Supporting Information for "A Mammalian Reductive Deiodinase has Broad Power to Dehalogenate Chlorinated and Brominated Substrates."

by

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Materials All reagents were obtained at the highest grade available commercially and used without further purification. 3-Methyltyrosine (Me-Tyr) was prepared previously using a literature protocol.[1]

General Methods Iodotyrosine deiodinase was expressed and isolated from Sf9 cells according to a method described previously.[2] Protein concentrations were determined using an ε_{280} of 57,600 M^{-1} cm^{-1} for holoenzyme IYD[3] and an ε_{450} 12,500 M^{-1} cm^{-1} for flavin (FMN).[4]

Anaerobic Reduction and Single Turnover of IYD. Anaerobic analysis of IYD was adapted from Goswami and Rosenburg.[5] Absorbance measurements were made using a Hewlett-Packard 8453 spectrophotometer (Palo Alto, CA). IYD solutions (36 μM based on FMN unless otherwise noted) containing 100 mM potassium phosphate pH 7.4 were made anaerobic by purging the head space of a sealed air tight cuvette using ultra-pure nitrogen at 4 °C while stirring for 6 hrs. Solutions were purged for an additional one hr at 25 °C while stirring and were subsequently maintained under these conditions throughout the assays. IYD was reduced by addition of an anaerobic stock solution of dithionite (~10 mM) until A_{450} no longer decreased and A_{317} (an isobestic point of oxidized and reduced flavin) began to increase. A minimum of excess dithionite (5 %) was added to ensure complete reduction of the cofactor. Anaerobic solutions of tyrosine derivatives (10 mM stock) were added in increments of 0.33 equiv. to the reduced IYD. Reaction was monitored spectrophotometrically for 15 min between each addition. A total of 2.0 molar equiv. of the respective Tyr derivatives was added before the assays were completed.

Product Detection after Anaerobic Turnover of IYD. Samples were analyzed by HPLC using a JASCO PU-908/MD1510 diode array instrument (Easton, MD), a reverse-phase C-18 analytical column (Varian, Microsorb-MV 300, 5 μm particle size, 250 mm x 4.6 mm) and a solvent program (1 mL/min) of 10 mM triethylammonium acetate (TEAA) pH 5.5 for 0-5 min followed by a linear gradient of 0-83 % methanol in10 mM TEAA pH 5.5 over 30 min. Aliquots (100 μL) of the anaerobic samples described above were analyzed directly by HPLC after filtration through a 10 kD centrifugal membrane filter. Solutions of each amino acid standard (36 μM) were examined similarly. The integrated signal of the standards at 280 nm were used to quantify the products of each enzymatic assay.

Equilibrium Binding of Ligands to IYD. Ligand binding was monitored by the change in fluorescence of the flavin bound to IYD using λ_{ex} of 450 nm and λ_{em} of 527 nm and a Hitachi F-4500 fluorescence spectrophotometer (Schaumburg, IL). Solutions of IYD (1.5 μM) in 100 mM potassium phosphate pH 7.4 (25 °C) were titrated with the Tyr derivatives over a range of four log units centered at the concentration that quenches 50% of the fluorescence (from 0.015 to 415 μM depending on the ligand). A minimum of three independent titrations were recorded for each ligand. Fluorescence intensities were corrected for the slight dilution caused by addition of ligand, normalized by dividing the observed fluorescence by the initial fluorescence (F_0), and plotted against ligand concentration [S_0]. Dissociation constants (K_D) were calculated as prescribed by the literature from a nonlinear best fit to eq. 1 (Tyr and Me-Tyr) and eq. 2 (I-Tyr, Br-Tyr, Cl-Tyr, and F-Tyr) using Origin 7.0.[6]

$$F = F_0 + \Delta F[S]_0/([S]_0 + K_D) \qquad \text{(eq. 1)}$$

$$F = F_0 + \Delta F \times \left\langle \frac{(K_D + [E]_0 + [S]_0) - \sqrt{(K_D + [E]_0 + [S]_0)^2 - 4[E]_0[S]_0}}{2[E]_0} \right\rangle \qquad \text{(eq. 2)}$$

X-band EPR Spectroscopy. EPR data were collected on a Bruker EMX 6/1 spectrometer equipped with a microwave frequency meter. Spectra of IYD were collected with the following experimental parameters: microwave frequency, 9.415 GHz; microwave power, 0.054 mW; modulation amplitude, 10 G; time constant, 40.96 ms; conversion time, 81.92 ms; gain, 1×10^4; 16 scans; temperature, 77 K. Quantitative spectra were collected using the following modification of the parameters above: microwave frequency, 9.41 GHz; gain, 5×10^4 and 5×10^3 for IYD and TEMPO, respectively; 4 scans. An anaerobic sample of IYD as described above was concentrated 14-fold using a 10 kD centrifugal membrane filter and then diluted by 50% with glycerol. This sample was stored for 16 hrs at 4 °C prior to EPR measurements. A calibration curve was generated with TEMPO (50, 100 and 250 μM) to quantify the radical signal maintained by IYD.

1. (a) Friedman, J. E. Ph.D. Dissertation, University of Maryland, 2001. (b) Kim, K.; Parang, K.; Lau, O. D.; Cole, P. A., *Bioorg. Med. Chem.* **2000**, *8*, 1263-1268.
2. Thomas, S.; McTamney, P. M.; Adler, J. M.; LaRonde-LeBlanc, N.; Rokita, S. E., *J. Biol. Chem.* **2009,** *284*, 19659-19667.
3. McTamney, P. M.; Adler, J. M.; Rokita, S. E., in preparation.
4. Koziol, J., *Methods Enzymol.* **1971,** *18*, 235-285.
5. Goswami, A.; Rosenberg, I. N., *J. Biol. Chem.* **1979,** *254*, 12326-12330.
6. Warner, J. R.; Copley, S. D., *Biochemistry* **2007**, *46*, 13211-13222.

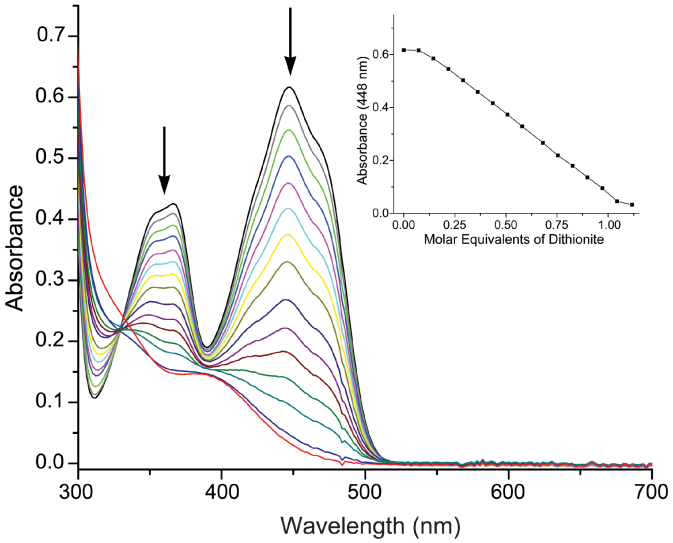

Figure S1. Reduction of flavin (FMN) within the active site of IYD as monitored by UV-Vis spectroscopy. Serial additions of dithionite (0.075 equiv. each) were combined with the oxidized IYD (50 μM) (–) under anaerobic conditions to generate the fully reduced form of the enzyme (–). The inset demonstrates that 1 equiv. of dithionite is sufficient to reduce each flavin (two per IYD dimer).

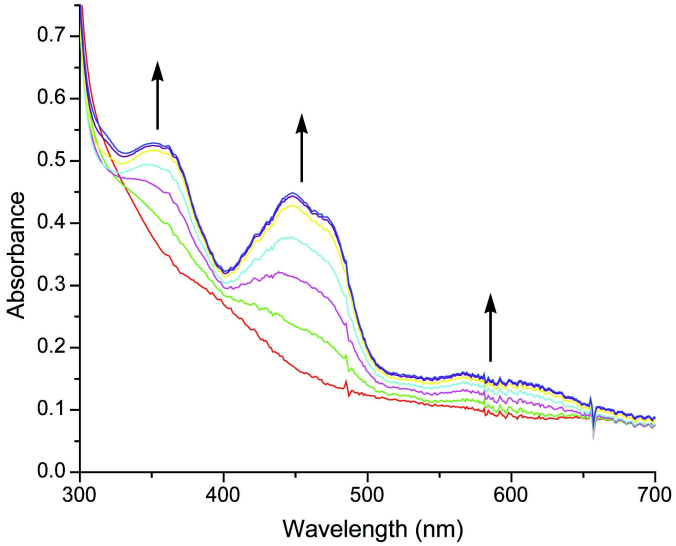

Figure S2. Reoxidation of the reduced form of IYD as monitored by UV-Vis spectroscopy. The fully reduced flavin (FMN) (–) of IYD (36 μM in FMN concentration) was oxidized under anaerobic conditions by serial additions of I-Tyr in increments of 0.33 equiv. to yield the final spectrum of oxidized IYD (–).

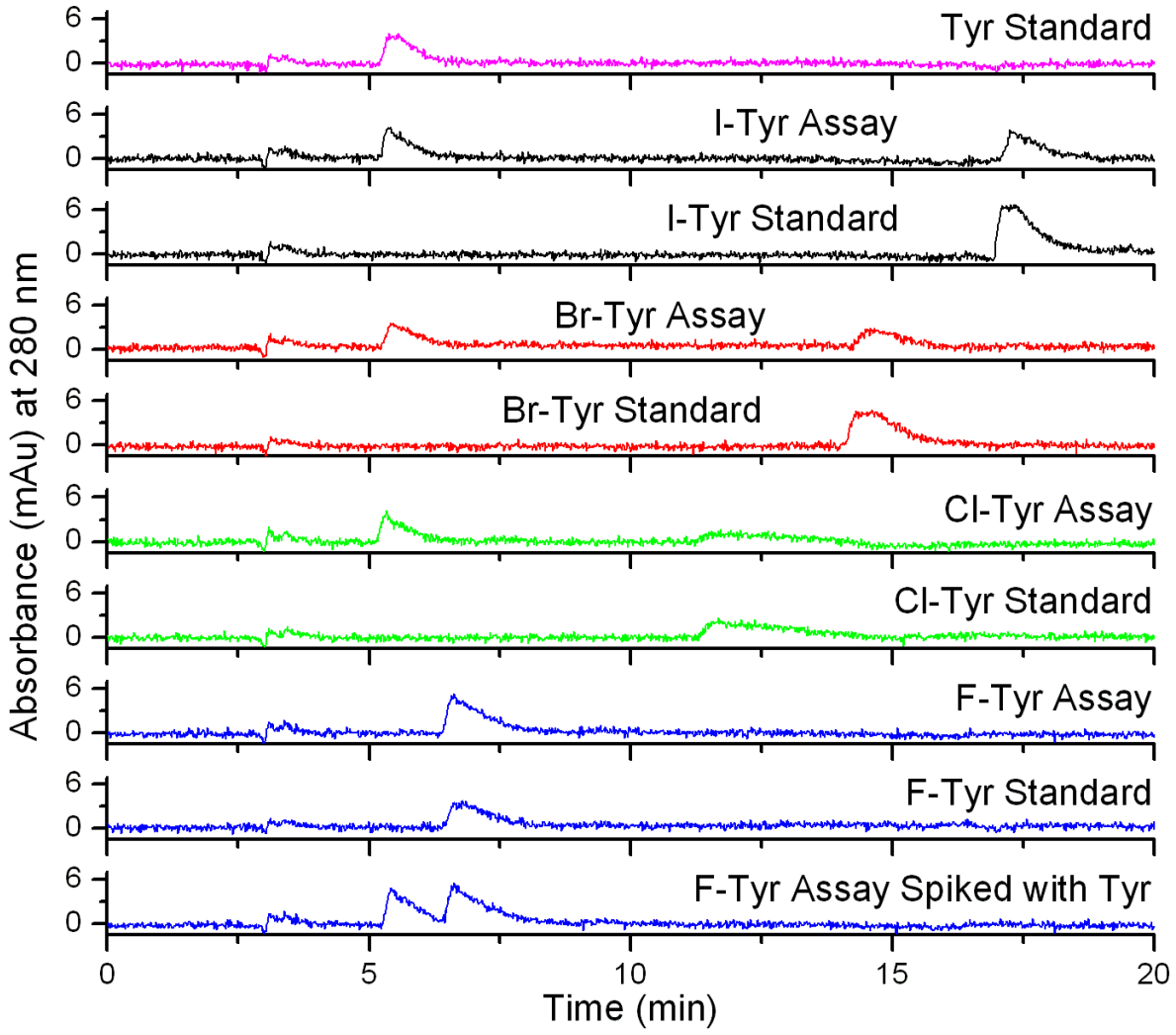

Figure S3. Reverse-phase (C-18) HPLC analysis of substrate dehalogenation by reduced IYD under anaerobic conditions. Aliquots of the anaerobic single turnover experiments described in the methods section containing 2 equiv. of substrate were compared to the appropriate standards at the same concentration using a methanol gradient for elution. Additionally, a sample of the incubation containing F-Tyr and IYD was spiked with a Tyr standard as a positive control for detecting Tyr.

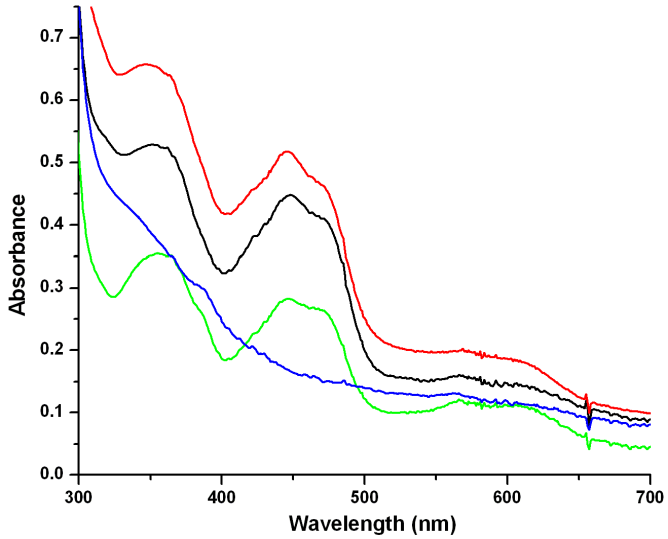

Figure S4. Absorbance spectra of IYD after its reduced form is oxidized by substrates. IYD containing fully reduced FMN (36 μM in FMN) was treated (25 °C) with 2.0 equiv. of I-Tyr (▬), Br-Tyr (▬), Cl-Tyr (▬), and F-Tyr (▬) alternatively for 2.5 h and then characterized by UV-Vis spectroscopy.

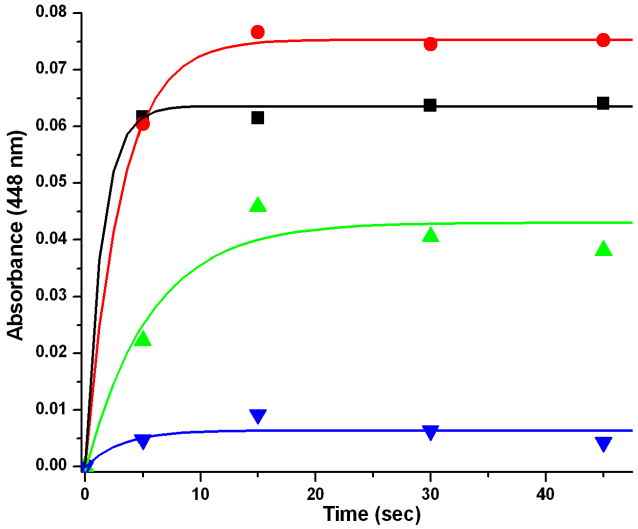

Figure S5. Rate of electron discharge from reduced IYD after addition of halotyrosines. A_{448} was monitored over time after 0.33 equiv. of I-Tyr (■), Br-Tyr (●), Cl-Tyr (▲), and F-Tyr (▼) was added to reduced IYD (36 μM in FMN) under anaerobic conditions (25 °C). Lines represent best fits of the data to a first order process (Origin 8.0) and provide estimated rate constants of ≥ 0.70 s^{-1}, 0.33 s^{-1} and 0.18 s^{-1} for I-Tyr, Br-Tyr and Cl-Tyr, respectively.

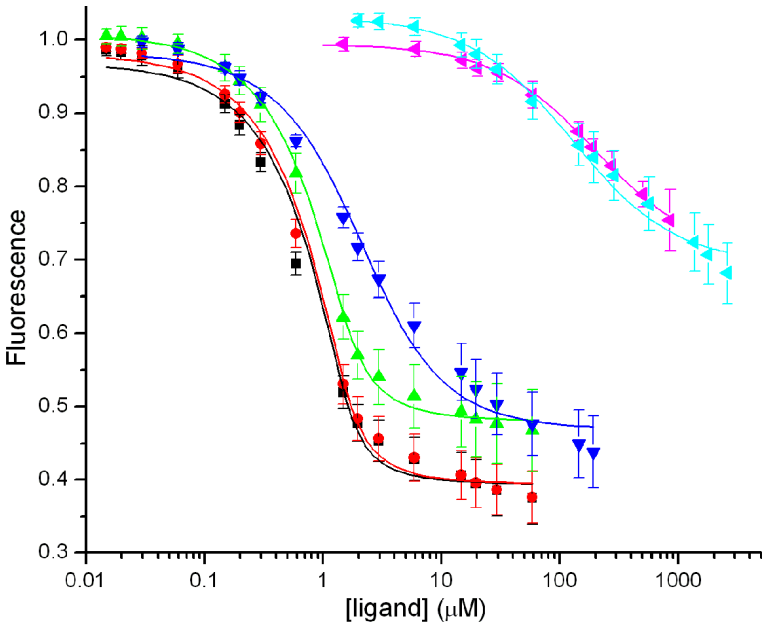

Figure S6. Fluorescence quenching of the FMN bound to IYD by substrates and ligands. Fluorescence emission (λ_{em} 527 nm; λ_{ex} 450 nm) was monitored during titration with tyrosine derivatives I-Tyr (–), Br-Tyr (–), Cl-Tyr (–), F-Tyr (–), Tyr (–) and Me-Tyr (–). Data points are an average of 3 or more independent sets of measurements, and the indicated errors represent standard deviation from each average value. Dissociation constants (K_D) derive from fits to eq. 1 (Tyr, Me-Tyr) and eq. 2 (I-Tyr, Br-Tyr, Cl-Tyr, and F-Tyr) as described above. Poor solubility of Tyr and Me-Tyr prevented accurate measure of binding and hence the minimum values based on the available data are reported.