Supporting Information

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for

The Flavoprotein Iodotyrosine Deiodinase Functions without Cysteine Residues

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Experimental Section

General materials. Oligodeoxynucleotide primers were obtained from Integrated DNA Technology (Coralville, IA). $\text{Na}^{125}$ used for radiolabeling diiodotyrosine (DIT) was obtained from Perkin-Elmer (Waltham, MA). All other reagents were obtained at the highest grade available and used without further purification.

General methods. DNA isolation was performed using Qiagen Mini, Midi, or Maxiprep Kits or a Fermentas GeneJet Plasmid Miniprep Kit. PCR reactions were performed by using an Eppendorf Mastercycler. All horizontal DNA agarose gel electrophoresis included a sample of a Fermentas Mass Mix DNA Ladder and was performed under standard conditions at 125 V using the appropriate percentage of agarose.$[^1]$ $E. \text{coli}$ transformations utilized an Eppendorf Electroporator 2510 (1700 V, 1 mm gap cuvette) and were plated on antibiotic selective LB plates.$[^1]$ All DNA sequencing was performed by Geneway Research (Hayward, CA). Protein concentrations were determined using the Pierce BCA assay. Vertical protein gel electrophoresis was performed at 200 V using the Bio-Rad Mini Protean 3 gel electrophoresis system. Denaturing dis- continuous
SDS-PAGE gels (12 % acrylamide resolving and 5 % stacking) and 1 X Laemmli running buffer were prepared and used according to standard protocols.\textsuperscript{[S1,S2]} Protein gels were stained by Coomassie Brilliant Blue. All ligations and dephosphorylations were performed under standard conditions using T4 ligase, and Antarctic phosphatase, respectively, purchased from New England Biolabs (Ipswich, MA).

**Mutagenesis of IYD for mammalian expression.** The IYD gene (\textit{M. musculus}, I.M.A.G.E. clone 5064638 from ATCC) was cloned into the mammalian expression vector pcDNA3.1(+)Zeo-IYD as described previously.\textsuperscript{[S3]} Mutagenesis was performed according to the Stratagene Quickchange Kit. The C217A mutant was generated with oligonucleotides 5'-CAGTGTGTCCATCGCC\textit{GCAGGCCCTTGCTGGC-3'} and 5'-GCCAGCGAGGCGCTTGGCGATGGGTGGC-3' (mutations in bold and introduced/removed restriction sites in italics). The C239A mutant was generated with oligonucleotides 5'-CACTACCACTCCCCCTCAACGCTGGTCTAGACTGAGGGGTGCTCC-3' and 5'-GGAGCACCCTCAGTCTAGGACCAGCGTTGAGGGGAGTGGTACTG-3'. The C217A/C239A double mutant was generated by sequentially applying the two sets of oligonucleotides above. Quickchange reaction mixtures were digested with Dpn I and then used to transform One Shot Top10 (Invitrogen) \textit{E.coli} according to the manufacturer's instructions. Each vector DNA was isolated by miniprep procedures from Zeocin resistant cells and screened for the C217A and C239A mutations by restriction analysis for the addition of a Stu I site and changes to the banding pattern created by Bfa I and Hpy181 I, respectively, before the genes were submitted for sequencing. The truncated mutant IYD Δ2-33 was generated from PCR of the parent IYD-containing plasmid and primers 5'-AAGCTTAAAGCTTGAGAATCCGCCACC\textit{ATGGCCTCAAGTTCAGCCC-3'} and 5'-ATTC\textit{TCCAGCTAATGGATGGGATGGGATGGTACTGCTACCATGAT-3'}. The resulting DNA product and plasmid were digested by Bam HI/Xho I, ligated together and then processed equivalently to the other mutants.

**Expression of IYD in mammalian cells.** Mammalian cells (HEK 293 and CHO) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Atlanta Biologicals) and 1% penicillin-streptomycin-glutamine (PSG, Invitrogen). Plasmids containing the individual constructs of IYD (12 µg) were incubated for 20 min with 30 µL of Lipofectamine 2000 in Opti-MEM (Invitrogen)
and then added to cells (~90 % confluent) in 10 cm dishes. After 6 h, the liquid medium was exchanged to DMEM, 10 % FCS and 1 % PSG. 48 h after transfection, the cells were washed twice with 10 mL Dulbecco’s phosphate buffered saline (DPBS, Invitrogen) and finally resuspended in DPBS again. The cells were centrifuged (300 g) for 5 min at 4 °C, and the resulting cell pellet was resuspended in 50 mM sodium phosphate pH 7.2 (1 mL/plate of harvested cells) supplemented with 0.25M sucrose and 0.1 mM dithiothreitol. The cells were then lysed by three cycles of freezing (liquid N₂) and thawing (37 °C) followed by three passages through a 20 gauge needle.

**Deiodinase activity.** IYD turnover and kinetic constants were measured using a standard assay described previously in the presence of NADPH (10 mM) or dithionite (1% w/v) as reductants. Concentrations of [¹²⁵I]-diiodotyrosine (DIT) were varied under standard conditions. Reaction mixtures were incubated under ambient temperature for 30 min and then passed through a cation exchange column (AG 50W-X8). The eluant containing [¹²⁵I]-iodide was quantified using a Packard 1600 TR liquid scintillation analyzer. Data from multiple independent trials (Figure S1) were fit to Michaelis-Menten kinetics using Origin 7.0. V_max values were calculated by estimating the concentration of IYD by electrophoretic separation of crude isolates and subsequent staining and densitometry (ImageQuant) as illustrated in Figure S2. These values were then divided by the predicted molecular weight of the appropriate enzymes to yield the corresponding k_cat values.

**Subcellular fractionation of IYD.** Lysates (4 mL) from HEK293 cells (50 mg) transfected with plasmids encoding IYD ?2-33 were centrifuged (500 x g, 15 min) to remove unlysed cells. The supernatant was transferred to polycarbonate tubes and further centrifuged for 30 min at 30 000 g (4 °C) to pellet cellular debris. The supernatant was finally centrifuged for 1.5 h at 100 000 g (4 °C). The supernatant was decanted and the pellet resuspended in an equal amount of cell lysis buffer.
Figure S1. Catalytic constants for wild type and mutant enzyme expressed in HEK 293 cells. Initial rates of deiodination were measured at varying $^{125}$I-diiodotyrosine (DIT) concentration in the presence of the indicated enzyme. Each color represents an independent set of measurements, and the indicated error represents the standard deviation at each substrate concentration. Kinetic constants derive from the best fit (line)
to Michaelis-Menten kinetics as calculated by Origin 7.0.

Figure S2. Estimation of IYD concentration in cell lysates. (A) HEK 293 cells (~ 50 mg) were harvested after transformation with the gene for IYD (wild type) and then lysed. A sample (15 µg) of total lysate was separated by denaturing polyacrylamide gel electrophoresis and stained by Coomassie Brilliant Blue. Equivalent samples were analyzed for cells transformed with each variant of IYD. Illustrated here are the lysates of IYD (lane 1), IYD Δ2-33 (lane 2), and IYD C217A/C239A (lane 3). Molecular weight markers are indicated in lane M. (B) The relative abundance of each protein in lanes 1-3 were determined by densitometry. Constitutive proteins indicated as “a” and “b” were used as controls and found to vary by less than 3 % in each lysate. The concentration of wild type IYD was calculated as the difference in “c” between lanes 1 and 2. The concentration of IYD Δ2-33 was calculated as the difference in “d” between lanes 2 and 1 as well as 2 and 3.

Figure S3. Catalytic constants for wild type IYD expressed in CHO cells. Initial rates of deiodination were measured at varying [125I]-diiodotyrosine concentration (DIT) in the presence of the indicated reductant. Kinetic constants derived from the best fit (line) to Michaelis-Menten kinetics for reaction driven by dithionite. Maximum rate values measured in the presence of NADPH are connected by a line to establish the maximum threshold of possible activity.
References


