Characterization of a Propylthiouracil-Insensitive Type I Iodothyronine Deiodinase*

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ABSTRACT

Mammalian type I iodothyronine deiodinase (D1) activates and inactivates thyroid hormone by outer ring deiodination (ORD) and inner ring deiodination (IRD), respectively, and is potently inhibited by propylthiouracil (PTU). Here we describe the cloning and characterization of a complementary DNA encoding a PTU-insensitive D1 from teleost fish (Oreochromis niloticus, tilapia). This complementary DNA codes for a protein of 248 amino acids, including a putative selenocysteine (Sec) residue, encoded by a TGA triplet, at position 126. The 3’ untranslated region contains two putative Sec insertion sequence (SECIS) elements. Recombinant enzyme expressed in COS-1 cells catalyzes both ORD of T4 and rT3 and IRD of T3 and T3 sulfate with the same substrate specificity as native tilapia D1 (tD1), i.e. rT3 ≫ T4 ≫ T3 sulfate ≫ T3. Native and recombinant tD1 show equally low sensitivities to inhibition by PTU, iodoacetate, and gold thioglucose compared with the potent inhibitions observed with mammalian D1s. Because the residue 2 positions downstream from Sec is Pro in tD1 and in all (PTU-insensitive) type II and type III iodothyronine deiodinases but Ser in all PTU-sensitive D1s, we prepared the Pro128Ser mutant of tD1. The mutant enzyme showed strongly decreased ORD and somewhat increased IRD activity, but was still insensitive to PTU. These results provide new information about the structure-activity relationship of D1 concerning two characteristic properties, i.e. catalysis of both ORD and IRD, and inhibition by PTU. (Endocrinology 138: 5153–5160, 1997)

THE MAJOR secretory product of the thyroid is a prohormone, T4, which is activated in peripheral tissues by outer ring deiodination (ORD) to T3. Both T4 and T3 are inactivated by inner ring deiodination (IRD) to rT3 and 3,3′-diiodothyronine (3,3′-T2), respectively (1–3). Three homologous iodothyronine deiodinases catalyze these reactions (1–3). Type I deiodinase (D1) is located in liver, kidney, and thyroid; has both ORD and IRD activities; prefers rT3 as substrate; and provides most of plasma T3. Type II deiodinase (D2) and type III deiodinase (D3) show distinct tissue distributions and contrasting enzyme activities; D2 catalyzes only ORD and D3 only IRD (1–3). The different deiodinases that have recently been characterized are ≈30 kDa proteins, featuring in corresponding positions a selenocysteine (Sec) residue that is important for catalysis (4–15). The catalytic cycle of D1 appears to consist of two half reactions: first, transfer of an I− from the substrate to the selenolate (Se−) anion of Sec, and second, reduction of the selenenyl iodide (SeI) generated by a thiol cofactor. In vitro, dithiothreitol (DTT) substitutes for the endogenous cofactor (1–3). 6-n-Propyl-2-thiouracil (PTU) is a thyroid peroxidase-blocking drug that is used for treatment of hyperthyroidism. PTU is also a potent inhibitor of mammalian D1 but has no effect on D2 and D3 (1–3). PTU is an uncompetitive D1 inhibitor that is thought to react with the Sec126Cys mutant of D1. The residue 2 positions downstream from Sec is Pro in tD1 and in all (PTU-insensitive) type II and type III iodothyronine deiodinases but Ser in all PTU-sensitive D1s, we prepared the Pro128Ser mutant of tD1. The mutant enzyme showed strongly decreased ORD and somewhat increased IRD activity, but was still insensitive to PTU. These results provide new information about the structure-activity relationship of D1 concerning two characteristic properties, i.e. catalysis of both ORD and IRD, and inhibition by PTU.

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and dog D1s and Xenopus laevis D3 sequences (4, 5, 7, 8). The resultant RT-PCR products were sequenced to confirm that they were related to known deiodinases and used as probes for cDNA library screening. Expression of the full-length cDNA clone obtained resulted in production of a functional deiodinase, the catalytic properties of which were examined.

Materials and Methods

Materials

Tilapia (Oreochromis niloticus) were obtained from CERER-University of Liège (Tihange, Belgium) and kept as described before (21). TRIzol reagent was obtained from Gibco BRL (Breda, The Netherlands); oligo(dT) cellulose was from New England Biolabs (Beverly, MA); Super-FIG. 1. Nucleotide and deduced amino acid sequence of cDNA clone TN12. Sec residue is denoted by X. Putative SECIS elements in 3' UTR are underlined.
DNA polymerase was from HT Biotechnology (Cambridge, UK); AMV reverse transcriptase and pCI-Neo were from Promega (Madison, WI); Klenow DNA polymerase was obtained from Boehringer Mannheim GmbH (Mannheim, Germany); pCR-II was from Invitrogen (San Diego, CA); synthetic oligonucleotides were from Pharmacia Biotech (Roosendaal, The Netherlands) or Gibco BRL; Hybrid membranes and [α-35S]deoxyATP were purchased from Amersham (Buckinghamshire, UK); polyethylene glycol (PEG6000) was from Merck (Hohenbrunn, Germany); diethylaminoethyl (DEAE)-dextran and Sephadex LH-20 were from Pharmacia. Nonradioactive iodothyronines were obtained from Sigma Chemical Co., St. Louis, MO; diethylaminoethyl (DEAE)-dextran and Sephadex LH-20 were from Pharmacia. [3,5-125I]T3 sulfate (T3S) were prepared in our laboratory as described previously (21). Total RNA was isolated from tilapia kidney using TRIzol reagent, and Poly(A+) RNA was isolated on oligo(dT) cellulose. cDNA was obtained by oligo(dT)-primed reverse transcription using AMV reverse transcriptase. PCR was performed using the primers 5'-AATTTGTGCGT-TGTACCTGACC-3' and 5'-RTGCGCTTCCCTCATTGATA-3' and Super Taq DNA polymerase. The PCR products were TA-cloned into pCR-II and sequenced. The tilapia kidney cDNA library was constructed in Lambda ZAP-Express (Stratagene, La Jolla, CA). The library was blotted on Hybond-N* and screened with the RT-PCR product labeled by primer extension using Klenow DNA polymerase in the presence of [α-35S]deoxyATP. The phagemids were sequenced by using the MOPRH kit (5prime→3prime, Boulder, CO) and the oligonucleotide 5'-ACACCCACCCCTCTTATTTATGTATAA-3'; the mutation was confirmed by sequencing. RNA secondary-structure prediction was done using the MFOLD program provided by Dr. M. Zuker (Institute for Biomedical Computing, Washington University, St. Louis, MO) on the Internet (http://www.ibc.wustl.edu/~zuker) (24).

Expression

cDNA was subcloned into pCI-Neo and expressed in COS-1 cells grown in DMEM/F12 containing 10% FCS (Gibco BRL) and 40 μM Na2SO3. After 3 days, the cells were rinsed with PBS; collected in 0.3 ml medium was replaced by DMEM/F12 containing 100 μM 125I-labeled T4 or rT3 or by IRD of inner ring 125I-labeled T3 or T3S were analyzed by quantitation of radioiodide released by ORD of outer ring 125I-labeled T3 or rT3, or by IRD of inner ring 125I-labeled T3 or T3S (22, 23). In short, appropriate amounts of enzyme protein were incubated in triplicate for 30–60 min at 37 C with 10 nm 125I-labeled substrate in 0.2 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, and 10 mM DTT; sonicated; snap-frozen on dry-ice/ethanol; and stored at −80 C.

Northern analysis

Tilapia tissues and microsomal fractions thereof were obtained as described previously (21). Northern blots were prepared using 20 μg total tissue RNA and Hybond-N membranes, which were hybridized for 16 h at 60 C with random hexamer-labeled full-length cDNA in 6× SSC, 0.5% SDS, 5× Denhardt’s, and salmon sperm DNA (100 μg/ml). Blots were washed twice for 15 min at 55 C in 0.1× SSC, 0.5% SDS, and twice for 15 min at 60 C in 1× SSC, 0.5% SDS. Autoradiographs were scanned with a Hewlett Packard Scanjet IIcx (Amstelveen, The Netherlands), and signals were quantified using software developed by Dr. R. Docter (Department of Internal Medicine III, Erasmus University Medical School, Rotterdam, The Netherlands). Results were standardized relative to the total amount of RNA applied, which was determined similarly by photography and quantitation of the fluorescence of the ethidium bromide-stained gel.

Enzyme assays

Deiodinase activities of native and recombinant enzyme preparations were analyzed by quantitation of radiiodide released by ORD of outer ring 125I-labeled T3 or rT3, or by IRD of inner ring 125I-labeled T3 or T3S (22, 23). By RT-PCR of tilapia kidney mRNA using oligonucleotide primers corresponding to the conserved amino acid sequence

Fig. 2. Predicted secondary structures of two putative SECIS elements in 3′ UTR of tD1 mRNA. Structure on right is exactly as predicted by the MFOLD program (26), whereas possible basepairing in loop structure on left has been omitted to enhance homology between secondary structures. Essential nucleotides identified in consensus SECIS elements (27, 28) are presented in bold.

920 994 1264 1338
quences NFGSCTSecP and YIEEAH, a 117-bp cDNA fragment was obtained, the sequence of which showed high homology with the corresponding region in mammalian D1s (4–7). The labeled PCR product was used as a probe to screen a tilapia kidney cDNA library (200,000 independent clones). Thirty double-positive clones were identified after plating 500,000 plaque-forming units of the amplified library. Using vector- and PCR product-specific primers, several possibly full-length clones were identified by PCR. One clone (TN12) was found to be 2401 bp long with a reading frame coding for a 248-amino acid protein, assuming that TGA at codon position 126 was translated as Sec (Fig. 1). Like the mammalian D1s, TN12 contains a large 3′ untranslated region (UTR). RNA secondary structure prediction (24) and comparison with published consensus sequences (27, 28) revealed two almost identical, putative Sec insertion sequence (SECIS) elements (Fig. 2), which are essential for Sec incorporation at the UGA stop codon.

The deduced amino acid sequence of TN12 showed 48% identity with rat D1, 48% identity with human D1, and 45% identity with dog D1 (Fig. 3), including the Sec residue (4, 5, 7). The N-terminal region of TN12 showed a large divergence from the hitherto described mammalian D1s. We have, therefore, sequenced the 5′ region of another, independent cDNA clone that proved to be identical. The TN12 protein appeared to be much more acidic (pI 7.0) than other D1s (pI 8.7–9.8), the biological significance of which is unknown. The amino acid sequence of TN12 showed only 31%, 33%, and 28% identity with human (13, 14), rat (13, 14), and Fundulus heteroclitus (teleost fish) (15) D2, respectively, and 38% identity with rat (10) and human (11) D3.

A 2.5-kb mRNA was detected on Northern blots by hybridization with labeled TN12 cDNA. The tissue distribution of this mRNA corresponded very well with that of tD1 activity, with highest mRNA and activity levels observed in kidney (Fig. 4). Shorter exposure of the Northern blot shown in Fig. 4 revealed only one prominent band of approximately 2.5 kb in kidney.

Enzyme activity expressed in COS-1 cells after transfection with TN12 cDNA was characteristic for D1 (1–3), showing 1) catalysis of both ORD and IRD, 2) a clear preference for rT3 as the substrate, and 3) increased IRD of T3S vs. T3 (Fig. 5).

Comparison of TN12-transfected COS-1 cell lysates with the native enzyme in tilapia kidney microsomes revealed the same patterns of inhibition of the ORD of [125I]rT3 by (in decreasing order of potency) rT3, T4, and T3 (Fig. 6). The apparent Michaelis-Menten constant (Km) value of rT3 for both native and recombinant enzyme amounted to 2 μM, which is 5- to 10-fold higher compared with rat and human D1 (22, 23). Native tD1 and the TN12-encoded enzyme showed identical patterns of inhibition by (in decreasing order of potency) GTG, IAc, and PTU (Fig. 7). These results indicated that both native and recombinant enzyme activities are approximately 10-fold less sensitive to PTU inhibition, and approximately 10-fold less sensitive to GTG and IAc inhibition than rat and human D1 (5, 21).

From the above findings we concluded that TN12 cDNA encodes the D1 expressed in tilapia kidney. Therefore, the relative insensitivity of tD1 to PTU, IAc, and GTG does not appear to be caused by the absence of Sec in its active center. The amino acid sequence around this Sec residue is highly conserved among the D1s cloned to date, including chicken D1 (29). However, the amino acid residue two positions downstream from Sec is Pro in tD1 but Ser in all other, PTU-sensitive D1s. Therefore, we produced the Pro128Ser mutant of tD1 by site-directed mutagenesis, and expressed the mutant protein in COS-1 cells. It was found that the mutant catalyzed the ORD of rT3 and T4 at only 2–3% of the activity of the wild-type enzyme, whereas IRD of T3S was increased by the mutation (Fig. 8). IRD of T3 by both wild-
type tD1 and the mutant was too slow to allow accurate measurements. Addition of 1 mM PTU had little effect on the ORD of rT3 as well as on the IRD of T3S by the Pro128Ser mutant (Fig. 9). T4 ORD by the mutant was too slow to exactly determine the effect of PTU. These results suggest that the PTU insensitivity of tD1 is not due to the presence of Pro instead of Ser at position 128.

Discussion

The evidence that the TN12 clone characterized in this study represents the cDNA coding for tD1 can be summarized as follows: 1) the amino acid sequence of TN12 is much more homologous with reported D1 sequences from other species (45–48% identity) than with the D2 sequences (27–31% identity) or D3 sequences (33–38% identity) from other species; 2) the tissue distribution of mRNA hybridizing with radiolabeled TN12 cDNA.

![Fig. 4. A. Tissue distribution of D1 activity and D1 mRNA levels in tilapia tissues. Error bars represent SDs of triplicate determinations (activity) or of two separate experiments (mRNA). B. Northern blot hybridized with radiolabeled TN12 cDNA.](image)

![Fig. 5. ORD of T4 and rT3 and IRD of T3 and T3S by native tD1 in tilapia kidney microsomes or by recombinant tD1 expressed in COS-1 cells. Conditions were: 10 nM substrate, 10 mM DTT, 1 (lysate) or 0.1 (microsomes) mg protein/ml, and 60 min of incubation at 37 C.](image)

![Fig. 6. Inhibition of ORD of [125I]rT3 by native tD1 in tilapia kidney microsomes (A) or recombinant tD1 expressed in COS-1 cells (B) by increasing concentrations of unlabeled rT3, T4, and T3. Conditions were: 10 nM [125I]rT3, 10 mM DTT, 1 (lysate) or 0.1 (microsomes) mg protein/ml, and 60 (lysate) or 30 (microsomes) min of incubation at 37 C.](image)
a TN12 cDNA probe and that of tD1 activity are identical, with kidney as the major site of enzyme production; 3) the enzyme activity expressed in COS-1 cells transfected with TN12 cDNA shows identical catalytic potential and substrate specificity as native tD1 present in tilapia kidney microsomes; and 4) recombinant and native enzymes show identical susceptibilities to inhibition by PTU, IAc, and GTG.

Although we have not directly shown that the enzyme expressed after transfection of COS-1 cells transfected with TN12 cDNA is a selenoprotein, indirect evidence strongly suggests that this is the case. First, mRNA transcribed from tD1 cDNA contains the opal UGA stop codon at a position identical to that in other D1 mRNAs shown to be translated as Sec. Furthermore, like the cDNAs for the other D1s cloned to date, tD1 cDNA contains a large 3′ UTR. Comparison with reported consensus sequences for SECIS elements, which are essential for Sec incorporation at the UGA codon (27, 28), and RNA secondary structure prediction (24) reveal that two almost identical, putative SECIS elements are present in the 3′ UTR of tD1. Although multiple SECIS elements have been described in selenoprotein P mRNA (27, 30), tD1 is the first deiodinase mRNA having more than one putative SECIS element. The function of multiple SECIS elements is unknown, but it may increase the efficiency of UGA codon read-through. Mutational analyses of mammalian D1s have indicated that the Sec residue is essential for enzyme activity. Replacement of Sec by Cys reduces catalytic activity approximately 100-fold, whereas replacement with Leu results in a complete loss of enzyme activity (4). Also, the protein truncated at the site of the Sec residue, which takes place in the absence of a SECIS element and, hence, if the UGA codon functions as a translation termination codon, is enzymatically inactive (4). Therefore, it is impossible that expression of TN12 cDNA yields a functional deiodinase if the UGA codon were not translated as Sec.

The most remarkable property of both native and recombinant tD1 is their insensitivity to PTU inhibition. Only weak inhibition is observed with as high as 1 mM PTU, whereas 1 mM of this drug strongly inhibits rat and human D1 (5, 21).
to inhibition by GTG and IAc than rat and human D1 (5, 21). We have demonstrated that this low sensitivity of tD1 to PTU, IAc and GTG is not caused by the absence of Sec, as previously hypothesized (21). Therefore, other structural determinants should be responsible for this anomaly, one of which could be the Pro residue two positions downstream from Sec. All PTU-sensitive deiodinases, i.e. human, rat, mouse, and dog D1 (4–7), have Ser, and all PTU-insensitive iodothyronine deiodinases, i.e. all D2s (12–15), all D3s (8–11), and tD1, have Pro at this position. If PTU inhibition is dependent on the nature of this residue, this could be explained by differences in effects of Pro and Ser on protein structure and, thus, on enzyme-inhibitor interaction, or by the requirement of the OH group of Ser for reaction of the inhibitors with Sec. Therefore, we studied the effect of the substitution of Pro128 by Ser on the catalytic properties of tD1, expecting that this mutation would increase the PTU sensitivity of the enzyme. One mM PTU was found to have as little effect on the Pro128Ser mutant as on the wild-type enzyme, suggesting that the PTU insensitivity of the latter is not solely due to the presence of Pro instead of Ser at position 128. However, in particular the low rT3 ORD activity of the Pro128Ser mutant hampers the interpretation of the lack of its inhibition by PTU (see below). It would be interesting to determine the effect of the reverse Ser128Pro mutation of other D1s on their inhibition by PTU.

The lower sensitivities of D2, D3, and tD1 to PTU, IAc, and GTG compared with mammalian D1s may be caused by the lower reactivity of the Sec residue. It is unknown whether other amino acid residues enhance Sec reactivity, much like Cys and Ser are activated by His and Asp residues in cysteine and serine proteases (31, 32). Essential His residues have indeed been identified in D1 (33), and these residues (His158 and His174) are also conserved in tD1. Because PTU supposedly reacts with an enzyme Sel group, the rate of formation of this intermediate also determines susceptibility to PTU inhibition. Our findings suggest that tD1 has a lower kcat than mammalian D1s, because the deiodinase activity expressed by transfection of COS-1 cells with tD1 is consistently approximately 10-fold lower than that produced by transfection with rat D1. This could be explained by a lower reactivity of Sec in tD1 than in mammalian D1, although the amount of tD1 protein expressed is unknown. In this regard it should be mentioned that the basic Arg and Lys residues at positions 11 and 12 of mammalian D1, which are important for membrane insertion (34), are lacking in tD1. Interestingly, a Phe residue that has been shown to be involved in rT3 binding to mammalian D1 (7, 22) is also conserved in tD1 (Phe65).

The Pro128Ser mutation in tD1 results in a selective and strong reduction of its ORD activity, whereas its IRD activity is not affected or even increased. These findings suggest that protein structural changes induced by the Pro128Ser mutation interfere in the interaction with substrates undergoing ORD, whereas they do not affect the interaction with substrates undergoing IRD. This is remarkable, because all other D1s that have Ser at this position catalyze ORD very effectively. Therefore, the catalytic specificities of the different deiodinases must be determined by additional structural elements in these proteins. Further mutational analyses should reveal the molecular basis for D1 having both ORD and IRD activity as opposed to D2 and D3, which have only ORD or only IRD activity, respectively, as well as for the different inhibitor susceptibilities of the different deiodinase isoenzymes. This may lead to the rational design of new and potent deiodinase inhibitors for research and clinical application.

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