Iodotyrosine Deiodinase Is the First Mammalian Member of the NADH Oxidase/Flavin Reductase Superfamily*

Received for publication, September 21, 2005, and in revised form, November 7, 2005 Published, JBC Papers in Press, November 29, 2005, DOI 10.1074/jbc.M510365200

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The enzyme responsible for iodide salvage in the thyroid, iodotyrosine deiodinase, was solubilized from porcine thyroid microsomes by limited proteolysis with trypsin. The resulting protein retained deiodinase activity and was purified using anion exchange, dye, and hydrophobic chromatography successively. Peptide sequencing of the final isolate identified the gene responsible for the deiodinase. The amino acid sequence of the porcine enzyme is highly homologous to corresponding genes in a variety of mammals including humans, and the mouse gene was expressed in human embryonic kidney 293 cells to confirm its identity. The amino acid sequence of the deiodinase suggests the presence of three domains. The N-terminal domain provides a membrane anchor. The intermediate domain contains the highest sequence variability and lacks homology to structural motifs available in the common databases. The C-terminal domain is highly conserved and resembles bacterial enzymes of the NADH oxidase/flavin reductase superfamly. A three-dimensional model of the deiodinase based on the coordinates of the minor nitroreductase of Escherichia coli indicates that a Cys common to all of the mammal sequences is located adjacent to bound FMN. However, the deiodinase is not structurally related to other known flavoproteins containing redox-active cysteines or the iodothyronine deiodinases containing an active site selenocysteine.

Iodotyrosine deiodinase (IYD)4 facilitates iodide salvage in thyroid tissue by catalyzing deiodination of mono- and diiodotyrosine (DIT), the halogenated byproducts of thyroid hormone production (Scheme 1) (1, 2). IYD has remained poorly characterized for more than 50 years despite its significant role in intrathyroidal iodine metabolism (3, 4). Patients with deficient IYD suffer from goiter, enlarged thyroid, and other symptoms associated with hypothyroidism because iodotyrosine cannot be directly reutilized for thyroid hormone synthesis (5).

The dehalogenation reaction catalyzed by IYD is unusual for aerobic organisms since the carbon-iodine bond is broken through a reductive process. More commonly, dehalogenation is accomplished by hydrolytic or oxidative pathways (Scheme 2). Considerable effort has been directed at these latter reactions in particular due to their potential utility in bioremediation of organohalides that persist in the environment seem to have emerged from existing enzyme superfamilies soon after widespread dispersal of halogenated compounds (9–12).

The best characterized examples of reductive dehalogenation in aerobic organisms are tetrachlorohydroquinone dehalogenase from Spingomonas chlorophenolica (13, 14) and iodothyronine deiodinase (ID) from a variety of higher organisms including human (15). Both enzymes catalyze reduction via similar chemical strategies. Tetrachlorohydroquinone dehalogenase appears to promote tautomerization of tetrachlorohydroquinone and transient oxidation of an active-site cysteine to yield the reduced product, trichlorohydroquinone (13, 14). Similarly, ID appears to act through an analogous tautomerization of thyroxin (3-[4-(4-hydroxy-3,5-diodophenox)-3,5-diodophenylalanine) and transient oxidation of an active-site selenocysteine to yield the reduced product, triiodothyronine (16–18). Both of these enzymes use the reducing power of exogenous thiols, and both contain a nucleophilic and redox-active residue in their active sites. Despite these similarities, the enzymes are unrelated structurally. The bacterial dehalogenase belongs to the glutathione S-transferase superfamily (19), and the mammalian ID belongs to the thioredoxin superfamily (20).

Our laboratory has proposed a mechanism for IYD that is related to the catalytic strategy used by tetrachlorohydroquinone dehalogenase and ID (21). Tautomeration of iodotyrosine to its keto form is followed by transfer of an iodonium equivalent to an active-site Cys generating the reduced tyrosine (Scheme 3). Delivery of reducing equivalents to the proposed cysteinyl iodide via FMN results in regeneration of the reduced Cys residue. Currently, the ability of IYD to bind tightly to stable analogs of the keto intermediate provides the primary support for this mechanism (21). Further details on the mechanism await ready access to both the protein and its gene. We now report purification of IYD after its proteolytic release from porcine microsomes and expression of a homologous protein from mouse in human embryonic kidney (HEK) 293 cells. Database analysis identifies IYD as the first mammalian representative of the NADH oxidase/flavin reductase superfamily, and quite distinct from mammalian iodothyronine dehalogenases (20, 22, 23).

**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine thyroids were obtained from Roth Products and Hatfield Quality Meats. Igepal, Triton X-100, octylglucoside, octylthioglycolic acid, deoxycholate, trypsin, trypsin inhibitor, lactate dehydrogenase (rabbit muscle), pyruvate, oxamate, phenyl-Sepharose, Dase I, and Cibacron blue 3G A were obtained from Sigma. Hydroxyapatite was obtained from Bio-Rad. CHAPS, GelCode glycoprotein staining kit, and BCA assay protein reagents were purchased from Pierce-Endogen. Q fast flow anion exchange resin was obtained from Amersham Biosciences. Oligodeoxynucleotide primers were synthesized by Integrated DNA Technologies, Inc. All restriction enzymes, Vent polymerase, and DNA T4 ligase were obtained from New England Biolabs unless specified otherwise. All other reagents were molecular biology grade or the highest grade available and were used without

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4 The abbreviations used are: IYD, iodotyrosine deiodinase; CHAPS, [3-[3-cholamidopropyldimethylammonio]-1-propanesulfonate; DIT, diiodotyrosine; HEK, human embryonic kidney; ID, iodothyronine deiodinase; LDH, lactate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
Further purification. [125I]DIT was prepared from DIT as described in the literature (24).

**General Methods**—Protein concentration was measured with a BCA assay according to the manufacturer’s (Pierce) instructions (25). Discontinuous Laemmli gels (26) were used to monitor protein purification employing a Bio-Rad Mini Protein 3 gel electrophoresis system (8 cm (length) × 0.75 mm (width) × 8.5 cm (height)). The resulting distribution of proteins was detected alternatively by Coomassie Brilliant Blue or silver (Silver Stain Plus kit, Bio-Rad).

**Preparation of Microsomes from Porcine Thyroids**—Frozen thyroids (300 g) stored at −20 °C were thawed at 4 °C for 1 h and then minced, washed, and homogenized as described previously for bovine thyroids (24). The homogenate was transferred to 2 × 500-ml polypropylene centrifuge bottles, and cell debris was removed by centrifugation for 30 min at 1800 × g in a Beckman 10.500 rotor in a Beckman Avanti J-25 at 20 °C. The supernatant was decanted, and the precipitate containing solubilized IYD was stored at 4 °C.

**Solubilization of IYD**—Microsomes (1 g) were suspended in the appropriate detergent in 10 mM potassium phosphate (pH 7.4, 5 ml) gently agitated overnight (4 °C) on a shaker and then centrifuged for 1 h at 100,000 × g. Pellets were resuspended and combined in 60 ml of soaking solution (24), divided into two 60-ml centrifuge tubes, and centrifuged for 1 h at 100,000 × g. The supernatant was decanted, and the mitochondrial/microsomal pellets (10 g) were stored at −20 °C.

**Purification of the Soluble Fragment of IYD Generated by Trypsin Proteolysis**—Microsomes from three pounds of thyroids were prepared and digested as described above. The resulting soluble protein was loaded by gravity on a Q fast flow anion exchange column (50 ml, 1.5 × 30 cm, 0.5 ml/min) previously equilibrated with 10 column volumes of 150 mM NaCl in 10 mM potassium phosphate (pH 7.4). The column was washed with 5 column volumes of this initial buffer (1 ml/min) and eluted with a 4-column-volume linear gradient of 150 mM NaCl to 500 mM NaCl in 10 mM potassium phosphate (pH 7.4) at a flow rate of 0.5 ml/min as controlled by a peristaltic pump. Fractions containing IYD activity were pooled and loaded directly onto a 14-ml Cibacron blue 3GA column (1.5 × 8 cm) previously equilibrated with 10 column volumes of 600 mM NaCl in 10 mM potassium phosphate (pH 7.4). After washing the column with 5 column volumes of this buffer, IYD was eluted with 5 column volumes of 1.5 mM NaCl in 10 mM potassium phosphate (pH 7.4) at a flow rate of 1 ml/min (gravity). The NaCl concentration of the eluate containing IYD was increased from 1.5 to 3 M by the addition of solid NaCl and then loaded on a phenyl-Sepharose column (0.8 × 4 cm, 2 ml) previously equilibrated with 10 column volumes of 3 M NaCl in 10 mM potassium phosphate (pH 7.4). The column was successively washed with 5 column volumes of each 2 M NaCl and 1 M NaCl in 10 mM potassium phosphate (pH 7.4). Finally, IYD was eluted at low ionic strength with 10 mM potassium phosphate (pH 7.4).

**Assay of IYD Activity**—Release of [125I]I − (nmol/h) from [125I]DIT was used to assay catalytic activity according to the literature (24). Briefly, protein fractions (100 μl) were added to 300 μl of a solution containing (1.66 mM methimazole, 0.1 mM FAD, 666 mM KCl, 333 mM potassium phosphate (pH 7.4), and 1.66 mM 2-mercaptoethanol), 100 μl of 100 μM DIT, 300 μl of deionized water, and 100 μl of [125I]DIT (50 μCi/μmol). The reaction was initiated by the addition of 100 μl of 10% sodium dithionite (w/v) in 5% sodium bicarbonate (w/v). Sample incubation was maintained at 30 min at 25 °C and then quenched by the addition of 100 μl of 0.1% unlabeled DIT (w/v) in 0.1 N NaOH. The final volume of each assay was 1.1 ml, and 250 μl of this was used to determine the total amount of [125I]I − in each assay. The remaining 850 μl was loaded onto Bio-Rad cation exchange AG 50W-X8 resin (4 ml) eluted in two fractions (4.15 and 5.00 ml) with 10% acetic acid and used to determine the amount of [125I]I − released from DIT. The rate of catalysis was then calculated by the fraction of iodide released above background levels, the mol of DIT, 300 μl of 10% sodium dithionite (w/v) in 5% sodium bicarbonate (w/v). Samples were incubated for 30 min at 25 °C and then quenched by the addition of 100 μl of 0.1% unlabeled DIT (w/v) in 0.1 N NaOH. The final volume of each assay was 1.1 ml, and 250 μl of this was used to determine the total amount of [125I]I − in each assay. The remaining 850 μl was loaded onto Bio-Rad cation exchange AG 50W-X8 resin (4 ml) eluted in two fractions (4.15 and 5.00 ml) with 10% acetic acid and used to determine the amount of [125I]I − released from DIT. The rate of catalysis was then calculated by the fraction of iodide released above background levels, the mol of DIT present initially (multiplied by two to account for the two sites for deiodination), and the duration of the assay (24). An additional factor of 1.3 included in the original calculations (24) was not included in our analysis since it is redundant when determining the fractional iodide released. One unit of enzyme has been defined historically as the amount necessary to catalyze release of 10 nmol of iodide per h (24).

**Native Gel Electrophoresis and Subsequent Analysis for Catalytic Activity and Protein Composition**—Native gel electrophoresis was performed using a Mini Protein 3 electrophoresis system and precast Ready Gels containing an acrylamide gradient of 8–16% (Bio-Rad). Protein samples were diluted with an equal volume of buffer (0.12 M Tris-HCl (pH 6.8), 20% glycerol, 0.05% bromphenol blue) before loading, and gels were run at 150 V (4 °C) using pre-chilled electrode buffer containing 0.025 M Tris (pH 8.3) and 0.19 M glycine for 70 min. A vertical strip...
of gel (2.5 × 7.5 cm) was excised and stained by Coomassie Brilliant Blue. This strip in turn guided fractionation of a second strip (2.5 × 7.5 cm) into 6 pieces (1 × 2.5, 1.5 × 2.5, 0.5 × 2.5, 1 × 2.5, 2 × 2.5, 1.5 × 2.5 cm). Each piece was then added to a standard activity assay for detecting the presence of IYD. The remaining 2 × 7.5-cm strip was soaked in 60 mM Tris-HCl (pH 6.8), 0.35 mM 2-mercaptoethanol, 2% SDS, 10% glycerol, and 0.025% bromphenol blue for 15 min. A 12% denaturing gel was cast with dimensions of 8 cm (length) × 1.5 mm (width) × 8.5 cm (height). A 5% stacking gel was then cast using a comb containing a single well (2 × 7.5 cm). The gel piece from the native gel was placed directly in this well, and electrophoresis was performed (150 V) for 1 h. Proteins were then visualized by silver staining.

**Lactate Dehydrogenase Assay**—The presence of lactate dehydrogenase (LDH) was detected by its catalytic activity as described previously (27, 28).

**Protein Sequencing**—Fragments of the denaturing polyacrylamide gels containing protein were sequenced by the Molecular Structure Facility at the University of California, Davis, CA. Samples were digested in situ by trypsin. The resulting peptides were then separated by high performance liquid chromatography and sequenced by either tandem mass spectrometry (MALDI-TOF) or Edman degradation.

**Subcloning of Mus musculus IYD**—The IYD gene (I.M.A.G.E. clone 5064638 from ATCC) was amplified by PCR using oligodeoxynucleotide primers 5’-AAGCTTAAAGCTTGAATTCGAACCACTGTTC-TCCTACACCACT-3’ and 5’-GGCCCGGGCGCGGCTATACTGTTACCATGAT-3’ to generate blunt-ended DNA with BamHI and NotI restriction sites at its termini. The PCR product and (+)−pDNA3.1/Zeo (Invitrogen) were digested with BamHI and NotI, and the vector was dephosphorylated with Antarctic alkaline phosphatase (New England Biolabs). The insert and vector were then ligated using T4 DNA ligase and transformed into One Shot TOP10 cells (Invitrogen) following the manufacturer’s instructions. Plasmid DNA was isolated from ampicillin-resistant colonies using a QIAprep spin mini-prep kit (Qiagen) and characterized by digestion with EcoRI, PstI, or StyI. Plasmids exhibiting the expected digestion pattern after gel electrophoresis (1% agarose, 125 V, 30 min) were sent for DNA sequencing (Gene Gateway).

**Expression of M. musculus IYD in HEK293**—Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Atlanta Biologicals) and 1% penicillin-streptomycin-glutamine (Invitrogen). The plasmid containing IYD (12 µg) was mixed with 30 µl of Lipopectamine 2000 in Opti-MEM (Invitrogen) and then added to cells (~90% confluent) in 10-cm dishes. After 6 h, the media were exchanged to Dulbecco’s modified Eagle’s medium. After 48 h of incubation, the cells were washed with Dulbecco’s phosphate-buffered saline (Invitrogen) and harvested into the same solution. After centrifugation (300 g) for 5 min at 4 °C, the cell pellet was resuspended in 1 ml of 50 mM sodium phosphate (pH 7.2) supplemented with 0.25 M sucrose and 0.1 mM dithiothreitol. The cells were lysed by 3 cycles of freezing (liquid N2) and thawing (37 °C) followed by 3 passages through a 20-gauge needle. The lysate was used in IYD assays without further purification. Catalytic constants for IYD were determined by plotting the initial rate of iodide release versus DIT concentration, and the resulting data were fit to the Michaelis-Menten equation with Origin 7.0 (Microcal).

**Homology Model of Iodotyrosine Deiodinase Structure**—The three-dimensional model of mouse IYD was calculated using the crystal structure coordinate set of the minor oxygen-insensitive nitroreductase of *Escherichia coli* (PDB code 1ICR). All steps of homology model building were performed by the program MODWEB, available at alto.compmbi.ucsf.edu/SAVS (31–35).

### RESULTS

**Extraction of IYD from Porcine Microsomes Using Detergents**—Previous isolation of native IYD from calf thyroid microsomes began with solubilization by cholate, ammonium sulfate precipitation, and ion exchange chromatography (36). Equivalent solubilization was not successful in our laboratory when applied to porcine thyroid microsomes. IYD lost activity when treated with cholate or deoxycholate. Consequently, a series of alternative detergents representative of zwitterionic bile salts, polyoxyethylene derivatives, and alkyl glucosides was tested for an ability to (i) solubilize IYD preferentially, (ii) maintain its catalytic activity, and (iii) support chromatographic purification. CHAPS (0.1%) was capable of extracting 30% of IYD from the microsomes, as detected by deiodinase activity. This activity persisted for at least 1 week of storage at 4 °C, but use of higher concentrations of CHAPS (0.5–3.0%) led to enzyme inactivation.

Equivalent evaluation of Triton X-100 was not pursued despite its ability to stabilize and solubilize IYD (21). Preliminary attempts to separate the Triton-solubilized enzyme with various ion exchange resins indicated that IYD was associated with heterogeneous aggregates of protein. Tween 20 and Igepal also efficiently solubilized IYD, and Igepal maintained activity without loss for more than 1 week (4 °C). Fractionation of Igepal-solubilized

### TABLE 1

| Sample                  | Total activity | Total protein | Specific activity | Fold purification |
|-------------------------|----------------|---------------|-------------------|-------------------|
| Microsomal suspension   | 12,600         | 2,030         | 6.2               | 1                 |
| Trypsin supernatant     | 10,400         | 960           | 11                | 1.8               |
| QFF anion exchange      | 8,460          | 95            | 92                | 15                |
| Cibacron blue           | 7,460          | 4.2           | 1,800             | 290               |
| Phenyl-Sepharose        | 1,920          | 0.62          | 3,100             | 500               |

* *Typical values obtained from 3 lbs of porcine thyroids.*
IYD using Q fast flow anion exchange, hydroxyapatite, phenyl-Sepharose, and various dye columns provided modest increases in specific activity, but protein aggregation still limited isolation of homogeneous enzyme. Finally, alkyl glucosides such as octyl glucoside and its thiol analog seemed promising due to their ability to extract IYD in high yields (90%) from microsomes while retaining specific activities close to the other detergents. Enzyme stability under these conditions could not be maintained after even a single anion exchange column.

**Purification of a Trypsin-solubilized Fragment of IYD**—A previous approach to purifying IYD was based on initial solubilization with steapsin, a crude pancreatic preparation of lipases that may have also contained proteases (36). Because the catalytic activity of IYD survived such treatment, a more formal investigation of proteases was conducted to test whether a soluble and active domain of IYD could be released from microsomes. Limited exposure to trypsin (0.1 mg of trypsin/mg of microsomal protein) successfully solubilized more than 70% of the IYD activity and only 16% of the total protein. The soluble fraction was then subjected to various chromatographic procedures.

Large scale digestion of microsomes containing IYD was not as efficient as the small scale digestion, but this treatment still provided sufficient material for enzyme purification. Proteolyzed samples were first fractionated by Q fast flow anion exchange (Table 1). Further purification was achieved using Cibacron blue and phenyl-Sepharose columns. Cibacron blue was chosen for its ability to bind enzymes that utilize cofactors such as NADH (37). However, IYD likely bound to this column in an adventitious manner since neither NADH (1 mM) nor ATP (10 mM) could elute IYD from this dye column. Similarly, binding was not based on interactions with the active site because a competitive and tight binding inhibitor of IYD, 3,5-dinitrotyrosine (1 mM) (38), also did not elute the enzyme. Although the phenyl-Sepharose provided only a small increase in purity of IYD, this procedure was successful in diminishing the sample heterogeneity as shown by the decrease in low level
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smear of proteins distributed throughout a polyacrylamide gel (Fig. 1). The major protein isolated from these sequential procedures had an estimated molecular mass of ∼30 kDa and was 70% pure by scanning densitometry. This protein did not stain using a GelCode glycoprotein staining kit and, thus, is not likely glycosylated in vivo.

**Protein Sequencing and Electrophoretic Characterization**—The major protein was excised from a denaturing polyacrylamide gel equivalent to that illustrated in Fig. 1 and submitted for limited proteolysis and sequencing. The resulting peptides did not coincide with any known pig proteins as determined by their molecular ions (MALDI-TOF mass spectrometry). Peptide fragments were also separated by reverse-phase chromatography, and four were selected for Edman degradation, yielding sequences of ARPVVDLEDKDSTDV, RSQEFYELLNK, LLMLPVGYPSK, and VPMEVIDNVIK. All of these sequences corresponded to a single protein that had previously been annotated as a flavoprotein from genomic sequencing of various mammals (Fig. 2A).

Identification of a flavoprotein gene was gratifying since IYD had previously been recognized as an FMN-dependent enzyme (39). However, the presence of additional proteins in the preparation of IYD (Fig. 1) made correlation between this flavoprotein and IYD less than definitive. Confirmation of their relationship was provided by tandem native and denaturing gel electrophoresis. The fraction of protein with IYD activity that was isolated from the Cibacron blue column was further separated by electrophoresis under native conditions. Only the segment of gel containing the fastest migrating proteins supported catalytic deiodination of DIT as summarized in Fig. 3. The activity measured within this gel fraction represented no less than 27% of the maximum possible activity from the protein applied to the gel. No other fraction contained a detectable level of IYD activity.

Analysis of the native gel electrophoresis by subsequent electrophoresis in a second dimension under denaturing conditions provided correlation between the putative IYD (∼30 kDa) and the major protein isolate (Fig. 3, band b). The assignment was also confirmed by identifying a sequence of IYD (EATVPD(I/L)PR) within this protein using tandem mass spectrometry. The second (band a, 33.4 kDa) and third (band c, 15.6 kDa) proteins were also submitted for peptide sequencing (Fig. 3). Edman degradation of a tryptic peptide derived from protein c yielded a sequence (KPLDQIMVT) common to IYD. Thus, protein c was also a fragment of IYD that formed during proteolytic solubilization of the parent enzyme. Mass spectrometry identified four sequences (MVVE-SAYEVIK, FIIIPQIVK, IVVVTAGV9, NSADTLWGIQK) from protein a. These all correspond to porcine LDH H4 (accession number 229620).

**Selective Inhibition of LDH Versus IYD**—The presence of LDH was confirmed in the preparation of IYD using a standard assay of NADH oxidation and pyruvate reduction (27, 28). LDH was not expected to contribute to the deiodinase activity since eukaryotic LDH is not a flavoprotein (40). Regardless, its inability to promote deiodination was equivalent to that illustrated in Fig. 1 and submitted for limited proteolysis and sequencing. Another section was divided into the regions indicated and tested for IYD activity as shown. Another section was divided into the regions indicated and tested for IYD activity (41). LDH activity present in the trypsin-solubilized fraction of thyroid proteins was also sensitive to this inhibitor. LDH activity decreases by almost 70 and 90% in the presence of 100 and 300 μM oxamate, respectively. In contrast, IYD activity decreased by less than 8% under each condition. Consequently, the putative IYD is most likely responsible for catalyzing deiodination of DIT.

**Expression and Activity of Mouse IYD**—When peptide sequences of IYD from pig were first determined, the cDNA sequence of the mouse gene was most complete. Consequently, the mouse cDNA was cloned and expressed in HEK 293 cells to verify that the gene for IYD had been properly assigned. Transfected cells (280 mg) expressed a protein with the appropriate molecular mass (32.8 kDa). The lysate of these cells exhibited deiodinase activity in contrast to a control lysate derived from

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**FIGURE 3. Two-dimensional electrophoretic analysis of porcine IYD.** An aliquot of protein exhibiting deiodinase activity after elution from a phenyl-Sepharose column (20 mg, 180 units of IYD activity) was first separated on a native polyacrylamide gel (gradient of 8–16%). A representative section of this gel was stained with Coomassie Brilliant Blue as shown. Another section was divided into the regions indicated and tested for IYD activity in situ. The remaining section was analyzed by denaturing polyacrylamide gel electrophoresis (12% resolving), and protein was visualized with silver stain. Amino acid sequencing was subsequently performed on the three protein fractions indicated by a, b, and c.

**FIGURE 4. Phylogenetic relationship of mammalian IYD and bacterial members of the NADH oxidase/flavin reductase superfamily.** A phylogenetic tree was constructed with ClustalW 1.83 (51) using IYD sequences obtained from GenBank™ that were homologous to domain 3. Bacterial sequences were additionally selected based on available crystal structures. The brackets indicate the common protein abbreviation/PDB identifier, and horizontal branch lengths are drawn to scale, with the scale bar indicating the number of amino acid substitutions per site. NIX, NADH oxidase; Frasel, NAD(P)H-utilizing flavin reductase; NR, nitroreductase; FRP, (NADPH-utilizing) flavin reductase P.
Conserved cysteine residues (217 and 239) are highlighted in yellow, and alignments were rendered using ESPript 2.2, available at espript.ibcp.fr/ESPript/ESPript/index.php (69).

The majority of protein isolated from a 500-fold purification of IYD cells that lacked the IYD gene. A $K_m$ value of $4.4 \pm 1.7 \mu M$ was measured for DIT using dithionite as the reductant. This value is consistent with those ranging from 2.0 to 9.3 $\mu M$ previously reported for IYD isolated from calf, sheep, pig, rat, and human (21–23, 38, 39). $V_{\text{max}}$ (12 ± 1 nmol/h/μg of IYD) and $K_{\text{cat}}$ (6.4 ± 0.7 min$^{-1}$) were also estimated based on the molecular mass of IYD, the total concentration of protein, and the fractional concentration of IYD as determined by gel electrophoresis and densitometry. Additionally, the expressed protein promoted deiodination of DIT in the presence of the native electron donor, NADPH (42), although the $K_m$ for DIT (2.0 ± 1.9 $\mu M$) was slightly lower than the $K_m$ determined in the presence of dithionite. The $V_{\text{max}}$ value (1.1 ± 0.4 nmol/h/μg of IYD) was substantially lower than the $V_{\text{max}}$ measured in the presence of dithionite and likely reflected a limited concentration of a reductase in HEK293 cells that is thought to be necessary for shuttling reducing equivalents between NADPH and IYD (43). The complete absence of this reductase has recently been used to explain the lack of NADPH-dependent deiodination by human IYD expressed in Chinese hamster ovary cells (22).

DISCUSSION

Enzyme Solubilization, Isolation, and Identification—Purification and detailed mechanistic study of both IYD and ID have been hindered by their association with cell membranes (36, 44). For ID, excision of its predicted transmembrane anchor still did not yield soluble protein (44). For IYD, limited proteolysis by trypsin did generate a soluble and active protein, presumably by hydrolysis of a single terminal peptide anchor. This procedure subsequently allowed purification of IYD sufficiently for identifying its gene. Alternative use of detergents to solubilize native IYD was frustrated by heterogeneous protein aggregation.

The majority of protein isolated from a 500-fold purification of IYD had a molecular mass of 30 kDa, as indicated by denaturing gel electrophoresis. Peptide sequencing matched the protein to a gene that had originally been annotated as an oxidoreductase in the human genome based on its homology to the NADH oxidase/flavin reductase superfamily of flavoproteins (45). This same gene was later suggested to be IYD based on a serial analysis of genes preferentially expressed in the thyroid (46, 47). Subsequent expression of the human gene in HEK293 cells confirmed this assignment (22). The gene identity is also now supported by our isolation and characterization of the porcine protein.

Homology of IYD to Members of the NADH Oxidase/Flavin Reductase Superfamily—The amino acid sequence of IYD is highly conserved throughout mammals ranging from mouse to pig to human (Fig. 2A). Amino acid identity within this group is greater than 80%, and similarity is greater than 90%. The known IYDs contain three cysteines, and at least one is expected to play a central role in catalysis (21). The protein sequence can be divided into three domains (Fig. 2B). The largest domain (3) extends from residue 82 to the C terminus (residue 285) and exhibits homology to NADH oxidase/flavin reductase superfamily of proteins that is common in bacteria (45). The intermediate domain (2) is not related to known folding patterns or sequences but is predicted to adopt an α-helical structure between residue 49 and 67 by 3D-PSSM (Web Server version 2.6.0, www.sbg.bio.ic.ac.uk/~3dpsmm/index2.html) (48).

The N-terminal domain (1) is very lipophilic and predicted to act as the transmembrane anchor by TMHMM (49). Consequently, C13 within this domain is not expected to participate in catalysis. Although SignalP (50) identified Ala-23—Asp-24 as a possible site for signal peptide cleavage, the probability for this hydrolysis is low (22%). No alternative regions could likely substitute as a membrane anchor if this domain was removed. Residues 213–229 of human IYD had previously been proposed as a membrane anchor based on an expectation that the N-terminal signal sequence would have been hydrolyzed (22). However, peptides from both sides of the central 213–229 region were identified while sequencing the trypsin-solubilized form of the porcine enzyme using dithionite as the reductant. This value is consistent with the known IYDs contain three cysteines, and at least one is expected to play a central role in catalysis (21). The protein sequence can be divided into three domains (Fig. 2B). The largest domain (3) extends from residue 82 to the C terminus (residue 285) and exhibits homology to NADH oxidase/flavin reductase superfamily of proteins that is common in bacteria (45). The intermediate domain (2) is not related to known folding patterns or sequences but is predicted to adopt an α-helical structure between residue 49 and 67 by 3D-PSSM (Web Server version 2.6.0, www.sbg.bio.ic.ac.uk/~3dpsmm/index2.html) (48).

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(52). However, the greatest sequence identity (24%) is shared between IYD and NADH oxidase from Thermus thermophilus, a protein from a different subset of proteins within the superfamily.

The bacterial proteins are not expected to exhibit dehalogenase activity. These proteins lack the conserved cysteines at positions 217 and 239 of the mammalian proteins. Instead, bacterial homologues within this superfamily are associated with a variety of different activities including reduction of nitroaromatics (NfsA and NfnB-NfsB, Fig. 4) (53–55) and generation of reduced flavin for use by luciferase and other enzymes (NAD(P)H-utilizing flavin reductase) (56–58). The physiological role of other homologues such as NADH oxidase remains to be determined (59). A Rossman fold is absent in this superfamily, yet the bacterial proteins still utilize NADH and/or NADPH as a source of hydride for reduction of nitroaromatics (NfsA and NfnB-NfsB, Fig. 4) (53–55) and generation of reduced flavin for use by luciferase and other enzymes (NAD(P)H-utilizing flavin reductase) (56–58). The physiological role of other homologues such as NADH oxidase remains to be determined (59). A Rossman fold is absent in this superfamily, yet the bacterial proteins still utilize NADH and/or NADPH as a source of hydride for reduction of FMN or FAD. Numerous electron acceptors may in turn oxidize the reduced flavin (54, 60).

Phylogenetic analysis of IYD provided the expected relationship between the various mammals (Fig. 4) (51). IYD also exhibited a surprising level of similarity to bacterial proteins within the NADH oxidase/flavin reductase superfamily. Representatives of this superfamily are widely distributed in bacteria, but IYD is the only example as yet discovered in mammals. Analysis by ClustalW suggested greatest similarity between IYD and the subset of proteins in the superfamily exemplified by the well studied flavin reductase P from Vibrio harveyi (52). However, the greatest sequence identity (24%) is shared between IYD and NADH oxidase from Thermus thermophilus, a protein from a different subset of proteins within the superfamily.

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FIGURE 6. A three-dimensional model of the NADH oxidase/flavin reductase domain of mouse IYD. Residues 88–285 of IYD were modeled by MODWEB after initial threading through the coordinates of NfnB-NfsB, PDB code 1ICR) and its bound FMN. This flavin and the two conserved cysteine residues (Cys-217 and Cys-239) found in all mammalian IYD sequences are shown as ball and stick representations. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by National Institutes of Health Grant P41 RR-01081).

completed. However, the current model of IYD indicates that Cys-217 and Cys-239 are the only nucleophiles within the proposed active site near FMN. As part of a highly acidic region, Glu-153 may possibly serve as a general acid/base to promote the aromatic tautomerization suggested in Scheme 3. This same residue is too far from the cysteines to facilitate deprotonation, and no alternative residues are easily ascribed to this function.

Convergent Strategy for Reductive Dehalogenation—Nature has developed at least three distinct but chemically related mechanisms for reductive cleavage of aromatic carbon-halogen bonds. Tetrachlorohydroquinone dehalogenase and IYD likely utilize the nucleophilicity and reducing power of a cysteine residue for dehalogenation (13, 14, 21), and ID appears to use an analogous selenocysteine (15). Despite the general similarity of the strategies of the enzymes for catalysis, their structural origins are quite diverse. IYD derives from neither the glutathione S-transferase superfamily that includes tetrachlorohydroquinone dehalogenase (19) nor the thioredoxin superfamily that includes ID (20). Instead, IYD derives from the NADH oxidase/flavin reductase superfamily and is the only mammalian representative discovered to date.

Exogenous thiol is sufficient to regenerate the reduced form of tetrachlorohydroquinone dehalogenase and ID as expected from their transient formation of an oxidized Cys and selenocysteine intermediate. An equivalent intermediate is also suggested for IYD (Scheme 3), but this enzyme is not sensitive to exogenous thiols. IYD activity is not detected
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in the presence of thiols as the sole reductant (62). Similarly, dithionite-dependent activity of IYD is not affected by the thiol included in standard assays. Further investigations will be necessary to identify the origin of this surprising lack of reaction with thiols.

The individual importance of a Cys residue and FMN had previously been shown for IYD activity (39, 43), and now these results can be connected by the Cys-flavin interactions predicted from the structural model of IYD. If these interactions are confirmed, then IYD will define a new class of proteins containing flavin and a redox-active Cys. Most examples described previously contain two redox-active Cys in their active site and belong to a distinct pyridine nucleotide-disulfide oxidoreductase superfamily (63, 64). Flavoproteins such as NADH peroxidase containing a single redox active Cys are also related to this superfamily (65). A second class of enzymes was identified more recently and includes sulfhydryl oxidase from chicken egg white (66), Erv2p from the Schizosaccharomyces pombe, and several enzymes linked to a different progenitor lacking an active site Cys, this flavoprotein provides an excellent system for studying an evolution of function.

Acknowledgments—We thank the Molecular Structure Facility at the University of California, Davis for protein sequencing and M. Winn and Dr. J. Hansza for help with IYD expression in HEK293.

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14. K regulates IYD activity and is expressed in the mammalian thyroid. If these interactions are confirmed, then IYD will define a new class of enzymes linked to a different progenitor lacking an active site Cys, this flavoprotein provides an excellent system for studying an evolution of function.