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Global stability of an α -ketoglutarate-dependent dioxygenase (TauD) and its related complexes

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Abstract

Background—TauD is a nonheme iron(II) and α -ketoglutarate (α KG) dependent dioxygenase, and a member of a broader family of enzymes that oxidatively decarboxylate α KG to succinate and carbon dioxide thereby activating O₂ to perform a range of oxidation reactions. However before O₂ activation can occur, these enzymes bind both substrate and cofactor in an effective manner. Here the thermodynamics associated with substrate and cofactor binding to FeTauD are explored.

Methods—Thermal denaturation of TauD and its enzyme-aurine, enzyme- α KG, and enzyme-aurine- α KG complexes are explored using circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC).

Results—Aurine binding is endothermic (+ 26 kcal/mol) and entropically driven that includes burial of hydrophobic surfaces to close the lid domain. Binding of α KG is enthalpically favorable and shows cooperativity with aurine binding, where the change in enthalpy associated with α KG binding (ΔH_{cal}) increases from -30.1 kcal/mol when binding to FeTauD to -65.2 kcal/mol when binding to the FeTauD-aurine complex.

Conclusions—The intermolecular interactions that govern aurine and α KG binding impact the global stability of TauD and its complexes, with clear and dramatic cooperativity between substrate and cofactor.

General Significance—Thermal denaturation of TauD and its enzyme-aurine, enzyme- α KG, and enzyme-aurine- α KG complexes each exhibited increased temperature stability over the free enzyme. Through deconvolution of the energetic profiles for all species studied, a thermodynamic cycle was generated that shows significant cooperativity between substrate and cofactor binding which continues to clarify the events leading up O₂ activation.

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Conflict of interests

The authors have no conflict of interests to declare.

Keywords

Differential scanning calorimetry; circular dichroism; nonheme iron(II); alpha-ketoglutarate; taurine; and thermal stability

1. Introduction

Metalloenzymes catalyze a wide-range of chemistries, including some that utilize dioxygen to oxidize substrates [1–7]. Some of these enzymes require that a cosubstrate is oxidized in conjunction with dioxygen activation to generate a high valent intermediate that reacts with the targeted substrate [8–10]. For example, the α -ketoglutarate (α KG) dependent, nonheme iron(II) dioxygenases use the cosubstrate α KG, which is oxidatively decarboxylated to form succinate and carbon dioxide in concert with O₂ activation to generate an iron(IV)-oxo species in the enzyme active site pocket. This high-valent species attacks the primary substrate, generally at a C-H bond with relatively high bond dissociation energy [1].

The α KG-dependent oxygenases contain a characteristic cupin fold (also known as double-stranded β -helix or jellyroll fold), made up of eight β -strands arranged in two β -sheets opposing each other and generating a barrel shape, around the active site cavity that supports a common 2-His-1-carboxylate metal binding motif (Figure 1, *A* and *B*) [11–13]. This facial triad binds a nonheme iron(II) ion through the imidazole rings of two histidine amino acid residues and one carboxylate of either an aspartate or glutamate residue occupying one face of the nonheme iron(II) octahedral coordinating sphere (Figure 1*B*). This geometry leaves the adjacent face available for binding of α KG (Figure 1*C*) and an open site for O₂ coordination. Although α KG-dependent oxygenases are well studied mechanistically [1,9,14–19], there are little thermodynamic data on the enzymes and enzyme-substrate complexes that lead to this O₂ activation event. In this study, we probe the global stability of the α KG-dependent dioxygenase TauD, a model α KG-dependent enzyme from *Escherichia coli* that hydroxylates taurine leading to its decomposition into aminoacetaldehyde and sulfite [20]. Specifically, we employ circular dichroism (CD) and differential scanning calorimetry (DSC) methods under anaerobic conditions to study the thermal stability of TauD apoprotein, the active holoenzyme (FeTauD), and relevant enzyme-substrate complexes. In addition, we measure enthalpies of unfolding for each complex, and present a complete summary of the substrate contributions to the global stability of this α KG-dependent system.

2. Materials and methods

2.1. Reagents and general procedures

Taurine (99 % purity) and α KG disodium salt (98 % purity) were purchased from Sigma-Aldrich and used as received. Buffering systems, ethylenediaminetetraacetic acid disodium salt (EDTA), and metal salts were purchased at the highest grade available and used as received. Buffer was made in 18 M Ω ultrapure water filtered through a Millipore Ultrapurification system.

2.2. Overexpression and purification of TauD

The recombinant α KG-dependent dioxygenase TauD was produced in *E. coli* BL21(DE3) [pME4141] cells grown at 37 °C in terrific broth containing kanamycin (50 μ g/mL) and induced by addition of 0.1 mM isopropyl- β -D-1-thiogalactose with further growth at 30 °C [20]. The cells were harvested, cell-free extracts were prepared, and TauD apoprotein was purified by sequential DEAE-Sepharose and phenyl Sepharose chromatographies as previously described [21]. Purified TauD apoprotein in 25 mM Tris, pH 8.0, buffer containing 1.0 mM EDTA was stored in aliquots at -80 °C.

2.3 TauD and TauD complex solution preparation

Solutions for CD and DSC analysis were prepared in the following manner: TauD apoprotein stock was dialyzed for 3 h against a buffering system of 50 mM ammonium acetate, 100 mM NaCl, and 20 μ M EDTA (pH 8.0) to remove residual metal. The TauD apoprotein stock was then dialyzed three times against buffer (1.0 L) containing 50 mM ammonium acetate and 100 mM NaCl (pH 8.0) for 3 h each, and one additional dialysis against 1.0 L of the buffer overnight. Stock solutions of taurine, α KG, and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ were prepared in the dialysate buffer, and all solutions were made anaerobic by purging with Ar to eliminate the various oxidation reactions generated when ferrous ions are present [22]. For FeTauD experiments, 0.9 equivalents of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ were added anaerobically to TauD apoprotein. To shift the equilibrium in favor of the substrate-enzyme complexes, taurine or α KG stock solutions were added anaerobically to enzyme samples to yield final concentrations of 1.0 mM substrates, concentrations that are much greater than the previously reported K_d values [11].

2.4. Circular dichroism

CD samples were diluted to a final concentration of 5 μ M (total protein) in the dialysate buffer and verified by absorption at 280 nm ($\epsilon_{280} = 46,410 \text{ M}^{-1}\text{cm}^{-1}$) [14]. TauD and its complexes were placed in a 1-cm pathlength quartz cell and sealed in an anaerobic environment. CD spectra of TauD and its complexes were collected using an Olis DSM-20 CD spectrometer (Bogart, GA) over a 200–250 nm scanning range. Thermal stability experiments were monitored from 25 to 85 °C with the samples incubated at each temperature for 5 min before the CD spectra were collected. The spectra were corrected for buffer contributions, and the data were analyzed using the Globalworks software package (Olis Inc., Bogart, GA). Melting profiles were generated by plotting the intensity data at 223 nm versus temperature.

2.5. Differential Scanning Calorimetry

TauD DSC samples were diluted to a final concentration of 0.5 mg/mL (16.7 μ M protomer for the dimeric protein) in the dialysate buffer and verified by UV spectroscopy. The N-DSC II (Calorimetry Sciences Corp) instrument was made anaerobic by constructing a gas bath or anaerobic well surrounding the reference and sample ports, and purging this system with Ar for at least 20 min. The dense Ar gas actively displaces the aerobic atmosphere, allowing both the sample and reference cells and their ports to remain anaerobic. With a constant Ar gas flow, the anaerobic samples and reference buffer were slowly added to the Ar-filled

cells. The N-DSC II pressure cap was sealed and pressurized to 3 atm before removing the inert gas flow from the contained environment. Enzyme and substrate-enzyme samples were subjected to temperature scans from 15 – 85 °C at a scan rate of 2 °C per min in both the forward and reverse directions (c.f. Supplemental Material Fig. S1.) Data analysis was performed using the CPCalc software available from the Calorimetry Sciences Corp data analysis software package. A final temperature scan was used as the baseline in each experiment, and was subtracted from the raw data. All data were fit to one or more two-state scaled models, where the best fits were assigned by directly comparing the goodness of fit values associated with the CPCalc software package. Experiments were replicated 2–3 times, and averaged. Error bars associated with the data are given as one standard deviation from the mean.

3. Results

The enthalpy change of a reaction, H , is a fundamental thermodynamic quantity that describes the amount of heat released or absorbed in the course of a reaction. For a reversible reaction it also describes the temperature dependence of the equilibrium constant, K , through the van't Hoff relationship, where R is the gas constant, T is the absolute temperature, and the partial derivative emphasizes the fact that other experimental variables such as pressure are held constant.

$$\Delta H = -R \left(\frac{\partial \ln K}{\partial \frac{1}{T}} \right)$$

The H of a reaction can be determined directly by using calorimetry (H_{cal}) or indirectly by measuring the temperature dependence of the equilibrium constant using the van't Hoff method (H_{vH}). In the van't Hoff method, the instantaneous slope of a plot of $\ln K$ vs $1/T$ multiplied by $-R$ is used to determine H_{vH} , this is important because reactions in aqueous solutions are usually accompanied by a significant change in heat capacity, C_p , which results in a temperature-dependent H . In theory, the H_{cal} and H_{vH} should be the same [23], however in many biological systems these terms differ due to the fact that the H_{cal} is a measurement of the change in enthalpy for all thermodynamic processes in the sample, whereas the H_{vH} is solely derived from spectroscopic changes taking place in the chemical system. Here we use both spectroscopic and calorimetric methods to study the thermodynamic properties of metal ion and substrates (taurine and α KG) binding to TauD. Both the van't Hoff and calorimetric analysis of these data give a better understanding of the thermodynamics of these processes and their impact to the global stability of the folded protein.

3.1. Circular Dichroism

The circular dichroism (CD) spectra (Figure 2) of the various folded (F) complexes of TauD show a prominent negative peak at 225 nm, which is characteristic of the cupin barrel, which surrounds the active site pocket of TauD [24–25]. The minor changes in the CD spectra for FeTauD and the related FeTauD-substrate(s) complexes suggest there are minimal changes to the cupin barrel when metal, substrate, and cofactor are added. The CD spectra show that

the cupin fold is completely unfolded (U) by 85 °C for TauD, FeTauD, and the FeTauD-substrate(s) complexes (Fig. 2). The iron(II) containing samples appear to form a weakly CD active species at 240-nm during unfolding, which could be related to the iron(II) species released into solution.

The melting profiles of TauD and TauD-complexes were collected using CD spectroscopy by monitoring the secondary structures of the TauD species during temperature scans. For all samples, intensities at 223 nm were plotted as a function of temperature (Fig. 3 and Fig. S2). The CD melting profiles were analyzed using a number of different models using OLIS Globalworks software, but the best correlation between fit and experimental data was observed with a two-state model. The average thermodynamic data (T_m and H_{vH}) determined from the CD melt experiments are shown in the supplementary materials Table S1. The T_m values obtained from the CD melting curves are tabulated along with the calorimetry data in Table 1. The T_m values obtained in the CD experiments exhibit the same trends as those obtained from the DSC measurements discussed below. However, the CD T_m values are typically shifted to a lower temperature by approximately 8 °C. This difference in the T_m values may be due to differences in how these data were collected. The CD data were collected at various temperatures, where the sample was equilibrated at a given temperature for 5 min before data were collected. The temperatures for the DSC data were scanned at a rate = 2 °C/min. One possibility is that the CD data are following the unfolding of the TauD cupin fold rather than the overall structure of the TauD protein as it denatures to a random coil. As described in more detail below, the CD melting profiles generally show increased temperature stability of TauD protein when iron(II) and/or substrates are complexed to the apoprotein. The H_{vH} data (shown in Table S1) obtained from the two-state fits of the CD melting profiles are simply not accurate enough to be considered further at this time.

The TauD apoprotein unfolding event occurs at a T_m of 51.1 (± 1.1) °C. In comparison, FeTauD denaturation occurs at a higher temperature with a T_m of 54.8 (± 2.7) °C. This apparent increase in thermal stability of the iron(II) TauD complex ($\delta T_m \approx 4$ °C) is consistent with the known exothermic binding of iron(II) by TauD [21].

Taurine, the primary substrate of TauD, was complexed with apoprotein TauD and the holoprotein, FeTauD. The thermal unfolding profiles of the TauD-aurine and FeTauD-aurine samples yield T_m values for the denaturation of the TauD-aurine and FeTauD-aurine complexes of $T_m = 53.3 (\pm 1.6)$ °C and $T_m = 58.8 (\pm 0.6)$ °C, respectively. The increase in the T_m of the ternary complex ($\delta T_m \approx 8$ °C) is approximately equal to the stability gained by binding iron(II) and taurine independently ($\delta T_m \approx 4$ °C and $\delta T_m \approx 2$ °C for iron(II) and taurine, respectively) to TauD. From these data it would appear that the binding of iron(II) and taurine are independent of each other.

When the co-substrate α KG was complexed to FeTauD, the T_m for the ternary complex (54.4 (± 2.7) °C) is unchanged in comparison to the T_m for binary complex FeTauD (54.8 (± 2.7) °C). However, the T_m for unfolding the quaternary complex, FeTauD- α KG-aurine shows a significant increase by more than 5 °C, FeTauD- α KG-aurine $T_m = 60.1 (\pm 0.1)$ °C. This result is consistent with an interaction between the binding of taurine and the binding of α KG to FeTauD.

In general, the CD data show that the binding of metal ions, cofactors, and/or substrates to TauD increases the thermal stability of the TauD protein (see CD determined T_m values in Table 1). They also suggest that the binding of iron(II) is independent of the substrate or cofactor but that the binding of the substrate (taurine) is not independent of cofactor (α KG) binding. As mentioned earlier, the differences in the T_m values obtained from CD experiments and DSC measurements may reflect differences in the temperature scan rate used in the two techniques or the fact that the CD experiments may be following a spectroscopic signal (the cupin fold) that does not reflect the overall structure of the TauD protein.

3.2. Differential Scanning Calorimetry

Typical DSC instruments designed for the study of biopolymers incorporate coin shaped sample and reference cells which exacerbate the tendency of protein systems to aggregate, and the associated heat effects often obscure the thermal denaturation data. In this study, we used a Calorimetry Sciences Nano-DSC that is constructed with gold capillary cells for reference and sample solutions. This type of cell provides greater spatial separation of unfolded protein molecules, reducing the apparent concentration of unfolded molecules and delaying aggregation. Moreover, because protein aggregation is typically a slower process than unfolding [26], we used a scan rate of 2 °C per min, which allowed us to observe the thermal denaturation of TauD before aggregation occurs. Indeed, initial data collection at a slower scan rate (1 °C/min) resulted in a large exotherm that consumed the unfolding curve (data not shown) and made quantitation impossible. An increase in scan rate eliminated the exotherm and allowed us to quantify the unfolding process. The data collected in this manner represent an approximation of the reversible unfolding of the protein (see Supplemental Materials Figs. S3–S8 and Table S2 and S3 for individual curves and fits) [27,28]. A complete list of thermodynamic data can be found in Table 1, and all relevant excess heat capacity (C_p) curves are found in Fig. 4.

The TauD apoprotein unfolding event was fit to a single two-state model, unfolding at $T_{m\text{TauD}}$ of 59.2 (± 0.1) °C with $H_{\text{TauD}} (= \int C_p T)$ of 132.3 (± 1.8) kcal/mol (Fig. 4 blue curve). For FeTauD (Fig. 4 orange curve), two unfolding events became evident; the first accounted for approximately 20 % of the enzyme in the cell and matches the $T_{m\text{TauD}}$ and H_{TauD} with values of 58.2 (± 0.03) °C and 133.0 (± 1.5) kcal/mol, respectively. The second unfolding event is assigned as FeTauD, which accounts for approximately 80 % of the protein in solution and unfolded with a $T_{m\text{FeTauD}}$ of 61.4 (± 0.4) °C and a H_{FeTauD} of 145.1 (± 1.8) kcal/mol. The difference in the unfolding enthalpies of the two forms of the protein yielded the enthalpy of binding of iron(II) to the enzyme: ($\delta H_{\text{binding}} = H_{\text{TauD}} - H_{\text{FeTauD}}$). This value ($-12.7 (\pm 2.6)$ kcal/mol) is in excellent agreement with our previously published iron(II) H_{binding} of $-11.6 (\pm 0.3)$ kcal/mol obtained by isothermal titration calorimetry [21]. The agreement of the irreversible unfolding events with the ITC-obtained reversible (equilibrium) values supports the justification that the thermodynamic values obtained herein for irreversible unfolding provide a good approximation of the equilibrium unfolding event.

Taurine, the primary substrate of TauD, was complexed with TauD apoprotein and the sample was subjected to thermal denaturation. Unfolding of the TauD-aurine sample fit to a single two-state model with a $T_{m\text{TauD-aurine}}$ of $60.6 (\pm 0.9) ^\circ\text{C}$ and a $H_{\text{TauD-aurine}}$ of $117.9 (\pm 0.6) \text{ kcal/mol}$ (Fig. 4, purple curve). Visual comparison of the heat capacity curves of unfolding of TauD-aurine and TauD indicates that the C_p of TauD-aurine is lower than that of TauD by approximately $4 \text{ kcal/mol } ^\circ\text{C}$. While the substrate-enzyme complex appeared to unfold at essentially the same temperature as TauD, the δH between TauD and TauD-aurine unfolding was $-14.4 \pm 1.9 \text{ kcal/mol}$, which corresponds to an enthalpic penalty during thermal unfolding for the sample with substrate. In comparison, the FeTauD-aurine complex produced a wide, slightly asymmetric curve with a lower C_p ($7 \text{ kcal/mol } ^\circ\text{C}$) than that of FeTauD (Fig. 4 green trace.) While the data could be fit to a single two-state function, tighter fits were obtained by using two two-state equilibria in which we observe a $T_{m\text{FeTauD}}$ and H_{FeTauD} that corresponds with approximately $5 (\pm 0.1) \%$ of the sample, and a second unfolding process, which we attribute to the unfolding of the enzyme-substrate complex with $T_{m\text{FeTauD-aurine}}$, and $H_{\text{FeTauD-aurine}}$ of $61.8 (\pm 0.2) ^\circ\text{C}$ and $118.9 (\pm 1.3) \text{ kcal/mol}$, respectively. Interestingly, we observed a δH of zero and essentially no melting temperature change between the TauD-aurine and FeTauD-aurine unfolding events, indicating the unfolding species are thermodynamically similar in both cases, and iron(II) binding seems to have minimal effect on the thermal denaturation of this species. In both cases, taurine binding appears to have an endothermic or positive H_{binding} . This result indicates an entropically driven binding event, where $S = 43 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ for the apoprotein, and $S = 78 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ for FeTauD.

The co-substrate αKG was bound to FeTauD and the sample was unfolded to produce a C_p curve that also fit well to two, two-state equilibria (Fig. 4, red curve). The first unfolding event involved approximately $38 (\pm 6) \%$ of the enzyme and occurred at a $T_{m\text{FeTauD-}\alpha\text{KG}}$ of $67.8 (\pm 0.2) ^\circ\text{C}$ with a $H_{\text{FeTauD-}\alpha\text{KG}}$ of $175.2 (\pm 1.1) \text{ kcal/mol}$. The additional feature in this sample represents approximately $65 (\pm 6) \%$ of the enzyme and occurred at a $T_{m\text{FeTauD-}\alpha\text{KG}^*}$ of $65.0 (\pm 0.1) ^\circ\text{C}$ with a $H_{\text{FeTauD-}\alpha\text{KG}^*}$ of $121.0 (\pm 0.01) \text{ kcal/mol}$. The two species in solution may be the result of a system in which the changes occurring in one monomeric unit of the dimer strongly influence the overall binding and conformational changes occurring in the other monomeric unit. We assign the higher melting curve to the FeTauD- αKG species, and the lower melting curve as a TauD species or related species in which αKG is bound, but has undergone changes (structure, coordination and/or solvent related) to produce a lower energy system. We have labeled this species FeTauD- αKG^* .

When the quaternary complex (consisting of iron(II), TauD, taurine, and αKG) was generated in an anaerobic environment and placed in the DSC, denaturation resulted in a highly asymmetric curve (Fig. 4, black curve). By comparison to the other substrate- and iron(II)-enzyme curves (Fig. 4), it becomes obvious that this asymmetry is the result of the sum of a number of the different TauD complex unfolding events. The first species, a small percentage of the total in solution, which corresponds with data collected for the unfolding of FeTauD, unfolds at a $T_m = 62.2 (\pm 0.2) ^\circ\text{C}$ and a $H = 143.0 (\pm 0.01)$. Sixty-three (± 8) percent of the enzyme is observed to unfold in a similar fashion to the FeTauD- αKG^* and/or (Fe)TauD-aurine species, as observed earlier these complexes in a lower enthalpy state. Perhaps this behavior is from a destabilization of one of the monomeric units of the dimeric

enzyme. The thermodynamic parameters for this species, which we are terming FeTauD- α KG-aurine* are T_m of 68.0 ($\pm$ 0.1) °C and a H of 116.3 ($\pm$ 1.4) kcal/mol. The remaining 33 ($\pm$ 2) % of the protein unfolded at a T_m of 71.9 ($\pm$ 0.02) °C and a H = 184.1 ($\pm$ 0.5) kcal/mol. We have assigned these parameters to the unfolding of the quaternary complex, FeTauD- α KG-aurine. In all enzyme-substrate complexes, an increase in T_m in comparison to free FeTauD or TauD was observed, but the enthalpy terms are more complex principally due to the endothermic binding of the substrate taurine and to the interaction between the binding of taurine and α KG.

4. Discussion

The data presented for the unfolding equilibria of TauD and a series of enzyme-substrate complexes by CD and DSC give a unique prospective into how this metalloprotein is stabilized by its metal ion and substrates. These linked equilibria include changes in solvation and ionization states of the protein, metal ion, substrate and cofactor, and buffer components, in addition to protein aggregation [21,28–31]. Many of these linked equilibria have no impact on the secondary structure monitored by CD (mainly the cupin fold), however the contributions of these effects on the overall denaturation process are better captured by monitoring the change in heat capacity of the enzyme complex using the DSC. This notion results in H_{vH} and H_{cal} values that are inconsistent with one another, just as we observe between the H_{vH} calculated from our CD experiments and the measured H_{cal} from the corresponding DSC studies. Indeed, deviations between the H_{vH} and H_{cal} values are commonly reported for unfolding equilibria [22]. For this reason, we have chosen to rely on DSC data rather than the van't Hoff analysis for more accurate enthalpies of denaturation. The reported enthalpies include all processes that take place in solution, unless deconvolution is noted. The CD spectra supply further evidence of the unfolding events occurring in solution, and provide crucial information on the enzyme structure during the unfolding process.

Comparisons of the heat capacity curves of TauD and FeTauD (Fig. 4, purple and orange curves) indicate that FeTauD is more stable than the apoprotein. More specifically, iron(II) confers approximately 2.2 °C in stability for the enzyme, as well as increasing the unfolding energy, H_{cal} , by approximately 12.7 kcal/mol (Table 2, Figure S9 reactions A and B1). Previous reports have shown there is no significant structural rearrangement when iron(II) binds to the 2-His-1-carboxylate facial triad [16,32,33], which suggests the overall increase in stability for the enzyme is primarily the change in enthalpy for binding iron(II). These data are supported by our CD data, which show no differences in structure between TauD and Fe-TauD; and by recently reported thermodynamic properties of metal binding to TauD, which indicate an overall binding enthalpy of –11.5 kcal/mol [21]. A thermodynamic profile of iron(II) binding to TauD is shown in Figure 5A.

The primary substrate taurine binds within a cavity of the active site pocket in proximity to the nonheme iron(II) center (Fig. 1B). Our FeTauD-aurine C_p curve shows no significant stabilization in the T_m for the complex over the FeTauD species, but a decrease in the enthalpy of unfolding for FeTauD-aurine over the FeTauD species. We believe this process is best understood by a model in which the binding of taurine is entropically driven,

therefore the protein-aurine complex has a lower enthalpy of unfolding plus aurine dissociation (Table 2, Fig. S9 reactions C and D1). The δH_{cal} between FeTauD and FeTauD-aurine is approximately 26.2 kcal/mol (or 14.4 kcal/mol for the TauD apoprotein), providing a favorable entropy term for the binding process: $S = 105 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$. The aurine-bound enzyme is stabilized by substrate interactions with several amino acid residues within the active site pocket (Fig. 1B, blue residues): the sulfonate moiety H-bonds with His70, Val102, and Arg270; the amine group forms H-bonds through two local water molecules; aurine's C2 has hydrophobic interactions with Tyr73; and both the C1 and C2 atoms have hydrophobic interactions with Phe159 and Phe206 to aid in positioning the aurine molecule for efficient hydroxylation [32–36]. Thus, a conformational change induced by aurine supports a H-bonding network that holds the active site closed and positions hydrophobic residues away from solvent [35]. The H-bonding network surrounding the iron(II) site is disrupted when aurine is bound, this perturbation destabilizes the enzyme which leads to the unfavorable H for this event. The resultant favorable entropy term for aurine binding is likely to be due to burial of hydrophobic residues that correspond with conformational changes taking place within the constraints of the active site pocket and the interface of the dimeric units. These conformational changes may be masked completely, or are at most the subtle changes seen on the edges of the large, broad, cupin barrel CD signal. Aurine does not directly bind to the iron(II) center of FeTauD, and TauD apoprotein can bind aurine within its active site cavity as well. The thermal unfolding of TauD-aurine yields T_m and H_{cal} values that are highly similar to FeTauD-aurine. Under the assumption that the irreversible unfolding approximates the reversible (equilibrium) reaction,[37] a thermodynamic profile was generated for aurine binding to FeTauD and is shown in Figure 5B.

Alpha-ketoglutarate binds in a bidentate fashion to the iron(II) center, displacing two solvent molecules in the process [18]. Secondary sphere residues Thr126 and Arg266 aid in the stabilization of the FeTauD- α KG complex through H-bonding and salt bridge formation, respectively (Fig. 1B, yellow residues). The T_m for the FeTauD- α KG complex suggests that it is significantly more stable than the enzyme alone, and the gain in stability could be associated with the intramolecular interactions between the substrate, iron(II), and the local amino acid residues. Moreover, the C_p curve is much more complex than observed for the free enzyme; deconvolution of the asymmetric curve results in two distinct unfolding processes. Interestingly, the first unfolding event occurs at a much lower enthalpy than that for unfolding of the free enzyme. We suggest this result fits either a model in which the binding of a molecule of α KG to one monomer induces a conformational change that destabilizes the second monomer in the dimeric unit, or a model in which α KG binds in two different modes; a productive mode where α KG is coordinated to the iron(II) center, and a nonproductive mode where α KG binds elsewhere. The productive α KG binding mode, or favorable enthalpy binding mode, (Table 2, Fig. S9 reaction E2) exhibits an increased T_m and H_{cal} over the FeTauD sample (Table 2, Fig. S9 reaction B2). It is likely that in either case, the α KG-bound species is contained in the cupin barrel, with the 2-His-1-carboxylate facial triad along with Arg266 and Thr126 providing favorable interactions within the enzyme-substrate complexes. The higher T_m process likely corresponds with denaturation of the enzyme-substrate complex, where the H_{cal} contains the enthalpy of unfolding of

FeTauD, along with the enthalpy of dissociation of α KG. Subtraction of H_{FeTauD} (145.1 kcal/mol) from $H_{\text{FeTauD-}\alpha\text{KG}}$ (175.2 kcal/mol) yields an α KG enthalpy of binding of approximately $-30.1 (\pm 2.1)$ kcal/mol. Using the previously reported K value and the enthalpy of binding for α KG determined here, we generated a full thermodynamic profile for the binding reaction of α KG to FeTauD (Fig. 5C). Although the favorable Gibbs free energy for this process is small ($K_d = 270 \mu\text{M}$) [14], the enthalpically driven binding event is balanced by an equally large, unfavorable entropy term.

When taurine binds to holoenzyme in the presence of α KG, the remaining solvent molecule in the iron(II) coordination sphere is lost, leaving a five-coordinate iron center and priming the site for O_2 coordination [13]. Additionally, the binding equilibrium for α KG coordination increases by two orders of magnitude ($K_d = 8 \mu\text{M}$) when taurine is present [14]. When this quaternary complex is unfolded, we see two noteworthy unfolding events. One event has an increased T_m over the FeTauD- α KG species by approximately 3°C , and it shows a decrease in the enthalpic stabilization for the complex. This corresponds with a FeTauD- α KG*-like species, as discussed above. The second noteworthy denaturation process observed is assigned to the FeTauD- α KG-aurine species, which shows both an increase in its T_m and H_{cal} over that for FeTauD- α KG species. It seems the presence of taurine induces a structural reorganization that allows for tighter binding of α KG [14], and thus an increase in the T_m for of the FeTauD- α KG complexes (71.9°C). This structural reorganization may be observed in the CD signal, where wavelength shifts within the major curve are seen, in addition to subtle changes on the outer edges of the signal. The H_{cal} of quaternary complex unfolding (184.1 kcal/mol) was deconvoluted using the $H_{\text{FeTauD-aurine}}$ to yield a value of -65.2 kcal/mol, which is the enthalpy of α KG binding in the presence of taurine. Using the more favorable K_d for α KG binding in the presence of taurine yields an increase in favorable free energy ($\Delta G = -6.94$ kcal/mol), and a ΔS term of $-195 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$. This large increase in thermodynamic terms includes additional heats besides the coordination of α KG to the iron(II), such as conformational change and solvent release. Additionally, the unfavorable entropy that balances the thermodynamic profile (Figure 5D) is likely due to conformational restraints such as those on the rotational and translational entropy that arises from locking the conformation in place through the Arg270 bridge between substrates. This quaternary complex creates a five coordinate iron(II) center by loss of a water molecule, which has been proposed to supply approximately 10 kcal/mol in energy for stabilization [36]. The water loss could be compensated by subtle structural changes within the enzyme to drive the reaction forward at the catalytic site. The thermodynamic profile associated with α KG binding to the FeTauD-aurine species is shown in Figure 5D.

From deconvolution of the DSC data, which approximate the equilibrium reaction, thermodynamic profiles for the binding reactions of substrates to FeTauD are depicted in Fig. 5. The binding reaction of α KG in the absence and presence of taurine is clearly favorable in enthalpy and Gibbs free energy; however, taurine binding is clearly entropically driven. Although the magnitude of the energy term for taurine binding is quite small, the overall stability of the substrate-enzyme complex is evident from the thermal denaturation experiments, and illustrates the cooperativity associated with substrate and cofactor binding. At this point, the molecular mechanism associated with this cooperativity is not clear, but

interestingly there is a single amino-acid (Arg270) that appears to link taurine to α KG (Figure 1). Arginine 270 forms a salt bridge or hydrogen bonding network between the sulfonate functional group of taurine and the guanidinium group of its side-chain; this side-chain also interacts with a terminal carboxylic acid of α KG in the quaternary structure of FeTauD- α KG. This linkage is just one possibility that could be involved in the cooperativity between substrate and cofactor binding in this well studied, but still intriguing nonheme iron(II) protein.

5. Conclusion

In summary, the thermal unfolding data for TauD and its substrate-enzyme complexes suggest that intramolecular interactions between substrates and TauD provide stability to the globular structure. Together these data provide the means to map a thermodynamic cycle associated with iron(II), α KG, and taurine binding to TauD (Figure 6). This cycle clearly shows the complexity of the interactions between the metal ion and substrates in the monomeric unit of TauD, where the presence of the substrate impacts the affinity of the cofactor. Although the mechanism of this cooperativity is unclear, the overall shift in melting temperature and enthalpy of denaturation (12 °C and 51.8 kcal/mol, respectively) suggest that binding of these two substrates in the active site cavity of TauD have a significant impact on the global stability of the substrate-enzyme complex.

Instrumental set-up, raw heat curves, and representative denaturation curves for each of the TauD species and the curve fits are available in the supplemental information. The supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.xxx.xx.xxx>

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

αKG	alpha-ketoglutarate
CD	circular dichroism
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
FeTauD	the holoprotein form of TauD

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Fig 1.

(A) The FeTauD monomer binds iron(II) in the center of the characteristic cupin barrel (red β strands). (B) The generalized 2-His-1-carboxylate binding motif, where the three endogenous ligands occupy one face of the iron(II) octahedral coordinating sphere. (C) The active site pocket of FeTauD with bound substrates (magenta carbon atoms) within the cavity. The carbons of the 2-His-1-carboxylate ligands are shown in green, the α KG binding domain contains secondary sphere residues that stabilize α KG (yellow carbon atoms), and the taurine binding domain includes a support network for positioning and stabilizing the primary substrate (blue carbon atoms). The crystal structure of the active site of FeTauD with bound substrates is taken from the protein data bank (PDB ID: 1OS7).

Fig. 2. CD spectra of folded (F) and thermally unfolded (U) TauD complexes at 25 and 85 °C, respectively. Individual traces are shown for TauD (black), TauD-aurine (purple), FeTauD (red), FeTauD-aurine (blue), FeTauD-αKG (orange), and TauD-αKG-aurine (green). Data were smoothed and corrected to remove a background signal.

Fig. 3. Thermal stability of TauD species: TauD apoprotein (black), FeTauD (red), TauD-taurine (purple), FeTauD-taurine (gray), FeTauD- α KG (green), and the quaternary complex (blue) consisting of TauD with bound Fe(II), taurine, and α KG. The unfolding transitions were monitored by CD at 223 nm after thermal equilibration for 5 min at each temperature from 25 to 85 °C.

Fig. 4. Heat capacity curves for the thermal denaturation of TauD species: TauD apoprotein (blue), FeTauD (orange), TauD-aurine (purple), FeTauD-aurine (green), FeTauD- α KG (red), and the quaternary complex (black) consisting of TauD with bound Fe(II), taurine, and α KG.

Fig. 5. Thermodynamic profiles for (A) iron(II) binding to TauD, (B) taurine binding to FeTauD, (C) α KG binding to FeTauD, and (D) α KG binding to FeTauD-aurine. G (red), H (blue) and $-T\Delta S$ (green) terms are taken from values listed in Table 2.

Fig. 6.

Summary of thermodynamic data collected as part of the DSC study. δH terms associated with each equilibrium are shown in parenthesis and have units of kcal/mol.

Table 1Thermodynamic data for the thermal denaturation of TauD ^a

Experiment	CD T _m (°C)	DSC Event	DSC T _m (°C)	H _{cal} (kcal/mol)	%	Proposed complex(es) unfolding
TauD	51.1 (±1.1)	A	59.2 (±0.1)	132.3 (±1.8)	100	TauD
FeTauD	54.8 (±2.7)	B1	58.2 (±0.03)	133.0 (±1.5)	22 (±1)	TauD
		B2	61.4 (±0.4)	145.1 (±1.8)	82 (±2)	FeTauD
TauD-<i>taurine</i>	53.6 (±1.6)	C	60.6 (±0.9)	117.9 (±0.6)	100	TauD- <i>Taurine</i>
FeTauD-<i>taurine</i>	58.4 (±0.6)	D1	61.9 (±0.2)	143.0 (±0.004)	5 (±0.1)	FeTauD
		D2	61.8 (±0.2)	118.9 (±1.3)	96 (±1)	FeTauD- <i>taurine</i>
FeTauD-<i>α</i>KG	54.4 (±2.7)	E1	65.0 (±0.1)	121.0 (±0.01)	65 (±6)	FeTauD- <i>α</i> KG*
		E2	67.8 (±0.2)	175.2 (±1.1)	38 (±6)	FeTauD- <i>α</i> KG
FeTauD-<i>α</i>KG-<i>Taurine</i>	60.1 (±0.1)	F1	62.2 (±0.2)	143.0 (±0.01)	3 (±2)	FeTauD
		F2	68.0 (±0.1)	116.3 (±1.4)	63 (±8)	FeTauD- <i>α</i> KG* (Fe)/TauD- <i>taurine</i>
		F3	71.9 (±0.02)	184.1 (±0.5)	33 (±2)	FeTauD- <i>α</i> KG- <i>taurine</i>

^a A visual summary of the data in Table 1 is available in the supporting materials as Fig S5.

Table 2Thermodynamic properties of substrate binding to TauD^a

Substrate	<i>G</i> (kcal/mol)	δH (kcal/mol)	$-T \Delta S$ (kcal/mol) ^b
Iron(II)	-10.1 ^c	-12.8 ^d (± 2.5)	2.6 (± 0.5)
Taurine (binding to FeTauD)	-5.1 ^e	26.2 (± 2.2)	-31.3 (± 2.6)
α -Ketoglutarate (binding to FeTauD)	-4.9 ^e	-30.1 (± 2.1)	25.3 (± 1.8)
α -Ketoglutarate (binding to FeTauD-aurine)	-6.9 ^e	-65.2 (± 1.4)	58.3 (± 1.3)

^aThermodynamic parameters derived from DSC data collected in this study.^bCalculated from *G* values and δH values reported here using the following equation $G = H - T \Delta S$, where the δH term is estimated to be equal to the enthalpy of binding. Error in these $-T \Delta S$ values is based on the uncertainty associated with the H values measured in this study; error in the *G* and temperature terms are small and not impactful to the expected error in the $-T \Delta S$ data.^cValue obtained from ITC measurements [21].^dThis DSC measured H value compares well with the -12.7 kcal/mol value collected by Henderson et al. [21]^eCalculated values based on the K_d reported by Ryle et al. [14].