A Switch between One- and Two-electron Chemistry of the Human Flavoprotein Iodotyrosine Deiodinase Is Controlled by Substrate

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Jimin Hu, Watchalee Chuenchor, and Steven E. Rokita

From the Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742 and Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

Background: Iodotyrosine deiodinase utilizes FMN to maintain iodide homeostasis by reductive deiodination of iodotyrosine.

Results: Crystallographic, pH, and redox studies demonstrate the role of substrate in organizing the active site for effective catalysis.

Conclusion: Stepwise single electron transfer is promoted only after coordination of a halotyrosine within iodotyrosine deiodinase.

Significance: A synergy between substrate selectivity and catalytic activity is created by the enzyme.

Reductive dehalogenation is not typical of aerobic organisms but plays a significant role in iodide homeostasis and thyroid activity. The flavoprotein iodotyrosine deiodinase (IYD) is responsible for iodide salvage by reductive deiodination of the iodotyrosine derivatives formed by byproducts of thyroid hormone biosynthesis. Heterologous expression of the human enzyme lacking its N-terminal membrane anchor has allowed for physical and biochemical studies to identify the role of substrate in controlling the active site geometry and flavin chemistry. Crystal structures of human IYD and its complex with 3-iodo-L-tyrosine illustrate the ability of the substrate to provide multiple interactions with the isosalloxazine system of FMN that are usually provided by protein side chains. Ligand binding acts to template the active site geometry and significantly stabilize the one-electron-reduced semiquinone form of FMN. The neutral form of this semiquinone is observed during reductive titration of IYD in the presence of the substrate analog 3-fluoro-L-tyrosine. In the absence of an active site ligand, only the oxidized and two-electron-reduced forms of FMN are detected. The pH dependence of IYD binding and turnover also supports the importance of direct coordination between substrate and FMN for productive catalysis.

Iodide is a micronutrient necessary for thyroid hormone biosynthesis and essential for human health. Efficient use of dietary iodide depends on its accumulation in the thyroid as managed by a sodium-iodide symporter. Iodide is also salvaged from iodinated tyrosine by-products as promoted by iodotyrosine deiodinase (IYD). Nature’s reliance on iodine for signaling is highly unusual, and thyroid hormones represent the only example of this in higher organisms. Much of the biochemistry associated with these hormones is similarly unusual. The enzymes responsible for deiodination of the thyroid hormones represent one of two known types of reductive dehalogenases in humans. These process the iodine-containing hormones and are members of the thioredoxin structural superfamily. Their active site contains a selenocysteine residue to promote a thiol-dependent deiodination. The second type of reductive dehalogenase is IYD. This enzyme contains a flavin mononucleotide (FMN) rather than a selenocysteine, and its reaction appears to be driven in vivo by NADPH rather than thiols (Scheme 1). In addition, IYD is a member of the nitroFMN reductase structural superfamily, which differs significantly from the thioredoxin superfamily. Thus, mammals maintain two quite distinct strategies for catalyzing a closely related set of unusual reactions.

Prior studies on mouse IYD (mIYD) identified three subdomains: an N-terminal membrane anchor, an intermediate domain, and a catalytic domain. A soluble construct could be expressed in human and insect cells after truncating the gene to remove the membrane anchor. More recent fusion constructs have allowed heterologous expression of IYD from many organisms in Escherichia coli. This same strategy has now been applied to generate sufficient quantities of human IYD (hIYD). Direct investigation of hIYD is particularly advantageous for understanding the molecular basis of genetic dis

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The atomic coordinates and structure factors (codes 4TB and 4TC) have been deposited in the Protein Data Bank (http://wwpdb.org/).§1

1 Present address: Laboratory of Molecular Biology, National Inst. of Diabetes and Digestive and Kidney Diseases, National Insts. of Health, Bethesda, MD 20892.

2 To whom correspondence should be addressed. Tel.: 410-516-8420; E-mail: rokita@jhu.edu.

The abbreviations used are: IYD, iodotyrosine deiodinase; mIYD, mouse IYD; hIYD, human IYD; AQDS, anthraquinone-2,6-disulfonate; Br-Tyr, 3-bromol-tyrosine; CI-Tyr, 3-chloro-L-tyrosine; FMN<sub>red</sub>, flavin mononucleotide in its oxidized form; FMN<sub>red</sub>, reduced FMN; hydroquinone; FMN<sub>sq</sub>, one-electron reduced FMN semiquinone; F-Tyr, 3-fluoro-L-tyrosine; I-Tyr, 3-iodo-L-tyrosine; I<sub>2</sub>-Tyr, 3,5-diiodo-L-tyrosine; NB, Nile blue; SFO, Safranin O; SUMO, small ubiquitin-like modifier; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; RMSD, root mean square deviation.

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The ability for NADPH to drive catalysis is lost once IYD is removed from its native membrane, and consequently reductants such as dithionite have been used for most studies in vitro and in vivo. Likely an NADPH reductase is recruited in vivo, but it has yet to be identified. Attention has instead focused on the unusual participation of flavin in a reductive dehalogenation process that supports dechlorination and debromination as well as deiodination. The enzyme activity exists for a mechanism by which FMN may promote this reaction despite the considerable knowledge amassed on FMN from many years of investigating the broad array of flavoproteins that catalyze many diverse reactions. Crystallographic studies of mIYD implicate a significant role for substrate in controlling enzyme activity. The substrates 3-ido-L-tyrosine (L-Tyr) and 3,5-diido-L-tyrosine (I2-Tyr) induce closure of an active site lid that is essential for catalysis. In addition, these substrates simultaneously and directly coordinate to the isoalloxazine system of FMN as well. Investigations now describe the consequences of these interactions on the oxidation and reduction properties of FMN and their significance to the catalytic mechanism and substrate selectivity of IYD.

**EXPERIMENTAL PROCEDURES**

**Materials**—Biochemical reagents including xanthine oxidase were purchased from Sigma and used without purification unless specified. 3-Fluoro-L-tyrosine and Nile blue were purchased from Sigma and used without purification unless specified. 3-Fluoro-L-tyrosine and Nile blue were purchased from Sigma and used without purification unless specified. 3-Fluoro-L-tyrosine and Nile blue were purchased from Sigma and used without purification unless specified.

**Cloning, Expression, and Purification of a Soluble Domain of hIYD**—The human iodotyrosine dehalogenase gene (GenBank accession number AY259176.1) was amplified by PCR using primers 5'-AAGCTTAAGTCCGCCACCATGGCTCAAGTTCAGCCC-3' and 5'-CTCGAGCCGAGCTATGTTGATGTGATGTGATGCTCCACATGATC-3' to generate a gene lacking codons for amino acids 1–31 and gaining a C-terminal His6 tag. The PCR product was digested with BamHI and XhoI and inserted into the pET28-SUMO vector (obtained from Dr. Christopher Lima) in which a SUMO tag is appended to the N terminus of the deiodinase. The resulting plasmid pET28-SUMO-JH1 containing the desired gene was transformed into Rosetta (DE3) E. coli (Novagen). Cells were grown in LB medium with kanamycin and chloramphenicol at 37 °C to an A600 of 0.6–0.8. The medium was then cooled to 18 °C, and protein expression was induced by addition of 0.2 mM isopropyl thio-β-galactoside. After 4 h at 18 °C, cells were harvested by centrifugation and resuspended in buffer N (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, and 10% glycerol). Cells were then flash frozen and stored at −80 °C until use. An alternative derivative of the hIYD gene was generated to truncate residues 1–69 by the appropriate PCR of pET28-SUMO-JH1, but this was abandoned after insoluble protein was observed to dominate expression.

To isolate the deiodinase, cells were thawed and subsequently lysed by three passages through a French press at 1000 p.s.i. Cell debris was removed by centrifugation at 40,000 × g at 4 °C for 2 h. The supernatant was fractionated with nickel ion affinity chromatography (Hispur nickel-nitrilotriacetic acid resin, Thermo Scientific) that was equilibrated with buffer N. Successive washes used increasing concentrations of imidazole (20 mM for 5 column volumes, 60 mM for 5 column volumes, 80 mM for 2 column volumes, and 100 mM for 1 column volume) in buffer N. The SUMO fusion of hIYD was finally eluted with 350 mM imidazole in buffer N and digested with Ulp1 protease (1:200, w/w) to release hIYD from SUMO. The resulting solution was concentrated to 3 ml and passed through a gel filtration column (Sephacryl S-200 HR, GE Healthcare) equilibrated with 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM DTT, and 10% glycerol. Fractions containing hIYD were pooled, and the concentration of hIYD was determined by the A280 after correcting for the contribution from bound FMN (A_{280}/A_{450} = 1.57) and an extinction coefficient (ε_{280} = 37,930 M⁻¹ cm⁻¹) estimated by the ExPaSy ProtParam tool (16). The concentration of FMN was determined by its A_{450} (ε_{450} = 12,500 M⁻¹ cm⁻¹) (17). The purified hIYD was concentrated to 10 mg/ml using Amicon Ultra centrifugal filters prior to storage at 4 °C.

**Deiodinase Activity Assay**—Catalytic deiodination was measured by release of [125I]iodide from [125I]I2-Tyr as reported previously (18). Sodium dithionite was used as the reducing reagent for enzyme turnover, and I2-Tyr concentration was varied between 0 and 70 μM. Each assay was performed in triplicate, and their average was fit to Michaelis-Menten kinetics using Origin 7.0. Reported error is based on the results of least square fitting.

**pH-Rate Profiles**—Steady-state kinetic constants were determined from the standard deiodinase assay at pH 6.0–7.5 (100 mM potassium phosphate) and pH 7.5–8.5 (100 mM Tris-HCl) using 0.08 μM hIYD. The values of V_{max} and V_{max}/K_m represent the average of three independent determinations. The log V_{max} and log(V_{max}/K_m) were plotted against pH and fit to Equation 1 (19).

\[ \log y = \log y_{\max} - \log(1 + 10^{pH - pK_s}) \]  
(Eq. 1)

**Affinity of Halotyrosines for hIYD**—Binding of halotyrosines to IYD was measured by their ability to quench the fluorescence of FMN (λ_{ex} = 450 nm and λ_{em} = 527 nm) as described previously (12). Briefly, hIYD (4.5 μM) was titrated with the Tyr derivatives over a range of concentrations centered around the value necessary for quenching 50% of the initial fluorescence signal (F_{0}). Fluorescence intensities were measured with a Hitachi F-4500 fluorescence spectrophotometer and normalized to the initial fluorescence (F_{0}) obtained in the absence of substrate. Dissociation constants (K_{D}) were obtained from the non-linear fit (Origin 7.0) of fluorescence versus ligand concentration using Equation 2 (20). Reported K_{D} values derive from
the average value of three independent determinations, and their error is based on the results of least square fitting. The pH dependence of ligand affinity was determined at pH 6.0–8.0 (100 mM potassium phosphate) and pH 8.0–9.0 (100 mM Tris-HCl). The values of log $K_D$ were plotted versus pH, fit to the sum of Equations 1 and 3, and optimized iteratively (19).

$$F = F_0 + \Delta F$$

$$\times \left( \frac{K_D + [E] + [S]}{\sqrt{K_D + [E] + [S]^2 - 4[E][S]}} \right)$$

(Eq. 2)

$$\log y = \log y_{max} - \log(1 + 10^{(\log E - \mathrm{pH})})$$

(Eq. 3)

**Protein Crystallization, Structure Determination, and Refinement**—Crystallization trials utilized a Phoenix crystallization robot and sparse matrix screening Wizard$^\text{TM}$ I, II, III, and IV (Emerald Biosciences), PEGSuite$^\text{TM}$ and CryoSuite$^\text{TM}$ (Qiagen), and Index$^\text{TM}$ (Hampton Research). After optimization, hIYD crystals grew in ~2 days at 20 °C by the hanging drop diffusion method with a ratio of 1 μl of hIYD (12 mg/ml; 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM DTT, and 10% glycerol) and 1 μl of precipitant (0.05 M ammonium sulfate, 50 mM BisTris, pH 6.5, and 25% pentaerythritol ethoxylate). Crystals were cryoprotected in the precipitant supplemented with 15% glycerol and 1 mM FMN prior to data collection. To generate co-crystals, hIYD was treated with 1.5 mM I-Tyr overnight and then subjected to the same hanging drop procedure using a well solution of 0.15 M sodium acetate, 85 mM Tris-HCl, pH 8.5, 25.5% (w/v) polyethylene glycol 4000, and 15% glycerol at 20 °C. Co-crystals of hIYD-I-Tyr formed within 24 h and were flash frozen in liquid nitrogen.

X-ray diffraction data were collected at Beamline 7-1 at the Stanford Synchrotron Radiation Laboratory and processed using the HKL2000 package (21). The structures of hIYD and mIYD (Protein Data Bank code 3TO0) (6) were used as the search model and yielded initial $R_{work}$ and $R_{free}$ values of 25.3 and 31.2%, respectively, from AutoBuild in PHENIX. A previous structure determined for mIYD (Protein Data Bank code 3TO0) (6) was used as the search model and yielded initial $R_{work}$ and $R_{free}$ values of 25.3 and 31.2%, respectively, from AutoBuild in PHENIX. The electron density maps for the hIYD and mIYD crystals were performed with Coot (23) and refined in PHENIX (22). The electron density maps for the hIYD-I-Tyr co-crystal using hIYD as the search model showed positive densities for I-Tyr. Final refinement statistics for both structures using hIYD as the search model showed positive den-

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**RESULTS**

**Expression and Purification of the Soluble Domain of hIYD**—Prior sequence analysis of the IYD gene indicated that the enzyme contains three domains: an N-terminal transmembrane region, an intermediate domain, and a catalytic domain (4). Removal of the transmembrane domain from mIYD generated a soluble, stable, and active derivative of the deiodinase (5). An equivalent truncation was performed on hIYD. The gene encoding hIYD but lacking the first (1–31) amino acids was inserted into the pET28-SUMO vector and expressed in *E. coli*. The soluble fraction of the fusion protein (~50%) was isolated by nickel-based affinity chromatography. The SUMO tag was then removed by the Ulp1 protease, and hIYD was separated by size exclusion chromatography in a yield of ~10–20 mg/liter of culture. Native protein exists as a homodimer and was isolated with the expected FMN to homodimer ratio of 1.9–2.1 as determined by A$_280$ and A$_450$. The purity of hIYD approached 97% as estimated by SDS-PAGE, Coomassie staining, and analysis by ImageQuant TL. The desired protein migrates similarly to that of a 30-kDa marker protein as expected for a monomer of 30.5 kDa as calculated by ExPASy.

**Deiodination Activity**—The standard $[^{125}]$iodide release assay (18) was used for initial characterization of hIYD expressed in *E. coli*. A $K_m$ of 31 ± 6 μM and a $k_{cat}$ of 12.5 ± 1 min$^{-1}$ were measured for I$_2$-Tyr. The resulting $k_{cat}/K_m$ of 0.40 min$^{-1}$ μM$^{-1}$ is very close to that reported for mIYD (0.95 min$^{-1}$ μM$^{-1}$) also lacking its N-terminal transmembrane region (5). The native sequence of hIYD had previously been expressed in HEK293 cells to yield a microsomal deiodinase exhibiting a $K_m$ of 2.67 μM and a $V_{max}$ of 53 pmol/min-μg of membrane fraction (27). The standard $[^{125}]$iodide release assay (18) was used for initial characterization of hIYD expressed in *E. coli*. A $K_m$ of 31 ± 6 μM and a $k_{cat}$ of 12.5 ± 1 min$^{-1}$ were measured for I$_2$-Tyr. The resulting $k_{cat}/K_m$ of 0.40 min$^{-1}$ μM$^{-1}$ is very close to that reported for mIYD (0.95 min$^{-1}$ μM$^{-1}$) also lacking its N-terminal transmembrane region (5). The native sequence of hIYD had previously been expressed in HEK293 cells to yield a microsomal deiodinase exhibiting a $K_m$ of 2.67 μM and a $V_{max}$ of 53 pmol/min-μg of membrane fraction (27).

**Affinity of hIYD for 3-Halotyrosines**—Binding of the various halogen-substituted tyrosines (F-Tyr, Cl-Tyr, Br-Tyr, and I-Tyr) to IYD was monitored by the decrease in fluorescence of the enzyme-bound FMN. Spectral changes were recorded every 2 min and compared with control samples containing no hIYD. Titrations were also repeated in the presence of F-Tyr (0.6 mM) to investigate the effect of ligand binding on the oxidation-reduction properties of the enzyme-bound FMN. Midpoint potentials of hIYD were calculated from concurrent reduction of a reference dye in situ (25) alternatively using anthraquin-one-2,6-disulfonate (AQDS; $E_m$ = −188 mV), Nile blue ($E_m$ = −116 mV), and Safranin O ($E_m$ = −280 mV) (26). Spectral changes were recorded every 2 min for over 2 h. All potentials are reported versus a standard hydrogen electrode and represent the average of three independent determinations.

**Substrate Control of FMN Redox Chemistry**

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phenolic proton. Further investigation shifted to hIYD as described below.

**pH Dependence of I₂-Tyr Deiodination and Binding with hIYD**—Steady-state kinetic analysis of I₂-Tyr deiodination by hIYD was repeated over a pH range of 6.0–8.5 (Fig. 1). The pH rate profiles of both log V and log(V/K) illustrate a basic limb with a slope of −1. Their respective pKₐ values of 7.5 ± 0.2 and 7.6 ± 0.2 as determined by Equation 1 are experimentally indistinguishable. The maximal activity at low pH is consistent with the protonation of a functional group within the active site complex. Furthermore, the similarity in the pH dependence of V and V/K indicates that their common parameter kₗ₉₅ dominates the profiles.

The influence of pH on substrate binding was determined independently by measuring the KD of I₂-Tyr in the absence of reductant (Fig. 2). Tightest binding was measured near a neutral pH of 7.6–7.7. Affinity diminished as the pH became alternatively more acidic and more basic. A plot of log KD versus pH suggests the importance of two ionizable groups with pKₐ values of 6.6 ± 0.2 and 8.7 ± 0.3 as estimated from non-linear least square fitting to the sum of Equations 1 and 3. Binding affinity decreases after protonation of an acidic group and deprotonation of a basic group. For catalysis, protonation of a group appears to enhance turnover despite the decrease of substrate affinity. Further studies will be necessary to establish the relationship between these pH profiles because binding was measured with enzyme in its oxidized form (FMNₙox), whereas the catalytic constants of deiodination were measured for its reduced form (FMNₙhq) by the necessity of using dithionite as the reductant.

**Structural Determination of hIYD**—The structures of hIYD and hIYD-I-Tyr were determined by x-ray diffraction and molecular replacement based on previous data for mIYD and mIYD-I-Tyr (6, 15). hIYD crystallized with one dimer per asymmetric unit, and the hIYD-I-Tyr complex crystallized with three dimers per asymmetric unit. These and additional parameters of diffraction and refinement are summarized in Table 2. Electron density for the first 38 residues (32–70) representing part of the intermediate domain was not observed for hIYD, nor was it evident in the previously reported structures of mIYD (6, 15). Attempts to truncate this entire region generated insoluble protein almost exclusively, and no further deletions were pursued. Consequently, this intermediate domain can be expected to stabilize the homodimeric structure or at least aid in its initial folding.
The two identical subunits of hIYD form an α-β fold and domain swaps at each N and C terminus consistent with the nitro-FMN reductase superfAMILY. Structures determined for hIYD and its co-cystal with I-Tyr are nearly superimposable (RMSD of 0.47 Å for a 347-atom comparison) (Fig. 3A). However, electron density for residues 161–178 and 199–211 was not observed in the absence of bound I-Tyr. This is presumably due to disorder and/or dynamics of these regions. However, these regions were evident from a co-crystal containing I-Tyr. The substrate I-Tyr was previously observed to template a closure of the active site of mIYD by concurrent association with side chains from the lid and the pyrimidine region of the isoxalozine ring (6, 15). An equivalent assembly is also evident within the co-crystal of hIYD and I-Tyr. The zwiterionic region of I-Tyr offers hydrogen bonding through its carboxylate to the N3 of FMN (2.5 Å). This same zwitterion coordinates to Glu-157, additionally stacks above the FMN with a closest approach of 4.9 Å and presents the potential for hydrogen bonding (Fig. 3B).

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FIGURE 3. Structure of hIYD. A, the structure of hIYD-I-Tyr (deep blue and deep purple to indicate the two monomers) is overlaid from a global fit to the structure of hIYD (light blue and light pink to indicate the two monomers). The active site lid composed of a loop (residues 199–211) and a helix-turn (residues 161–178) are only detected in the hIYD co-crystal. I-Tyr is illustrated with its carbon skeleton in green. FMN is indicated in yellow and gray for structures containing and lacking the substrate I-Tyr, respectively. B, binding of I-Tyr to hIYD induces a reorientation of Thr-239 to provide a hydrogen bond to the N5 of FMN. This is illustrated with the same coloring used for the full structure in A and aligned relative to FMN.

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coordination of the isoalloxazine ring suggested that substrate binding could significantly affect the oxidation-reduction properties of IYD.

Reduction of hIYD in the Absence and Presence of Halotyrosines—FMN is capable of participating in both one- and two-electron transfer processes that are typically controlled by the surrounding protein environment (13, 28–30). FMN may be reduced directly by two electrons to generate its hydroquinone form (FMNhq). Alternatively, reduction may occur via two stepwise single electron processes through an intervening semiquinone intermediate (FMNsq) (24). Each of these species has a unique absorbance signature for easy detection. The oxidized FMN bound to hIYD has an absorbance maximum at 446 nm and a shoulder near 470 nm. This absorbance decreased during reduction by xanthine and xanthine oxidase and ultimately generated a spectrum consistent with the fully reduced FMNhq (Fig. 5A). An isosbestic point at 325 nm was observed throughout the titration, indicating that no intermediates were observed during this reduction.

In contrast, the neutral FMNsq could be detected during turnover of the active halotyrosine substrates (12). This signified a dramatic change in the oxidation-reduction properties of IYD due to either catalytic turnover or perhaps just substrate binding. To focus on the effects caused by halotyrosine coordination alone, attention was directed to the effect of F-Tyr. This halotyrosine binds to the active site with reasonable affinity (Table 1) but is not subject to dehalogenation (12). Reduction of hIYD in the presence of F-Tyr generated a spectral intermediate with a $\lambda_{\text{max}}$ of 590 nm increase and then decrease during reduction. This is characteristic of a neutral FMNsq formed after single electron reduction of FMN (24). Accumulation of the neutral semiquinone illustrates the ability of the active site structure templated by F-Tyr to stabilize and promote the one-electron reduction.
chemistry of the deiodinase. Ultimately, the FMN$_{sq}$ is further reduced to FMN$_{hq}$ by the xanthine and xanthine oxidase system. Detection of the intermediate semiquinone suggests that a large separation is created between the midpoint potentials of the successive one-electron reduction steps.

Reduction Potential of FMN Bound to hIYD in the Absence and Presence of F-Tyr—Reductive titration of hIYD was repeated in the presence of suitable redox-active dyes to determine the midpoint potential of the FMN bound to IYD. AQDS was used as a reference dye because its midpoint potential is comparable with that of FMN$_{ox}$/FMN$_{hq}$ bound to hIYD in the absence of F-Tyr. The distinct spectral changes of FMN and AQDS during reduction also facilitated the measurements (Fig. 6). A plot of log(FMN$_{ox}$/FMN$_{sq}$) versus log(AQDS$_{ox}$/AQDS$_{red}$) produced a slope of 0.96. This value is close to the theoretical value of unity expected for the two-electron reduction of both AQDS and hIYD. From this analysis, a midpoint potential of $-200$ mV (pH 7.4) was determined for the FMN$_{ox}$/FMN$_{hq}$ couple. This potential is very similar to that for FMN$_{ox}$/FMN$_{hq}$ of free FMN ($-205$ mV, pH 7.0) in the absence of protein (31).

The presence of F-Tyr significantly stabilized the neutral semiquinone and promoted reduction of hIYD by two single electron transfer. To characterize the first transfer, Nile blue (NB) was found to be most appropriate to monitor the FMN$_{ox}$/FMN$_{sq}$ couple of hIYD. In this case, a slope of 1.9 was obtained from plotting log(FMN$_{ox}$/FMN$_{sq}$) versus log(NB$_{ox}$/NB$_{red}$) (24, 25) (Fig. 7). This slope is near the theoretical value of 2.0 based on the two-electron reduction of NB and the one-electron reduction of FMN$_{ox}$ to FMN$_{sq}$. The midpoint potential for FMN$_{sq}$/FMN$_{hq}$ under these conditions is $-156$ mV.

Safranin O (SFO) was selected as the dye to monitor the FMN$_{sq}$/FMN$_{hq}$ couple after screening a variety of alternatives. The plot of log(FMN$_{sq}$/FMN$_{hq}$) versus log(SFO$_{ox}$/SFO$_{red}$) generated a slope of 1.2 (Fig. 8). This differs from the expected slope of 2 based on the one-electron reduction of FMN$_{sq}$ to FMN$_{hq}$ and the two-electron reduction of SFO (26). Similar deviations were previously observed with an electron transfer flavoprotein.
and vanillyl-alcohol oxidase and ascribed to a kinetic barrier for reduction of FMN$_{sq}$ (32, 33). Still, a midpoint potential of $-310$ mV for FMN$_{sq}$/FMN$_{hq}$ could be estimated from this titration. The difference between the midpoint potentials of FMN$_{sq}$/FMN$_{hq}$ is $154$ mV, a value large enough to suggest that $\sim99\%$ of the FMN bound to IYD in the presence of F-Tyr is reduced by one electron prior to its reduction by a second electron (26, 34). These results are also consistent with the observed formation of FMN$_{sq}$ in the presence of either I-Tyr (12) or F-Tyr without a reference dye (Fig. 5).

**DISCUSSION**

Current studies on hIYD validate prior use of mIYD as a model for the human enzyme. Their sequences share $87\%$ identity, and their structures are nearly superimposable with a RMSD of $0.26$ Å (for a 399-atom comparison of Protein Data Bank code 4TTC versus code 3GFD, respectively). Both form active site lids consisting of a loop and helix that sequester their substrate-FMN complexes from solvent. This conformation is templated by coordination of substrate to multiple side chains of the lid and multiple sites on the isoalloxazine ring of the FMN. Additionally, the four known human mutations that compromise IYD activity and result in thyroid disease (8, 9) map to the hIYD structure as predicted earlier from the mIYD model (15). One of the most severe mutations is R101W, which effects a key interaction between the protein and the phosphate group of FMN.

The $k_{cat}/K_m$ of $0.40 \text{ min}^{-1} \mu\text{M}^{-1}$ for hIYD also falls within the range of $0.28$ to $3.5 \text{ min}^{-1} \mu\text{M}^{-1}$ that has been detected for IYD from a highly diverse series of organisms (7). Similarly, I-Tyr binds very tightly to hIYD as well as to mIYD with $K_D$ values below $1 \mu\text{M}$ (Table 1). Despite the potential for halogen bonding (35, 36), no evidence supports such an interaction between IYD and its substrates. Particularly for hIYD, the relative affinities of Br-Tyr $\geq$ I-Tyr $\geq$ F-Tyr are antithetical to the trend associated with halogen bonding.

The halogen substituent nonetheless appears to be critical for enzyme recognition as implied by prior investigations exploring substrate specificity (12). Neither tyrosine nor 3-methylyrosine exhibited measurable affinity for IYD despite the common presence of the zwitterion for chelation by the active site lid and FMN. The ability of the halogen to lower the $pK_a$ of the phenolic proton was used to rationalize the previous binding data (12) and was consistent with an earlier report on the high affinity of nitro- and dinitrotyrosine for IYD (37). These results together suggested preferential binding of the phenolate form of the tyrosine derivatives and a corollary of enhanced binding at alkaline pH. The pH dependence of I$_2$-Tyr binding now confirms an increase in affinity from pH 6.0 to 7.5 as measured by the decrease in the dissociation constant $K_D$ (Fig. 2). However, binding again weakens for I$_2$-Tyr above pH 7.5. The two regions of the pH profile suggest an apparent deprotonation of a group with a $pK_a$ of 6.6 and protonation of a group with a $pK_a$ of 8.7 for maximum affinity. The $pK_a$ of the phenol in I$_2$-Tyr has been reported as 6.4 and 6.5 (38, 39). These values are quite similar to the value measured from the pH dependence of binding to hIYD and consistent with a preferential association of hIYD with the phenolate form of the halotyrosines. The pH dependence of binding similarly explains the poor affinity of tyrosine because its phenol is not ionized under neutral conditions due to its corresponding $pK_a$ of 9.1 (39).

The phenolate form of the halotyrosines may act as a hydrogen bond acceptor by its close association with the amide backbone of Ala-130 (2.7 Å) and the 2'-hydroxy group of FMN (2.6 Å). Direct coordination between substrates and the ribose component of a flavin cofactor is not common but has precedence. This interaction serves a significant role in stabilizing the transition state of many acyl-CoA dehydrogenases (40, 41). In these examples, hydrogen bonding provided by the 2'-hydroxyl group polarizes the acyl carbonyl and stabilizes its developing charge. Nitroalkane oxidase is a member of the same acyl-CoA dehydrogenase superfamily and demonstrates an equivalent interaction between the 2'-hydroxyl of FAD and an oxygen of the nitro group (42). This same activation strategy may also be proposed for BluB, an enzyme closely related to IYD in structure but not function (7, 15). The structure of BluB reveals a similar interaction between its substrate molecular oxygen and the 2'-hydroxyl of its FMN (43). However, IYD remains unusual among these examples because the flavin side chain seems to associate with the initial electron-rich phenolate form of the substrate rather than a carbonyl group that gains charge during catalysis. There is little mechanistic logic for IYD to preferentially bind an electron-rich form of its substrate and then introduce an additional two electrons into this species. Still, the 2'-hydroxyl group of FMN could function in a manner complementary to that described for acyl-CoA dehydrogenase and BluB if the non-aromatic keto form of the I-Tyr was generated in the active site as an electrophilic acceptor of reducing equivalents (2). The only evidence currently supporting such an intermediate derives from the high affinity of IYD for non-aromatic pyridone analogs designed to mimic the keto form of the halotyrosines (44).

Another ionizable group that may affect binding is the $\alpha$-ammonium group of I$_2$-Tyr. The $pK_a$ of 8.7 estimated from the pH dependence of binding (Fig. 2) is higher than that reported for I$_2$-Tyr ($pK_a$ of 7.8) (39) but similar to the textbook values ranging from 9.0 to 10.8 for the $\alpha$-ammonium groups of all 20 common amino acids. Very few other ionizable groups are in the vicinity of the active site besides Lys-182 and Glu-157. Both of these are expected to be ionized for interaction with the $\alpha$-carboxylate and the $\alpha$-ammonium groups of I$_2$-Tyr, respectively (6). The only other candidate is the N3–H of FMN. The $pK_a$ of this group is $\sim10$ for FMN in solution but may vary when sequestered in the IYD active site (13). The necessity for the protonated $\alpha$-ammonium form of the tyrosine derivatives is easily rationalized from the structure of the hIYD-I-Tyr complex. This group provides electrostatic attraction and potential hydrogen bonding to the active site lid residue Glu-157 from a distance of 3.0 Å (6, 15) as well as the O$^4$ of FMN (2.5 Å; Fig. 9).

The pH dependence of both $V$ and $V/K$ for IYD turnover of I$_2$-Tyr suggests that protonation of a group with a $pK_a$ of 7.5–7.6 facilitates catalysis (Fig. 1). This is intermediate between the two $pK_a$ values apparent for substrate binding. However, these data are not necessarily comparable. Binding studies involve FMN$_{sq}$ whereas enzyme turnover involves an initial FMN$_{hq}$. Again, few candidates are immediately apparent for the ioniza-
tion important in catalysis. The origin of this pH dependence will most likely be revealed once the rate-determining step of catalysis can be determined. If the α-ammonium group of L2-Tyr remains crucial for transition state stabilization, then its interaction with the O₃ of FMN may help orient substrates with regard to the isoalloxazine system and modulate the oxidation-reduction properties of this system.

The midpoint potentials of flavin and its ability to promote one- and two-electron chemistry vary over the range of flavoproteins. Typically, flavin chemistry is controlled by the protein side chains surrounding the isoalloxazine ring that establish the active site polarity, hydrogen bonding, electrostatic environment, and π-stacking (13, 29). These features are all evident in IYD, but substrate rather than protein provides many of the necessary contacts (Figs. 3 and 9). The only exception is the interaction between the side chain of Arg-104 and the N1/O₂ region of FMN. The guanidinium group of this Arg is poised to stabilize the increase in charge at N1 after reduction of FMN to its FMNH₂ form (13). A similar interaction is common to many flavoproteins and is particularly prevalent in the nitro-FMN reductase superfamily (15, 45). The protein also helps to shield the FMN-substrate complex from solvent by creating a lid over the active site (Fig. 4). However, the aromatic ring of the substrate provides direct stacking onto the isoalloxazine ring (Fig. 9). The substrate further establishes direct electrostatic and hydrogen bonding interactions with the N3 and O₄ of the isoalloxazine system as described above.

Although all of these interactions may influence the redox chemistry of FMN, the region surrounding its N5 position is often considered the defining feature for many flavoproteins (29, 43, 46–48). Enzymes within a subclass of the nitro-FMN reductase superfamily that catalyze obligate two-electron reduction of their substrates typically maintain hydrogen bonding to the N5 position through a main chain amide proton (15, 45). In contrast, BluB relies on alternative hydrogen bonding from a side chain (Ser) to the N5 and represents a different subclass within this same superfamily (43). Such hydrogen bonding is critical for the function of BluB because mutation of this Ser to Gly reduces catalytic activity by ~30-fold (43). Side chain hydrogen bonding to N5 is also evident in electron transfer flavoproteins and is generally thought to facilitate one-electron chemistry (46). This is consistent with the ability of BluB to promote an initial one-electron transfer to its substrate, molecular oxygen. IYD contains a corresponding Thr-239, but its hydroxyl group remains 4.9 Å from the N5 of FMN in the absence of the substrate I-Tyr. Once substrate binds to IYD and templates a closure of the active site lid, the hydroxyl group of Thr-239 approaches the N5 position within hydrogen bonding distance (3.1 Å) (Fig. 3B).

The significance of hydrogen bonding by Thr-239 along with the many other substrate-induced changes around FMN is best demonstrated by their collective effect on the redox chemistry of IYD. For this analysis, the substrate analog F-Tyr was used in place of a halotyrosine substrate to focus on the effect of ligand coordination to the FMN reduction without catalytic turnover. IYD has little influence on FMN in the absence of an active site ligand. Its midpoint potential is similar to that of FMN in solution, and only FMN₂ and FMNH₂ could be observed during reduction (Figs. 5A and 6). The one-electron reduced FMN₂ was not evident and likely too unstable to accumulate under the experimental conditions. In contrast, a definitive switch to stepwise reduction of FMN was apparent after its association with F-Tyr. Under these conditions, FMN₂ became a distinct intermediate during reduction of FMNH₂ (Fig. 5B). The extent of semiquinone stabilization is illustrated by comparing the
midpoint potential of FNM_{ox} to FNM_{sq} when bound to hIYD (−156 mV) and free in solution (−314 mV) (49). In contrast, reduction of FNM_{sq} to FNM_{h} is destabilized when bound to IYD (−310 mV) versus free in solution (−124 mV) (49). These potentials of hIYD are similar to those of flavodoxin (50), ferredoxin-NADP⁺ oxidoreductase (51), and nitric-oxide synthase (52) for which FNM is involved in electron transfer reactions.

The ability of substrate to lower the reduction potential of FNM_{sq} bound to IYD suggests that substrate helps to promote its own reduction and initiation of deiodination. A related modulation of the electron donating ability of reduced flavin has been observed with certain acyl-CoA dehydrogenases. In these examples, the presence of an enoyl-CoA product affects the reduction potential of FMN to facilitate subsequent transfer of electrons to an electron carrier (53, 54). Both processes likely satisfy physiological necessities. For IYD, deiodination of I-Tyr and I₂-Tyr is required in the simultaneous presence of iodinated tyrosyl residues of thyroglobulin and the iodinated thyroid hormones. All of these would be subject to deiodination and loss of activity if the common iodophenol group were the only determinant for recognition and catalysis. Instead, the zwitterion unique to the free halotyrosines is essential for coordinating to FNM and promoting catalysis. The substrate-induced switch of FNM from two-electron to one-electron chemistry also supports the current proposal on the mechanism of IYD involving stepwise reduction of its substrate (Fig. 10) (2). A previous proposal based on a single two-electron transfer mechanism conceived in analogy to the thiol-dependent dechlorinases (20, 55) was dismissed as soon as IYD was shown to act independently of thiols and contain no Cys (nor SeCys) residues in its active site (5, 15).

Successful preparation of hIYD lacking its native membrane anchor now provides easy access to the enzyme most relevant to understanding its contribution to human health. The pH dependence of I₂-Tyr binding and turnover suggests key roles for its phenolate group and perhaps its α-ammonium group in substrate recognition and catalytic efficiency. An intimate relationship among I-Tyr, FNM, and the active site lid of hIYD is illustrated by crystallographic analysis and consistent with previous observations with mIYD. However, the extent to which ligand association can modulate the redox properties of the enzyme was not appreciated before the current characterization of hIYD. This flavoprotein provides a rare example in which substrate establishes direct and multiple interactions with the isoalloxazine system. Such coordination likely guides substrate selection in vivo and provides an opportune system for future studies in vitro to measure the contributions of individual functional groups in stabilizing the active site lid and controlling the redox chemistry of FNM.

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