (coating #1900, Acton Research, Trenton, NJ). Spectral dispersion and detection was performed by using a 30-cm polychromator (model Triax 322, Jobin-Yvon, Edison, NJ) equipped with a back-illuminated charged-coupled device camera (model 1024 $\times$ 256 BIUV-STE, Jobin-Yvon) for maximum UV sensitivity; the 300 lines/mm grating blazed at 250 nm provided a spectral slit width of 30 μ m and allowed detection of 0.26 nm/pixel. The high quantum yield of this spectrometer permitted use of minimal probe light intensity, thus reducing sample degradation due to protein UV photolysis.

The amplitude of the optical changes is proportional to sample concentration on the absorption scale. At lower sample concentrations the acquisition noise remains constant while the intensity of the optical changes decreases, thus degrading the S/N ratio. At higher sample concentration, the exponentially rising contribution of background signal (from detector noise, stray light, etc.) overwhelms the linear increase in the optical changes, also limiting the S/N ratio. Here the sample concentration was selected to produce a maximum optical density of 1.2 at 280 nm compared to the saturating optical density of 3. These conditions provided an optimal signal to noise ratio in the difference absorption spectra by maximizing signal intensity while maintaining sufficient light transmission to suppress noise due to strong background absorption.

The absolute spectra of protein samples and all additives in corresponding media were recorded separately for post-processing. Spectra of protein samples were recorded as a reference immediately prior to the titrations. During and after additions, spectra were continuously monitored, groups of 30 scans each of 10-ms acquisitions were averaged, and the results were recorded as a two-dimensional matrix. In subsequent processing, successive spectra were further averaged in 2 min intervals over the duration of the spectral changes as judged by temporal profiles at selected wavelengths.

Spectral corrections

Addition of reactant solutions led to a small but significant dilution of the sample and corresponding decrease of protein absorption. To minimize such dilution the volumes of all additions were kept small (2–10 μ l added to 1,500 μ l sample); nevertheless, addition of 10 μ l

aliquots of buffer alone led to a $\Delta A = 0.008$ at 280 nm (optical density 1.2), which was comparable to the metal- and substrate-induced changes under our conditions. To eliminate the effect of dilution from the observed difference spectra the following correction was applied:

$$\Delta A_{\text{corr}} = \Delta A_{\text{obs}} d + (d - 1) A_r \quad (1)$$

where ΔA_{obs} and ΔA_{corr} are observed and corrected difference spectra, respectively, and $d = V_o/V_r$ is the inverted dilution of sample volume V_o in which ΔA_{obs} was acquired relative to the volume V_r in which reference spectrum A_r was acquired. No additional corrections for baseline drifts or light scattering were applied to the observed spectra, but instead these factors were accounted for in the subsequent spectral analysis.

Spectral non-linear global regression analysis

Data were recorded by using xDSoft custom data acquisition software and processed using Igor Pro 6.02 software (Wavemetrics, Lake Oswego, Oregon). For a simple equilibrium between protein E and ligand L:

associated with the dissociation constant K_D . Substrate-dependent absorption changes at wavelength λ are determined by the concentration of enzyme-ligand complex $[EL_n]$ and changes in the coefficient of molar extinction $\Delta \epsilon_\lambda$ as follows:

$$\Delta A_\lambda = \Delta \epsilon_\lambda [EL_n] = \Delta \epsilon_\lambda ((K_D + [L_T] + n[E_T]) - ((K_D + [L_T] + n[E_T])^2 - 4[L_T]n[E_T])^{1/2})/2n \quad (3)$$

This expression can be used in traditional non-linear regression of an absorption profile at a characteristic wavelength λ to obtain binding parameters ($\Delta \epsilon_\lambda$, K_D , and n), although the reliability of such analysis is strongly contingent on the absence of interference from parallel processes. When interference is present (such as due to changes in light scattering) more reliable results are obtained by using a global, simultaneous nonlinear regression of two-dimensional spectra to equation 3. The wavelength-dependent spectrum of a mixture of several species can be described as a linear combination of their individual spectra: $S_{o,\lambda} = C_1 \epsilon_{1,\lambda} + C_2 \epsilon_{2,\lambda} + \dots + C_i \epsilon_{i,\lambda}$, where $S_{o,\lambda}$ is the observed spectrum over wavelength λ , while C_i and $\epsilon_{i,\lambda}$ are the concentrations and wavelength-dependent extinction coefficients of i -th species. In the case of substrate binding studies, binding parameters (K_D , and n) are global variable parameters which apply uniformly across all points and determine C_i per equation 3, while the extinction coefficient $\Delta \epsilon_\lambda$ is a local variable parameter which is specific for each wavelengths. Changes in the spectral background due to light scattering represent a separate wavelength-dependent process not related directly to substrate binding. The exact description of light scattering by particles is complex, but it can be empirically approximated as:

$$B_{\lambda,k} = B_{0,k} + B_{1,k}/\lambda^2 + B_{2,k}/\lambda^4 \quad (4)$$

where $B_{0,k}$, $B_{1,k}$, etc. represent polynomial coefficients specific for each spectrum k . Thus, the total optical changes observed at wavelength λ in the series of spectra k where substrate concentration was L_k can be written as:

$$S_{\lambda,k} = \Delta \epsilon_\lambda EL_{\lambda,k} + B_{\lambda,k} \quad (5)$$

where the first and second terms are found from (3) and (4) respectively.

Here, dilution-corrected spectra of TauD were assembled into 2-D difference absorption datasets of wavelength vs. titrant concentrations. Spectral changes in the entire dataset were modeled simultaneously as concentration-dependent ligand binding and ligand-independent changes in light scattering according to the equation (5).

Fluorescence spectroscopy

Fluorescence spectra were acquired using a Photon Technology International spectrofluorimeter (Birmingham, NJ) equipped with arc lamp A-101-B, an LPS 220B power supply, and a model 810 photomultiplier detection system. Anaerobic protein sample (10 μ M) in a septum-sealed fluorescence cuvette was placed into a model TLC 50 thermostated cuvette holder (Quantum Northwest, Inc., Liberty Lake, WA) with magnetic stirring. Stock solutions of Fe(II), α KG, or taurine were added using a gas tight syringe, with fluorescence intensities corrected for dilution as described above.

Results

Changes in the UV spectrum of TauD induced by metal binding

The addition of stoichiometric Fe(II) to anaerobic TauD resulted in perturbations of its UV absorption spectrum (Fig. 2A), as clearly seen in the resulting difference spectrum (Fig. 2B). Although small (ca. 1/50th of the absolute protein absorption), the spectral changes were reproducible between protein preparations in the presence of Fe(II). These features were not observed in samples lacking TauD or for buffer addition to TauD-containing samples, confirming that the effects are dependent on protein-metal interactions. The addition of Fe(II) to TauD caused an overall increase in the UV absorption, particularly toward shorter wavelengths, but with several distinctive troughs between 280 and 300 nm. The major trough at 297 nm exhibited a molar extinction coefficient of $\epsilon = \sim 0.4 \text{ mM}^{-1} \text{ cm}^{-1}$ and a half-width of $\sim 6.5 \text{ nm}$. A second, weaker trough was evident at 286 nm ($\epsilon = \sim 0.3 \text{ mM}^{-1} \text{ cm}^{-1}$, half-width of $\sim 3.9 \text{ nm}$) and additional minor difference features were present between 270 and 290 nm (Fig. 2B).

Four other divalent metal ions, Ni(II), Co(II), Cu(II), and Zn(II), yielded similar perturbations of the TauD UV spectrum (Fig. 3). The difference spectra of all metal ions exhibited two major troughs, with those for Co(II), Ni(II), and Cu(II) being slightly red shifted ($\sim 298 \text{ nm}$ and $\sim 288 \text{ nm}$) compared to the Zn(II) and Fe(II) features. A 3-fold molar excess of Ni(II), Co(II), and Zn(II) was required for full development of the 297 nm absorption differences, consistent with lower apparent affinities of these metal ions, and the maximum intensities varied from $0.35 \text{ mM}^{-1} \text{ cm}^{-1}$ for Ni(II) and Co(II) to $0.5 \text{ mM}^{-1} \text{ cm}^{-1}$ for Zn(II). The relative intensities of the 297/286-nm troughs appeared to be altered for the Cu(II) sample, but this was probably due to overlap with a broad transition at 306 nm. Only the Zn(II)-form of TauD exhibited a clear feature at 277 nm. Weak and broad optical transitions observed at wavelengths greater than 300 nm varied among the metal ions, both in intensity and energy.

The kinetics of metal ion binding to TauD apoprotein was examined following the addition of equimolar amount of metals to TauD apoprotein. At the acquisition rate of 3.5 s per spectrum with manual injection and moderate sample stirring, the spectroscopic changes were too rapid to measure for Fe(II), Zn(II), and Co(II), consistent with a k_{on} of greater than $18 \text{ mM}^{-1} \text{ s}^{-1}$. In contrast, Ni(II) binding was significantly slower than for the other metal ions and a second-order binding rate constant was estimated to be $0.17 \text{ mM}^{-1} \text{ s}^{-1}$ (Fig. 4).

Binding affinity for metal ions

The spectroscopic changes associated with Fe(II) addition to a solution of TauD apoprotein were used to assess the thermodynamics of this interaction. As the amount Fe(II) in the sample increased, the spectral features noted above became more apparent in the difference spectra (Fig. 5A). Global regression analysis of these data (see Methods section) produced a set of extinction coefficients that represent a metal binding spectrum and identified the largest spectral differences at 298.5 nm (Fig. 5B). It also revealed that Fe(II) binding is not directly associated with an increase in the UV absorption, which is attributed to minor protein precipitation. The changes saturated after the addition of stoichiometric amounts of Fe(II), and the K_d of Fe(II) binding to apoprotein was estimated to be 90 ± 50 nM (Fig. 5C) although its accuracy is limited by a relatively high protein concentration. Binding of Ni(II) to apoprotein (K_D of $\sim 200 \pm 100$ nM) was ~ 2 -fold weaker than that of Fe(II) (Fig. 5C). A much larger difference between the two metals was described above for the kinetics of binding (Fig. 4). Prior studies had demonstrated that Ni(II) is a slow-binding competitive inhibitor of the enzyme [7], presumably due to its substitution for Fe(II) at the active site.

To further characterize the interaction between Fe(II) and TauD apoprotein, we examined several TauD variants with amino acid substitutions involving the metal-binding residues His99, Asp101, and His255. The addition of three equivalents of Fe(II) to the H255Q variant yielded spectral differences that were essentially identical to wild-type protein (Fig. 6A), coincident with the nearly fully active phenotypes of this enzyme form [15]. Clear spectral features, though much less pronounced, also were observed upon the addition of 3 equivalents of Fe(II) to D101E TauD (Fig. 6A). Titration studies provided a $K_d = 5.9 \pm 0.7$ μ M for the D101E variant (Fig. 6B) which is known to possess about 1/5 of the wild-type enzyme activity [15]. While no spectroscopic changes were apparent upon addition of three equivalents of Fe(II) to H99A TauD (Fig. 6A), greater concentrations of Fe(II) did result in spectral perturbations of small intensity and allowed estimation of the K_D of 380 ± 26 μ M (Fig. 6B). This mutant was previously found to be essentially inactive when purified by our standard procedure [15], whereas it exhibited $\sim 40\%$ of wild-type enzyme activity when bacterial growth, protein isolation, and activity assays were carried out at low temperature [16]. In a similar manner, three molar equivalents of Fe(II) added to D101Q TauD had no effect on the UV spectrum (Fig. 6A), consistent with the previously reported near absence of activity and poor Fe(II) binding ability of this variant [15], whereas two additional equivalents showed a small increase in the difference spectrum (Fig. 6B). Finally, Fe(II) had no effect on the spectrum of the essentially inactive H255A variant (Fig. 6A).

UV spectral changes due to substrate binding

The addition of taurine to TauD apoprotein resulted in the formation of a sharp positive difference spectral feature at 295 nm with a K_d of 175 ± 10 μ M (Fig. 7). To further confirm that this response was monitoring the binding of taurine to the active site, we also examined the behavior of H70A TauD, a variant previously shown to be incapable of binding taurine [14]. No spectral changes similar to those observed with the wild-type protein were detected for the H70A variant (Fig. 7, bottom trace).

In contrast to the situation with taurine, the addition of the co-substrate α KG produced no distinctive changes in the UV difference spectrum of TauD apoprotein (data not shown). When α KG was added to Fe(II)-TauD, however, complex synergistic changes were observed in the UV region in addition to the well known metal-to-ligand charge-transfer (MLCT) transitions at 530 nm [13]. This interplay between α KG, Fe(II), and various mutant forms of TauD will be the subject of a future report and is beyond the scope of the present study.

Fluorescence spectroscopy

To corroborate the UV difference absorption results as an approach to probe ligand binding interactions with TauD, the effects of metal and substrate addition on the protein fluorescence spectrum were examined using excitation at the maximal protein absorption (280 nm) and at the wavelength of maximal optical changes (297 nm). Changes in the fluorescence associated with metal binding were identified at 335 nm. The addition of Fe(II) led to a quenching of ~35% of the fluorescence signal for either excitation wavelength, and titration allowed for calculation of a K_d of 101 ± 36 nM (Fig. 5C, asterisks) in very good agreement with the absorption titration. Analogous studies on binding of taurine or α KG to TauD apoprotein produced no significant change in the fluorescence intensity.

Discussion

Ligand binding in TauD

Analysis of Fe(II) binding to TauD and other apoproteins has been confounded by the lack of an associated visible absorption change. Earlier titration measurements of metal binding to TauD (indicating a K_d of less than 8 μ M) made use of the MLCT transition that develops in the presence of α KG and is further altered by taurine [13], but these effects could be limited by the α KG binding. The 90 nM K_d of Fe(II) binding to TauD observed here represents the first direct measurement of binding by this metal to any member of the α KG-dependent dioxygenase superfamily. In addition to being independent of protein- α KG interactions, the current approach benefits from an increased sensitivity of over 100-fold due to the lower protein concentration. The 1:1 stoichiometry of metal binding noted here effectively excludes the possibility of metal binding outside the TauD active site. The loss of spectral changes or decreased affinity for Fe(II) in known metal ligands provide further evidence of active site binding by the metal ions.

The small spectral variations in the UV difference spectra between various metal ions are likely to be secondary in nature, mediated by a variation in metal binding geometry, ionic radius, electronic configuration, etc. The unusual binding properties of Ni(II) among other metals also may be attributed to such differences. The significantly slower binding rate with only moderately larger K_D observed here for Ni(II) suggest a very slow dynamics of the TauD-Ni(II) complex affecting both its binding and dissociation. This is consistent with the significantly slower exchange rates for the aqua complex of this metal compared to the other metal ions tested. For example, the measured exchange rate for Ni(II) is more than an order of magnitude slower than for Co(II), and the rate is two, three, and four orders of magnitude slower than for Fe(II), Zn(II) and Cu(II), respectively [17].

Our analysis of taurine-induced UV changes provides the first thermodynamic characterization of interactions of this ligand with TauD. Suppression of taurine-associated spectral changes in the inactive H70A variant provides evidence that the effects are associated with the catalytically relevant binding site. The taurine-induced absorption at 295 nm is not associated with His70, whose optical absorption occurs at shorter wavelengths ($\lambda < 220$ nm). The crystal structure of the taurine- α KG-Fe(II)-TauD complex [5;6] indicates that His70 stabilizes taurine binding by interacting with the sulfonate group of the substrate. We had previously shown that Fe(II) and α KG bind to the H70A variant forming MLCT transitions that are characteristic of a properly formed active site [14], but we had also noted that subsequent addition of taurine failed to perturb this spectrum as seen for the wild-type enzyme. This result was consistent with the mutant protein's inability to bind taurine substrate, thus accounting for its lack of activity [14]. Results presented herein provide direct evidence for this conclusion.

Origin of the UV difference spectral features

The binding of metal ions to TauD might be expected to give rise to *d-d* or MLCT transitions across the optical spectrum, including the UV region of interest. Such features are dependent on the electronic configuration of the metal ion and would be expected to differ significantly with the metal species, whereas we observe only minor absorption changes among the various metal ions examined. Furthermore, the changes noted are much narrower ($\sim 700\text{ cm}^{-1}$) and more intense ($\sim 1\text{ mM}^{-1}\text{ cm}^{-1}$) than would be expected for metal transitions [18]. More significantly, any electronic transitions associated with the various metal ions would increase the intrinsic protein absorption, whereas we observe a clear initial diminishment of the 297 nm absorption upon binding of metal ions. For this to occur, the spectral changes at 297 nm must reflect at least a partial loss of a chromophore that exists in apoprotein prior to metal binding. In contrast to the situation for added metal ions, we observe a positive absorption change associated with the binding of taurine to TauD. Taurine itself, however, lacks electronic transitions near 280–300 nm and cannot contribute to the observed difference spectra. Rather than assigning the observed UV changes directly to absorption by ligands, we attribute them to ligand-induced effects on the endogenous protein chromophores, specifically the aromatic residues as there are no other known cofactors in TauD.

The absolute spectra of TauD's aromatic residues, and those of proteins in general, add up to a quite broad ($\sim 40\text{ nm}$) spectrum with multiple transitions, while the UV spectral changes induced by ligand binding are confined to a narrow ($\sim 15\text{ nm}$) range. The distinctive optical shifts observed here argue against a collective change in multiple aromatic residues due to gross structural changes as their primary origin. The spectra of aromatic residues are not sensitive to their rotational configuration relative to the peptide bond [19], but they do exhibit perturbations related to the overall hydrophobicity of their environment. For example, the lowest energy $^1\text{L}_b$ transition of indole is a narrow feature that shifts from 287 nm to 284.5 nm when comparing sample dissolved in methylcyclohexane versus perfluorinated hexane [20]. Even larger shifts are noted for some of the broader $^1\text{L}_a$ transitions (261.5 nm to 257 nm and 266.5 to 262 nm) in these solvents [20]. Furthermore, the electronic transitions of tryptophan mimics are shifted by charges placed at different positions around the indole ring [21]. Similar effects could occur upon binding of a metal ion or a sulfonate group in the vicinity of a Trp residue (no aromatic residues directly bind metals or taurine, but multiple aromatic residues are located near the active site of TauD; Fig. 1). Given the high proton affinities of Trp and Tyr residues, especially in the low dielectric environment of the protein interior [22], indirect structural changes upon ligand binding are not likely to cause deprotonation of these side chains. The observed optical changes could, however, reflect milder perturbations of the electronic structure of aromatic residues caused by changes in the local environment and mediated by hydrogen-bonding interactions with their ionizable groups [19]. Spectral features observed for TauD appear to be dominated by a 3–5 nm change in a major transition around 297 nm indicative of its well structured origin.

Using the known structures of TauD apoprotein and taurine- $\alpha\text{KG-Fe(II)-TauD}$ [5;6], we can speculate on the identity of likely aromatic residues responsible for the observed spectroscopic changes. Given the close proximity of Trp248 and Trp128 to the metal-binding site of TauD (6.4 and 7.3 Å for Fe(II) to the indole nitrogen, respectively), it is reasonable to expect their contribution to the observed spectral changes. On the other hand, metal coordination to His99 could alter hydrogen bonding of the adjacent Trp98 thus leading to the observed effects. The closest aromatic residue to the taurine-binding site is Tyr73; however, one could rationalize the observed spectroscopic changes upon taurine binding from more distant effects. For example, it has been noted [6] that the absence of taurine leads to relaxation of residues 60–80 which exposes Tyr164 and Trp174 to solvent. Future studies will directly address the

identity of the key residues involved in the observed spectroscopic effects by experimentally substituting these residues.

UV difference spectroscopy of proteins

Our UV spectroscopic results with TauD showcase a novel approach to examine structural changes in proteins induced by substrate or cofactor binding. Any protein interactions that specifically affect aromatic residues, directly or indirectly via ligand binding, (de)protonation, oxidation [12], etc., can be examined by protein UV absorption. This approach is somewhat analogous to the fluorescence-based methods widely used to study ligand binding interactions [23;24]; however, the physical basis of the two methods is quite distinct. Whereas not all aromatic residues give rise to fluorescent signatures, all such residues will exhibit UV absorption. In the case of Fe(II) binding to TauD, both methods provided very similar K_d values, but only UV absorption allowed resolution of substrate binding. Other benefits of the optical absorption approach include the ease of quantification, resilience to interference (such as the intrinsic fluorescence of flavins in fluorescence and Raman studies or water absorption in the infrared spectrum), and accessibility. As demonstrated here, this method is capable of monitoring the binding of several different metal ions as well as the substrate taurine to the TauD apoprotein. Indeed, we have shown analogous behavior when applying this difference UV approach for examining ligand binding interactions to CsiD, another Fe/ α KG dioxygenase (data not shown), and this tool is likely to be more generally applicable to a large assortment of proteins and their diverse variety of ligands. Although most results presented here were obtained using a custom spectrometer that allowed for resolution of small optical shifts, our first observations of the reported phenomenon were made using a standard commercial spectrophotometer, as were the UV absorption changes in cytochrome oxidase [12], illustrating that this approach is accessible for most laboratories. The key considerations in experimental design are the balance between the light transmission and intensity of optical changes, careful correction for dilution effects and predictable optical properties of buffers, ligands and intrinsic chromophores (e.g., cofactors). The presence of one or more aromatic side chains at the ligand binding site, or being otherwise affected by ligand binding, also is necessary.

Conclusions

We have shown that the addition of divalent metal ions and substrate to anaerobic samples of TauD leads to the development of characteristic and reproducible UV absorption signatures. The observed UV perturbations arise from protein conformational changes induced by the binding of ligands to the active site, as shown by ligand variant studies, and are proposed to arise from ligand-induced changes in the environment of aromatic residues in the protein. UV spectral changes with different concentrations of Fe(II) and taurine allowed us to obtain K_d values for each ligand, providing new insight into this archetype α KG-dependent dioxygenase. Protein-based UV perturbations, as described here for TauD, can be exploited to investigate a host of protein-ligand interactions, including binding of spectroscopically silent metals such as Fe(II), Ni(II), and Zn(II), to many other metalloproteins.

Acknowledgments

We thank Allan TerBush for assistance with collecting the fluorescence data and Dr. Lisa Lapidus for use of the fluorimeter. These studies were supported by the National Institutes of Health (Grant GM063586 to R.P.H. and Grant GM070544 to D.A.P.).

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Fig. 1. TauD structure (PDB 1OS7). The protein is shown with its Fe(II) (magenta sphere) bound to His99, Asp101, and His255 (orange). The α KG co-substrate chelates the metal and its C5 carboxylate forms an ion pair with Arg266 (both yellow). Additional features highlighted include the substrate taurine and its stabilizing His70 residue (green), the nine Tyr residues (cyan), and the six Trp residues (blue) in the protein.

Fig. 2. Perturbations of the UV absorbance spectrum of TauD upon anaerobic addition of Fe(II). (A) Absolute spectra of anaerobic TauD apoprotein (Apo, 25 μ M subunit) in 25 mM Tris, pH 8.0 (solid line), and the same sample after addition of 25 μ M Fe(II) (dashed line). (B) Difference spectra associated with Fe(II) binding to TauD apoprotein, corrected for sample dilution.

Fig. 3. Spectral effects of various divalent metals on the difference UV absorption spectra of TauD. Spectral changes were examined upon addition of 25 μ M Fe(II) and Cu(II) or 75 μ M Ni(II), Co(II) and Zn(II) to 25 μ M TauD apoprotein. All spectra are corrected for dilution and are offset vertically for clarity.

Fig. 4. Kinetics of Fe(II) and Ni(II) binding to TauD apoprotein. One equivalent of Fe(II) (solid symbols) or Ni(II) (open symbols) was added to 25 μM TauD subunit. Optical changes averaged over the 296.7–298.0 nm range were measured relative to a linear baseline between 292.2–293.2 nm and 303.6–305.1 nm ranges. Solid lines represent exponential fits to the data.

Fig. 5. Thermodynamics of Fe(II) and Ni(II) interaction with TauD apoprotein. (A) Spectral changes in the wild-type TauD apoprotein (25 μM) at selected Fe(II) concentrations, corrected for dilution and offset for clarity. (B) Fe(II) binding spectrum obtained by regression analysis on the experimental spectra from panel A. (C) Concentration dependence of Fe(II)- and Ni(II)-induced spectral changes. The UV absorption changes for Fe(II) and Ni(II) (solid and open symbols, respectively) are compared to the changes in fluorescence emission for Fe(II) (asterisks). The lines represent simulated binding curves.

Fig. 6. Concentration dependence of Fe(II)-induced changes in the spectra of TauD metal-ligand variants. (A) Three equivalents of Fe(II) were added to H255Q, H255A, D101E, D101Q, and H99A variants of TauD apoprotein and the spectral changes were determined (vertically offset for clarity). (B) Spectral changes at 298 nm observed as Fe(II) was titrated into solutions containing the same variants (●, ○, ▲, △, and ◆, respectively) of TauD apoproteins (25 μM), after correction for dilution.

Fig. 7. Effect of taurine addition on wild-type TauD apoprotein and the H70A variant. (A) Difference spectra resulting from the addition of taurine to 25 μM of wild-type TauD or the H70A variant. (B) Taurine binding spectrum obtained by regression analysis on the wild-type TauD experimental spectra from panel A. (C) Intensity changes at 294.7 nm as a function of taurine added for the wild-type apoprotein (solid symbols) and the H70A variant (open symbols).

Scheme 1.