Supporting Information

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SI Materials and Methods

Materials. All chemicals were from Sigma-Aldrich, unless otherwise noted, and used as received. Restriction enzymes were from New England Biolabs, and oligonucleotide primers were from Operon Biotechnologies; competent cells were from Stratagene. Assays to determine the presence of partially reduced oxygen species (superoxide or hydrogen peroxide) as described in the main text were carried out using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt, horseradish peroxidase, and superoxide dismutase from Sigma, as recommended by the manufacturer.

Mutagenesis and Protein Purification. Mutagenesis was carried out on tauD at position 159 using a QuikchangeII mutagenesis kit (Stratagene) according to the manufacturer's protocol. The mutagenic primers are indicated in Table S1. Plasmid DNA was prepared using a Qiagen QIAprep Spin Miniprep Kit, and the sequence of the entire gene was confirmed according to sequencing from the University of California, Berkeley DNA sequencing facility. Protein expression and purification as in ref. 1 were accomplished similarly for wild-type and mutant enzymes. All protein concentrations were determined using Bio-Rad Bradford protein assay with BSA as standard.

Quenched Assays. Quenched assays to determine the stoichiometry of oxygen uptake to product formation were performed in the presence of 1 mM αKG, between 1 and 5 mM of the indicated isotopomer of *N*-methyltaurine [depending on the enzyme form used owing to differing K_m(substrate) values] and ambient dioxygen (237 μM at 30 °C). The reactions took place in a 1 mL volume contained within a jacketed reaction vessel and were monitored by a YSI model 5300 biologic oxygen electrode. Reactions were initiated with enzyme preloaded with substoichiometric Fe(II) (0.2–3.0 μM final concentration of loaded enzyme) and allowed to proceed until 20–75 nmol of oxygen had been consumed, at which point the reactions were quenched by the addition of 50 μL of 2.5 M trichloroacetic acid (TCA). Once oxygen uptake had ceased, the quenched assay was quickly neutralized by addition of 50 μL of 2.5 M NaOH. The concentrations of succinate or sulfite were determined as described below.

Succinate Determination. Succinate concentration was determined using a succinic acid assay kit from Megazyme International Ireland according to the manufacturer's protocol, adjusting the volumes to a final assay volume of 1 mL instead of the recommended 2.74 mL. Succinate concentration is determined via the kit by a coupled enzymatic assay using succinyl-CoA synthase (SCS), pyruvate kinase (PK), and lactate dehydrogenase (LDH), similar to a previously reported method (2). Succinate is converted to succinyl-CoA by SCS with concomitant production of ADP, which PK consumes in the production of pyruvate. The pyruvate produced stoichiometrically with respect to succinate is reduced to lactate by LDH using NADH. The decrease in the concentration of NADH is monitored spectrophotometrically at 340 nm. Mock assays spiked with 25 μM succinate were experimentally determined by this method to contain 26 ± 5 μM succinate, indicating no interference from assay components.

Sulfite Determination. Sulfite concentration was determined using Ellman's reagent. To quenched assays, 100 μL of 2.5 mM Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid), dissolved in 100 mM KP_i, pH 7.0] was immediately added and allowed to react with sulfite for ≈10 min (1). Protein precipitate was removed from quenched assays by centrifugation for 2 min at ≈17,000 × *g*. The concentration of sulfite present was determined by comparing the absorbance of the sample at 415 nm with a standard curve of sodium sulfite (Mallinckrodt) prepared under conditions of the assay. It was noted that stored sulfite stocks could oxidize or off gas as sulfur dioxide under acidic conditions, so that sulfite standards were made immediately before use. Sulfite concentrations were adjusted for any reactivity of compounds in the enzyme solution by subtracting an "enzyme blank" to which substrate was added after enzyme had been inactivated by TCA.

Turnover Assays. Assays were performed under saturating conditions as for quenched assays, but in various concentrations of buffer. The reactions were allowed to proceed until the rate of oxygen uptake equaled the background rate (the rate of oxygen uptake before the introduction of enzyme). The number of turnovers undergone was determined as the concentration of oxygen consumed divided by the concentration of enzyme added. Values in the limit of zero and infinite buffer were obtained by fitting the number of turnovers undergone vs. the inverse of the buffer concentration to an equation of the form $y = Ae^{(-bx)} + C$ [where y is turnovers undergone, and x is inverse buffer concentration (M^{-1})]. C will represent turnovers undergone in the limit of zero buffer concentration, and A + C will represent turnovers undergone in the limit of infinite buffer concentration. The form of this equation was chosen to allow determinations in the limits of very low and very high buffer concentrations, and the constants do not have any physical meaning beyond those outlined above. The raw data were fitted to the described exponential equation using KaleidaGraph (Fig. S1). It should be noted that the ratios of turnovers undergone at the highest buffer concentration (200 mM) to those at the lowest buffer concentration (10 mM) are very close to, but slightly lower than, those determined in the limits of infinite and zero buffer concentration. These ratios are shown in Table S2 along with the R^2 (square of the correlation coefficient) values of the described fits. It is also worth noting that even at the lowest buffer concentration (10 mM), the concentration of products produced initial rates in the presence of low enzyme concentrations (see "Quenched Assays" above) amounts to less than 1% of the buffer concentration, making a pH drift unlikely even under the most extreme conditions.

Stopped-Flow Kinetic Assays. Stopped-flow UV-visible spectra were obtained using a Hi-Tech Scientific model SF-61 DX2 stopped-flow spectrophotometer (xenon lamp, photodiode array detection mode, 1.0-cm path length, 1.5-ms integration time) in single-mixing mode. The system could be made sufficiently anaerobic by flushing the flow system with 50 mM bis-Tris buffer pH 6.2 made anaerobic by repeated vacuum/argon cycles. Enzyme/αKG/substrate solutions in 50 mM bis-Tris buffer were made anaerobic by repeated vacuum/argon cycles, and a minimal volume of anaerobic Fe(II) as ferrous ammonium sulfate in water was added using a gas-tight syringe. The anaerobic Fe(II) solution was prepared by exchanging the headspace over a Thermo Scientific Reacti-Vial containing ferrous ammonium sulfate with argon and adding an appropriate volume of water sparged vigorously with argon using a gas-tight syringe. One drive syringe of the stopped-flow apparatus contained 550 μM

enzyme, 500 μM Fe(II), 10 mM H or D substrate, and 10 mM αKG in 50 mM bis-Tris buffer pH 6.2. The other contained 50 mM bis-Tris buffer saturated with O_2 (1,200 μM). The stopping volume was ≈100 μL, and the drive pressure was 0.4 MPa. The temperature of the drive syringes and the observation cell was maintained at 10 °C using a Neslab recirculating bath. Apparent rate constants from kinetic traces were calculated from monoexponential fits using Kinetic Studio software (v. 1.0.5.19739, TgK Scientific), although biexponential fits to curves with well-defined accumulation phases yielded decay rates indistinguishable from those obtained using monoexponential fits. Representative traces and fits are shown in Figs. S2–S4. It should be noted that experiments were replicated 9–13 times, and although fits to single traces may appear less than ideal there was no reproducible bias in residuals, and the standard deviations of mean rate data never exceeded 16% (1 case) and were generally 5% or less.

1. Price JC, Barr EW, Tirupati B, Bollinger JM, Krebs C (2003) The first direct characterization of a high-valent iron intermediate in the reaction of an alpha-ketoglutarate-dependent dioxygenase: A high-spin Fe(IV) complex in taurine/alpha-ketoglutarate dioxygenase (TauD) from *Escherichia coli. Biochemistry* 42:7497–7508.

2. Luo LS, et al. (2006) An assay for Fe(II)/2-oxoglutarate-dependent dioxygenases by enzyme-coupled detection of succinate formation. *Anal Biochem* 353:69–74.

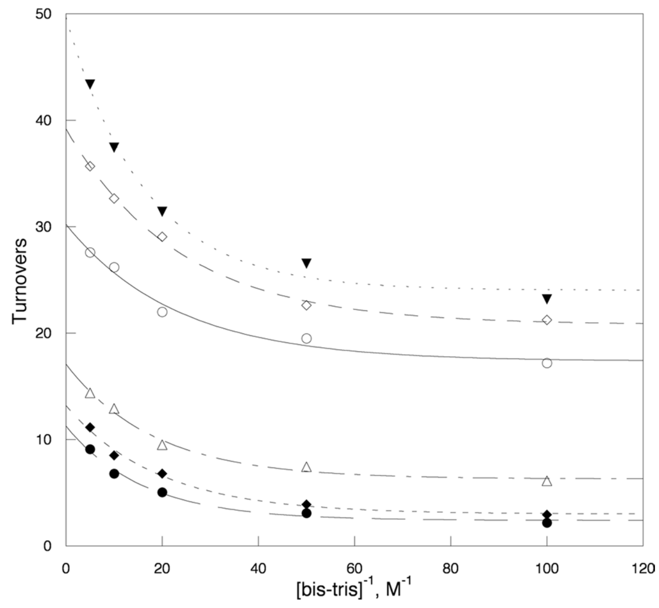

Fig. S1. Turnovers undergone by tauD and variants as a function of inverse buffer concentration. Inverted filled triangles, Wild-type with d_2 substrate; open diamonds, F159L with h_2 substrate; filled diamonds, F159L with d_2 substrate; open circles, F159V with h_2 substrate; filled circles, F159V with d_2 substrate; open triangles, F159A with h_2 substrate. The fits are as described in *SI Materials and Methods*.

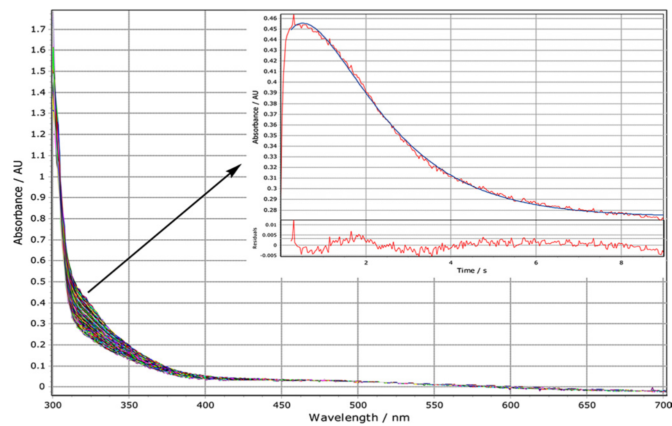

Fig. S2. Stopped-flow trace of wild-type tauD with 1,1-[$^2H_2$]-*N*-methyltaurine as substrate. The main panel shows the absorbance at all wavelengths over the course of the experiment. *Inset:* Absorbance at 318 nm as a function of time; the fit was performed as described above. The residuals are shown below the A_{318} vs. time inset.

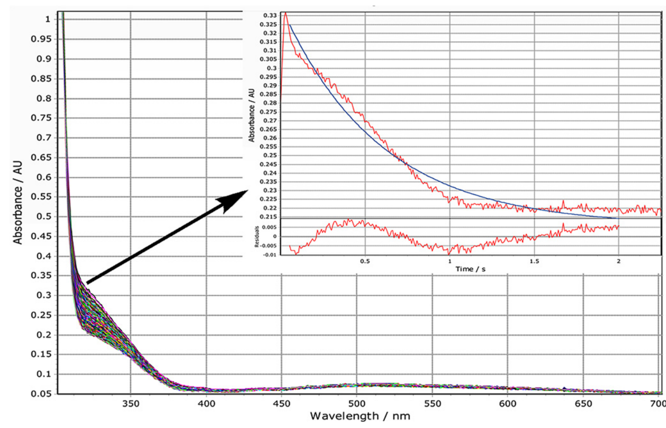

Fig. S3. Stopped-flow trace of F159L tauD with 1,1-[$^1H_2$]-*N*-methyltaurine as substrate. The main panel shows the absorbance at all wavelengths over the course of the experiment. *Inset:* Absorbance at 318 nm as a function of time; the fit was performed as described above. The residuals are shown below the A_{318} vs. time inset.

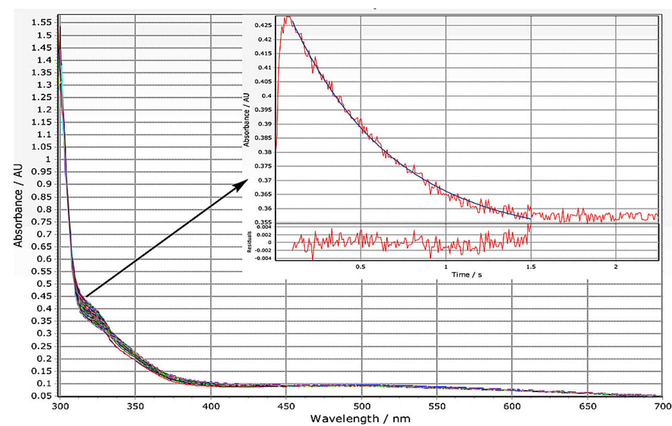

Fig. S4. Stopped-flow trace of F159V tauD with 1,1-[$^1H_2$]-*N*-methyltaurine as substrate. The main panel shows the absorbance at all wavelengths over the course of the experiment. *Inset:* Absorbance at 318 nm as a function of time; the fit was performed as described above. The residuals are shown below the A_{318} vs. time inset.

Table S1. Mutagenic primers used

Primer	Sequence*
F159Lfwd	CGGAGCATGATTTCCGTAAATCGTTACCGGAATACAAAT
F159Lrvs	ATTTGTATTCCGGTAACGATTTACGGAAATCATGCTCCG
F159Vfwd	AGCATGATTTCCGTAAATCGGTCCCGGAATACAAATACC
F159Vrvs	GGTATTTGTATTCCGGGACCGATTTACGGAAATCATGCT
F159Afwd	AGCATGATTTCCGTAAATCGGCCCCGGAATACAAATACCGC
F159Arvs	GCGGTATTTGTATTCCGGGGCCGATTTACGGAAATCATGCT
F159Gfwd	AGCATGATTTCCGTAAATCGGGCCCGGAATACAAATACCGC
F159Grvs	GCGGTATTTGTATTCCGGGCCCGATTTACGGAAATCATGCT

*The sequence is listed 3′ to 5′; the region leading to the mutated codon is underlined.

Table S2. Comparison of turnover ratios under highest and lowest experimental buffer concentrations with those in the extrapolated limits of infinite and zero buffer

Enzyme	Substrate	Hi/Lo*	∞/0†	$R^{2‡}$
Wild-type	D	1.9	2.1 ± 0.2	0.989
F159L	H	1.7	1.9 ± 0.1	0.998
F159V	H	1.7	1.7 ± 0.2	0.982
F159A	H	2.4	2.7 ± 0.4	0.989
F159L	D	3.9	4.4 ± 1.1	0.990
F159V	D	4.1	4.7 ± 0.9	0.990

H, 1,1-[$^1H_2$]; D, 1,1-[$^2H_2$]-2-methylaminoethane-1-sulfonic acid.

*Ratio of turnovers undergone in the highest buffer concentration used (200 mM bis-Tris) to those undergone in the lowest buffer concentration used (10 mM bis-Tris).

†Ratio of turnovers undergone in the extrapolated limits of infinite and zero buffer.

‡Square of the correlation coefficient of the exponential fit described in *SI Materials and Methods*.