

Characterization of a Propylthiouracil-Insensitive Type I Iodothyronine Deiodinase*

JO P. SANDERS, SERGE VAN DER GEYTEN, ELLEN KAPTEIN,
VEERLE M. DARRAS, EDUARD R. KÜHN, JACK L. LEONARD, AND
THEO J. VISSER

Department of Internal Medicine III (J.P.S., E.K., S.V.d.G., T.J.V.), Erasmus University Medical School, 3000 DR Rotterdam, The Netherlands; Laboratory of Comparative Endocrinology (S.V.d.G., V.M.D., E.R.K.), K. U. Leuven, 3000 Leuven, Belgium; and Department of Nuclear Medicine (J.L.L.), University of Massachusetts, Medical Center, Worcester, Massachusetts 01655

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 T_4 and rT_3 and IRD of T_3 and T_3 sulfate with the same substrate specificity as native tilapia D1 (tD1),

i.e. $rT_3 \gg T_4 > T_3$ sulfate $> T_3$. 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 pro-hormone, T_4 , which is activated in peripheral tissues by outer ring deiodination (ORD) to T_3 . Both T_4 and T_3 are inactivated by inner ring deiodination (IRD) to rT_3 and 3,3'-diiodothyronine (3,3'- T_2), 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 rT_3 as substrate; and provides most of plasma T_3 . 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*, dithiotreitol (DTT)

substitutes for the endogenous cofactor (1–3). 6-*n*-Propylthiouracil (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 SeI intermediate. The nucleophile-directed reagents iodoacetate (IAC) and gold thioglucose (GTG) also inhibit D1 more effectively than D2 and D3 (16–18). Both IAC and GTG are competitive D1 inhibitors that probably interact with the Se^- anion (1–3).

Before the amino acid sequences of D2 and D3 were known, the findings that PTU and GTG are less potent inhibitors of these enzymes and of the Sec126Cys mutant of D1 than of the wild-type D1 led to the proposal that Sec was absent in the catalytic centers of D2 and D3 (17, 18). This was supported by the relatively minor decreases in D2 and D3 activities compared with the strong decreases in hepatic and renal D1 activities in selenium-deficient rats (19, 20). A D1 variant has recently been identified in the kidney of the teleost fish *Oreochromis niloticus* (tilapia), showing characteristic catalytic and substrate specificities but low sensitivities to inhibition by PTU, IAC, and GTG (21). Similar to D2 and D3, it was proposed that Sec was not present in the active site of this tilapia D1 (tD1). To determine the molecular basis for the differences in catalytic mechanisms and inhibitor sensitivities between the deiodinase isoenzymes, we cloned and characterized complementary DNA (cDNA) coding for tD1. This involved RT-PCR of tilapia kidney messenger RNA (mRNA) using primers based on conserved amino acid sequences (NFGSCTSecP and YIEEAH) present in rat, human,

Received June 9, 1997.

Address all correspondence and requests for reprints to: Theo J. Visser, Department of Internal Medicine III, Erasmus University Medical School, Room Bd 234, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: visser@inw3.azr.nl.

* This work was supported by the Netherlands Organization of Scientific Research NWO (Grant 903–40-168), the Belgian National Fund for Scientific Research (FKFO 2.0114.94), and Onderzoeksraad K. U. Leuven (Project OT/94/11). The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number Y11109.

CGTCAACCTGCTAACATGTTTCTGCAAAAAATTGTGCTTTATTTGTGACAGCATGTATG	60
M F L Q K I V L Y L S T A C M	15
TTTTGCTACATGTTGGGAAAGTTTCTTATGCTGGTATATTGCAGTTTTTCTCACCAAGC	120
F C Y M L G K F L M L V I L Q F F S P S	35
CTTGCGAAGAAGTTCATACTTCGCATGGGTGAAAAGATCACCATGACCCAGAATCCAAGG	180
L A K K F I L R M G E K I T M T Q N P R	55
TTAACTATGAGGATTGGGTCTGACATTTATGCTCCTTGGCATTATTATAAAAGCTTCT	240
F N Y E D W G L T F M S L A F I K T A S	75
TCTCACATGTGGCTGTCTCTCGGAGAAGAAGCGTTTGTGGGAGGAGAAGCTCCAGACTCA	300
S H M W L S L G E E A F V G G E A P D S	95
CCTGTGGTCACTATGGACCGGAGAAAACCAGCATCTCCAAGTATTTAAAAGGCCAACAGA	360
P V V T M D R E K T S I S K Y L K G N R	115
CCGCTGGTGTGAGTTTTGGAAGTTGCACCTGACCCCGTTTATGTATAAAGTTGATGAG	420
P L V L S F G S C T X P P F M Y K L D E	135
TTAAGCAACTCGTCAAGGACTTCAGCGATGTTGCCGACTTTCTTGTGATCTACATCGCA	480
F K Q L V K D F S D V A D F L V I Y I A	155
GAGGCTCATTCAACAGATGGTTGGCCCTTTAAGAACAACTATGACATCAACCAGCACCAG	540
E A H S T D G W A F K N N Y D I N Q H Q	175
AGCCTGGAGGACAGGCTGTCTGCGGCACAGGTCTGGTTCAGAGTGAACCCCTGTGTCTCT	600
S L E D R L S A A Q V L V Q S E P L C P	195
GTGGTGTGGATGAAATGACCGATGTCCACCACATAAAGTACGGAGCTCTGCCTGAGAGG	660
V V V D E M T D V T T I K Y G A L P E R	215
CTTTTCATTCTGCAGGCTGGGAAAGTGCTCTACAAGGGAGGGAAAGGGCCTTGGGGCTAC	720
L F I L Q A G K V L Y K G G K G P W G Y	235
AACCAGCGGAGGTGCGATCCTTCTTGGAGAAGATAAAAATAAGAAGCTCTGCGCTTATAT	780
N P A E V R S F L E K I K *	248
GAATAAAGGTTTCTTCTTATTAAGGGTGGGTTTATCTACATTCTAACCGTATACATT	840
ACTTATATAGTGGGGGATTTTACAATCAGGCTCTCATGTAGTCTTAAAAAGATATCAG	900
CCTCTGAGCTTGAATCTCCTCTCAGTGTATTAAATGACGGCTACAGATTAAACCTTTA	960
<u>GCCTCTGGAGCCAGATGCATTCAAACACAGGGAGACAGTCCCACTCAGCGGTTTGTGATA</u>	1020
<u>AAACGGCGCGTACAGGAGATGTAATTTGTGTGAGCATGCATGTGTTTTTTGTCCATATTC</u>	1080
<u>TCTGGCCTTTTTGGTTCGGTGGTGGTTTATTATGGTGCAAGTTTGTCTGCATTGTACTG</u>	1140
<u>TAATCCACTACATCAGCATGTAGTCTATTGTCAGTCTTTCACATTAATCACAAAAGGT</u>	1200
<u>GTAGAAATGGTGTGTAACCTTTCTGTAGTCTTAAGAGATGTGAGCCTCTGAGCTTGCAAC</u>	1260
<u>CCCCTCTCTGTGTTTCTCAGTGAAGGCTACAGATTAAACCTCTGGCCTCTGGAGCCAGAT</u>	1320
<u>GCATTGAAACGCAGGGAGACAGCCAACCTCAGTCATGTTCATGTTCCGTTGTATCACAGAT</u>	1380
<u>AGACAGCAGATGTAGTTTGCTTCCATATGTGTTTTGCTTATGTTGCCTGGCTTCTTCAGT</u>	1440
<u>TAAAATCCCTGAAGATTTAAGGCAGTGAAGTTTTCCATTTCCAGTGTGAGTTATCAAG</u>	1500
<u>TGCACACTTATGAATTTCTCCACAGTTTCTACAGGTGGCTACTATTTACATGACAATGACA</u>	1560
<u>GTCAAAACCTTCTTAGCTGGAAGTCTTTGAACTATGCAGGTTAATCTATTATGGGACAC</u>	1620
<u>TTATTGATTTGAATATTCTTGGATGTGAAGTCATCTGGTAGCACTCCAACATAAGAGTAC</u>	1680
<u>TCAATTTGTCAAAGTAAGTCATAAACCCAGACATGCAGCAGCACCGTATGCTAGTTGCTT</u>	1740
<u>TGTGTTGTCACTGTGACTCACCCCTGACCCATTAAGAGAGAATATGATTGTTCCAAAGCT</u>	1800
<u>CGAGTTCTCCATTATTACATAATGTCTCTGCGTAGAAAAAAATGCTTTCAGAGTTTFA</u>	1860
<u>ATACCAACAAAGAGCAGATTTCTCCACATGAGAATCTGCCTGTTGTTTGCTGTCTTGTG</u>	1920
<u>TGGAAATGATAAACCTTCTGCAGTGTGAGTTGCGCAAGTTCAGATTCCTTTGAACACA</u>	1980
<u>CTTTGCAGTCTGCACCTTATACATGTGAGCTGTGAGCTGCAGCACTGCATTGAGCCTGATG</u>	2040
<u>AGTTAATATCTAACTGTTATCTGCACATCAGACGATTTTGGCAGCCAGTGCCTTGTTC</u>	2100
<u>ATAATCTAATCTAATGAGGGTCTTATCCAAGTGTAGTTGGTGAAGGGCACAAGAGAGC</u>	2160
<u>TTTCAAGTATTTTGATAAAATGTTGATTGGAGAAAACAATCTGATTTTAATTTCCATCC</u>	2220
<u>AAGCATGAATAATTTGTAGTTTAAGTACTTCTTTCATGTTTTTGTGATATCAGTGCT</u>	2280
<u>TAATTTACTTGGCAGAATACTTTGTCTCGTCTCAATAAACACGAGTGCTAATGCGAGCCT</u>	2340
<u>GTGGCAATATTTAAAGCAAACCTTGTGAACTGAAAAAAAAAAAAAAAAAAAAAAAAAAAA</u>	2400
A	2401

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.

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-

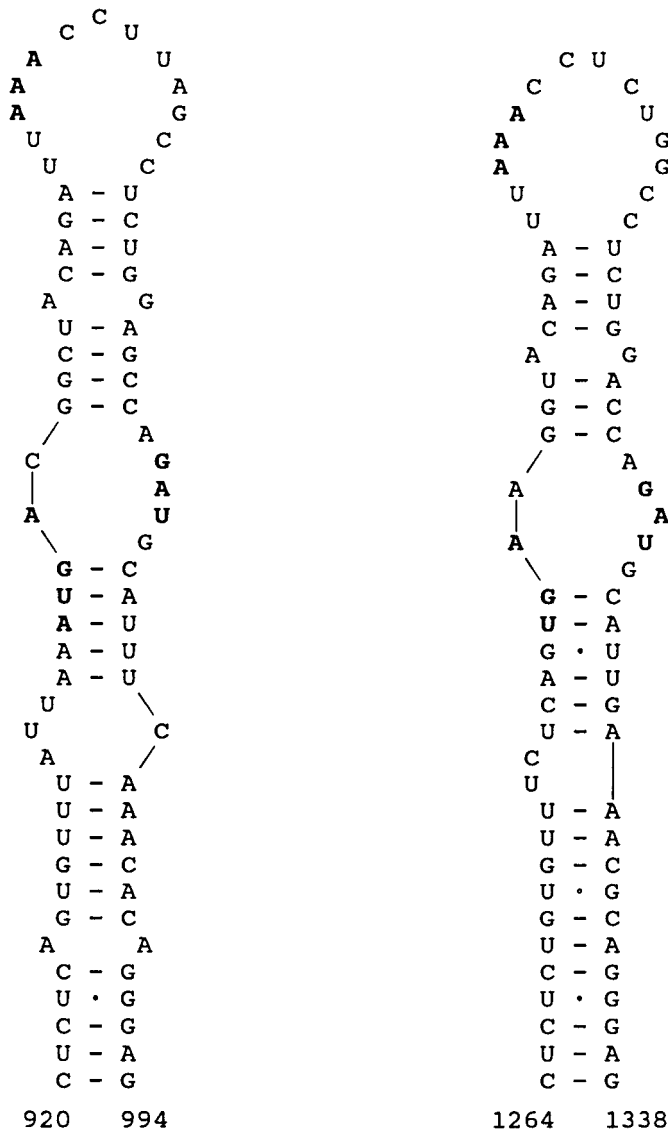


FIG. 2. Predicted secondary structures of two putative SECIS elements in 3' UTR of tD1 mRNA. Structure on *right* is exactly as predicted by the MFOLD program (26), whereas possible basepairing in loop structure on *left* has been omitted to enhance homology between secondary structures. Essential nucleotides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

Taq 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; Hybond membranes and [α - 32 P]deoxyATP were purchased from Amersham (Buckinghamshire, UK); polyethyleneglycol (PEG6000) was from Merck (Hohenbrunn, Germany); diethylaminoethyl (DEAE)-dextran and Sephadex LH-20 were from Pharmacia. Nonradioactive iodothyronines were obtained from Henning Berlin R & D (Berlin, Germany), [$3',5'-^{125}$ I]T₄ (\approx 1200 Ci/mmol) from Amersham, and [$3,5-^{125}$ I]T₃ (\approx 35 Ci/mmol) from Mr. R. Thoma (Formula GmbH, Berlin, Germany) courtesy of Dr. G. Decker (Hennig Berlin GmbH, Berlin, Germany). [$3',5'-^{125}$ I]rT₃ (\approx 2000 Ci/mmol) and [$3,5-^{125}$ I]T₃ sulfate (T₃S) were prepared in our laboratory as described previously (22, 23). PTU, IAC, GTG, DTT, and chloroquine were obtained from Sigma (St. Louis, MO). All other reagents were of the highest purity commercially available.

Cloning

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'-AATTTTGGCAGT-TGTACCTGACC-3' and 5'-RTGIGCTTCCTCIATGTA-3' and SuperTaq 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 - 32 P]deoxyATP. The phagemids carried in selected positive bacteriophages were excised, generating cDNA clones in pBK-CMV. The inserts were sequenced manually and by automatic sequencing in both directions using the dideoxy method of Sanger *et al.* (23a). Site-directed mutagenesis was carried out using the MORPH kit (5prime \rightarrow 3prime, Boulder, CO) and the oligonucleotide 5'-CACCTGACCCTGGTTTATGTATAA-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/~zucker>) (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 nM Na₂SeO₃ (25). One day before transfection, COS-1 cells were seeded at 50% confluency in 55-cm² cell culture dishes. Expression constructs (7 μ g), isolated by alkaline lysis and polyethyleneglycol precipitation (26), were added to serum-free DMEM/F12 medium containing 100 μ g/ml DEAE-dextran. After 2 h, the medium was replaced by serum-free DMEM/F12 medium containing 100 μ M chloroquine. Again 2 h later the medium was replaced by DMEM/F12 containing 10% FCS and 40 nM Na₂SO₃. After 3 days, the cells were rinsed with PBS; collected in 0.3 ml 0.1 M phosphate (pH 6.9), 1 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 μ g 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 \times SSC, 0.5% SDS, 5 \times Denhardt's, and salmon sperm DNA (100 μ g/ml). Blots were washed twice for 15 min at 55 C in 3 \times SSC, 0.5% SDS at 55 C, and twice for 15 min at 60 C in 1 \times SSC, 0.5% SDS. Autoradiographs were scanned using 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 radioiodide released by ORD of outer ring 125 I-labeled T₄ or rT₃ or by IRD of inner ring 125 I-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 125 I-labeled substrate in 0.2 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, and 10 mM DTT. Reactions were stopped and [125 I]iodothyronines were precipitated by successive addition of 0.1 ml 5% BSA and 0.5 ml 10% TCA. 125 I⁻ was further isolated from the supernatant on Sephadex LH-20 minicolumns (22, 23). Unless indicated otherwise, the results presented are taken from representative experiments and were reproduced in at least two other experiments.

Results

By RT-PCR of tilapia kidney mRNA using oligonucleotide primers corresponding to the conserved amino acid se-

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 opal 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 rT_3 as the substrate, and 3) increased IRD of T_3S vs. T_3 (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 [^{125}I]r T_3 by (in decreasing order of potency) rT_3 , T_4 , and T_3 (Fig. 6). The apparent Michaelis-Menten constant (K_m) value of rT_3 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 10^3 -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 rT_3 and T_4 at only 2–3% of the activity of the wild-type enzyme, whereas IRD of T_3S was increased by the mutation (Fig. 8). IRD of T_3 by both wild-

Til	MFLQKIVLYLSTACMFCYMLGKFLMLVILQFFSPSLAKKFILRMGEKITMTQNPRFNYED	60
Rat	•g•sqlw•w•krlvi•lqvalevatgkv•mtlf•erv•qn••a••q•tg••r•••••apdn	60
Hum	•g•ppqg•w•krlwvlllevavhvvgkv•lilf•drv•rn••a•••••tg••r••h•shdn	60
Dog	•g•prp••w•rrlwvllqvavqavqkvflklf•arv•qh•va•ng•-----h•s•dn	55
Til	WGLTFMSLAFIKTASSHMWLSLGEEAFVGGAPDSPVVTMDREKTSISKYLKGNRPLVLS	120
Rat	•vp••f•iqyfwfvlkvr•qr•edr•ey••l••nct••rlsgq•cnvwdfiq•s•••••n	120
Hum	•ip••f•tqyfwfvlkvr•qr•edttel••l••nc••rlsgqrcn•wefmq•••••n	120
Dog	•ap•ly•mqyfwfvlkvq•qr•edrtep••l••nc••rlsgqrcn•wdfmq•••••n	115
Til	FGSCTXPPFMYKLDEFKQLVKDFSDVADFLVIYIAEAHSTDGWAFKNNYDINQHQSLEDR	180
Rat	•••••s•ll•f•q•r•d•ast•••i••e••a•••••v•r•r•q••	180
Hum	•••••s•f•f•q•r•ie••si•••••e••as•••••m•rn•n•q••	180
Dog	•••••s•lf•f•q•r•ie•cst•••i••e••as•••••vn•rt•t•q••	175
Til	LSAAQVLVQSEPLCPVVDEMTDVTTIKYGALPERLFILQAGKVLYKGGKGPWGYNPAEV	240
Rat	•r••hl•lars•q•••••t•qnqssql•a•••••yvi•eric••kp••n••e••	240
Hum	•q••hl•lars•q•••••t•qnqssql•a•••••y•i•e•ri••ks••n••e••	240
Dog	•q••rl•ldra•p•••••t•rnqssqf•a•••••v•e•ri••kp••n•h•e••	235
Til	RSFLEKIK	248
Rat	•av•••lcippghmpqf	257
Hum	•av•••lhs	249
Dog	•av•••lhs	244

FIG. 3. Alignment of amino acid sequences of tilapia, rat, human, and dog type I iodothyronine deiodinases.

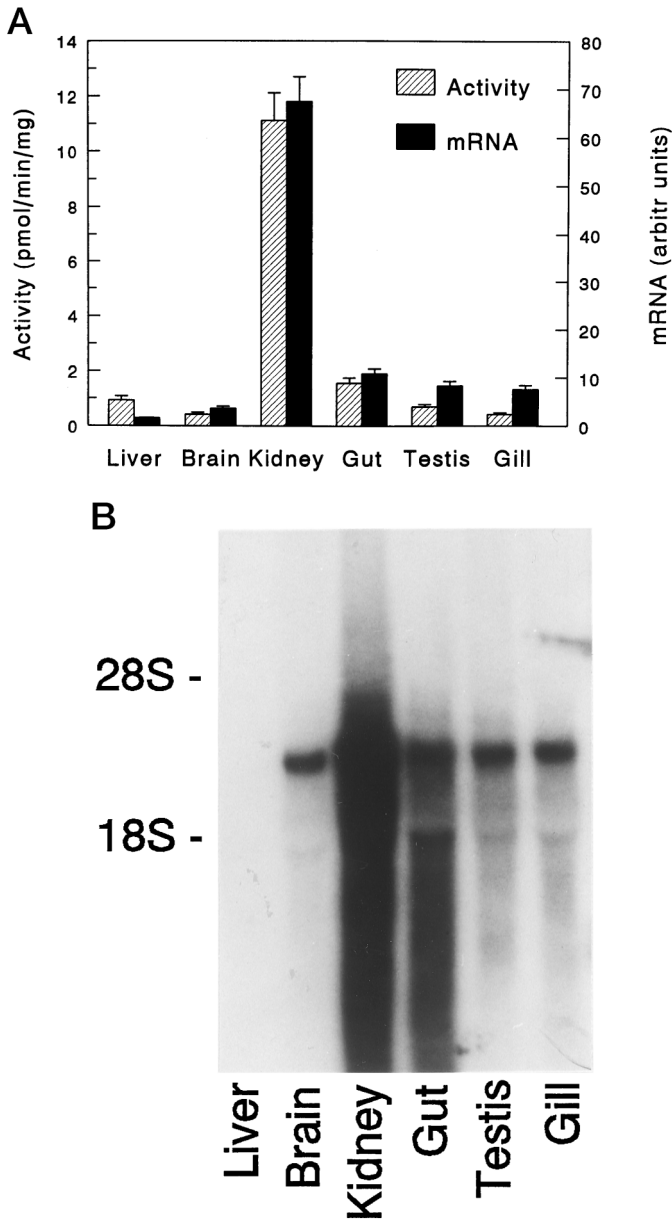


FIG. 4. A, Tissue distribution of D1 activity and D1 mRNA levels in tilapia tissues. Error bars represent SDs of triplicate determinations (activity) or of two separate experiments (mRNA). B, Northern blot hybridized with radiolabeled TN12 cDNA.

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

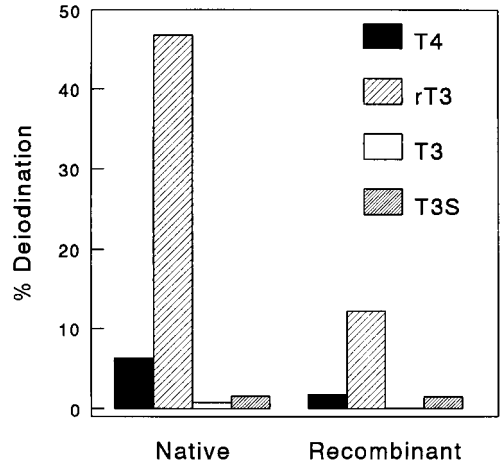


FIG. 5. ORD of T₄ and rT₃ and IRD of T₃ and T₃S by native tD1 in tilapia kidney microsomes or by recombinant tD1 expressed in COS-1 cells. Conditions were: 10 nM substrate, 10 mM DTT, 1 (lysate) or 0.1 (microsomes) mg protein/ml, and 60 min of incubation at 37 C.

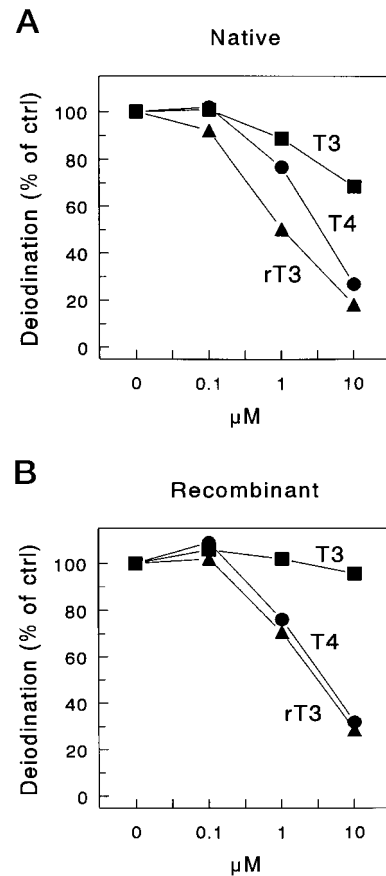


FIG. 6. Inhibition of ORD of [¹²⁵I]rT₃ by native tD1 in tilapia kidney microsomes (A) or recombinant tD1 expressed in COS-1 cells (B) by increasing concentrations of unlabeled rT₃, T₄, and T₃. Conditions were: 10 nM [¹²⁵I]rT₃, 10 mM DTT, 1 (lysate) or 0.1 (microsomes) mg protein/ml, and 60 (lysate) or 30 (microsomes) min of incubation at 37 C.

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

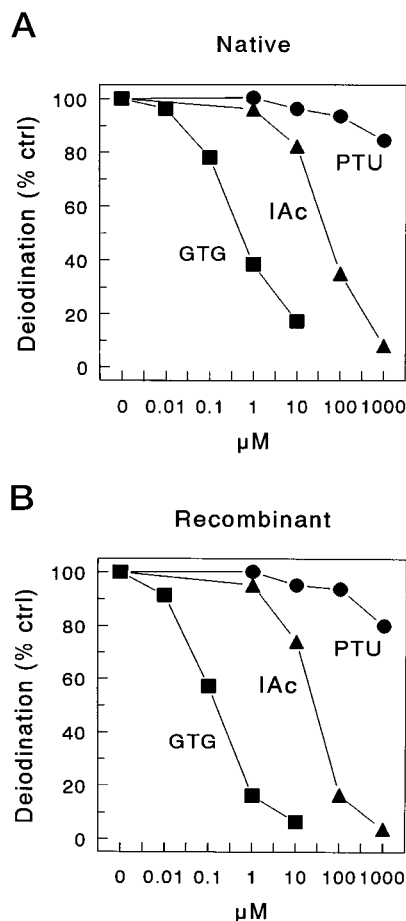


FIG. 7. Inhibition of ORD of [125 I]rT₃ by native tD1 in tilapia kidney microsomes (A) or recombinant tD1 expressed in COS-1 cells (B) by increasing concentrations of GTG, IAc, and PTU. Conditions were: 0.1 μ M [125 I]rT₃, 10 mM DTT, 1 (lysate) or 0.1 (microsomes) mg protein/ml, and 60 (lysate) or 30 (microsomes) min of incubation at 37 C.

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 with tD1 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

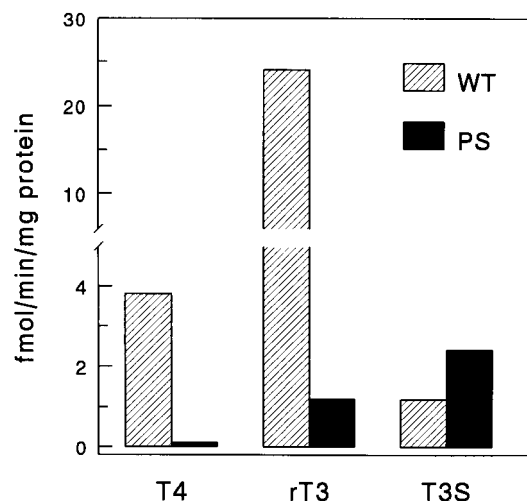


FIG. 8. ORD of T₄ and rT₃ and IRD of T₃ and T₃S by wild-type tD1 (WT) and Pro128Ser mutant (PS). Conditions were: 10 nM substrate, 10 mM DTT, 1 mg lysate protein/ml, and 60 min of incubation at 37 C. Percentage deiodination of T₄ by WT and PS amounted to 2.3 and 0.1, of rT₃ to 14.4 and 0.7, and of T₃S to 1.2 and 2.4, respectively.

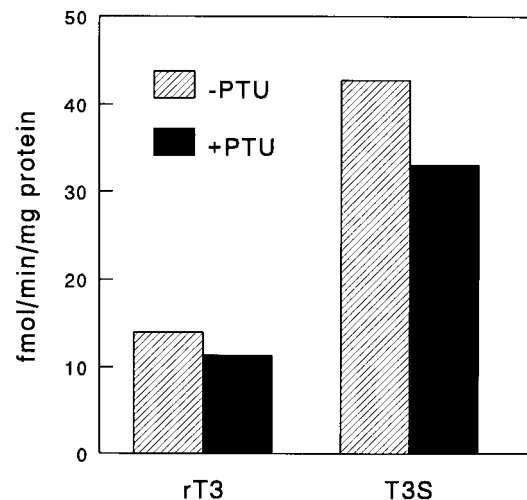


FIG. 9. Effects of PTU on ORD of rT₃ and IRD of T₃S by Pro128Ser mutant of tD1. Conditions were: 0.1 μ M substrate, 10 mM DTT, 1 mM PTU, 1 mg lysate protein/ml, and 120 min of incubation at 37 C.

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 μ M of this drug strongly inhibits rat and human D1 (5, 21). In addition, tD1 is also approximately 10-fold less sensitive

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 rT_3 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 SeI group, the rate of formation of this intermediate also determines susceptibility to PTU inhibition. Our findings suggest that tD1 has a lower k_{cat} 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 rT_3 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.

Acknowledgments

We thank Mr. R. Thoma (Formula GmbH, Berlin, Germany) and Dr. G. Decker (Hennig Berlin GmbH, Berlin, Germany) for supply of inner ring-labeled T_3 .

References

- Leonard JL, Köhrle J 1996 Intracellular pathways of iodothyronine metabolism. In: Braverman LE, Utiger R (eds) *The Thyroid*. Lippincott-Raven, Philadelphia, pp 125–161
- Larsen PR, Berry MJ 1995 Nutritional and hormonal regulation of thyroid hormone deiodinases. *Annu Rev Nutr* 15:323–352
- Visser TJ 1996 Pathways of thyroid hormone metabolism. *Acta Med Austr* 23:10–16
- Berry MJ, Banu L, Larsen PR 1991 Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. *Nature* 349:438–440
- Mandel SJ, Berry MJ, Kieffer JD, Harney JW, Warne RL, Larsen PR 1992 Cloning and *in vitro* expression of the human selenoprotein, type I iodothyronine deiodinase. *J Clin Endocrinol Metab* 75:1133–1139
- Maia AL, Berry MJ, Sabbag R, Harney JW, Larsen PR 1995 Structural and functional differences in the *diol1* gene in mice with inherited type I deiodinase deficiency. *Mol Endocrinol* 9:969–980
- Toyoda N, Harney JW, Berry MJ, Larsen PR 1995b Identification of critical amino acids for 3,3',5'-triiodothyronine deiodination by human type I deiodinase based on comparative functional-structural analyses of the human, dog and rat enzymes. *J Biol Chem* 269:20329–20334
- St. Germain DL, Schwartzman RA, Croteau W, Kanamori A, Wang Z, Brown DD, Galton VA 1994 A thyroid hormone-regulated gene in *Xenopus laevis* encodes a type III iodothyronine 5-deiodinase. *Proc Natl Acad Sci USA* 91:7767–7771, 11282
- Becker KB, Schneider MJ, Davey JC, Galton VA 1995 The type III 5-deiodinase in *Rana catesbeiana* tadpoles is encoded by a thyroid hormone-responsive gene. *Endocrinology* 136:4424–4431
- Croteau W, Whittemore SL, Schneider MJ, St. Germain DL 1995 Cloning and expression of a cDNA for a mammalian type III iodothyronine deiodinase. *J Biol Chem* 270:16569–16575
- Salvatore D, Low SC, Berry MJ, Maia AL, Harney JW, Croteau W, St. Germain DL, Larsen PR 1995 Type 3 iodothyronine deiodinase: cloning, *in vitro* expression, and functional analysis of the placental selenoenzyme. *J Clin Invest* 96:2421–2430
- Davey JC, Becker KB, Schneider MJ, St. Germain DL, Galton VA 1995 Cloning of a cDNA for the type II iodothyronine deiodinase. *J Biol Chem* 270:26786–26789
- Croteau W, Davey JC, Galton VA, St. Germain DL 1996 Cloning of the mammalian type II iodothyronine deiodinase: a selenoprotein differentially expressed and regulated in human and rat brain and other tissues. *J Clin Invest* 98:405–417
- Salvatore D, Bartha T, Harney JW, Larsen PR 1996 Molecular biological and biochemical characterization of the human type 2 selenodeiodinase. *Endocrinology* 137:3308–3315
- Valverde R, Croteau W, Lafleur GJ, Orozco A, St. Germain DL 1997 Cloning and expression of a 5'-iodothyronine deiodinase from the liver of *Fundulus heteroclitus*. *Endocrinology* 138:642–648
- Leonard JL, Visser TJ 1984 Selective modification of the active center of renal iodothyronine 5'-deiodinase by iodoacetate. *Biochim Biophys Acta* 787:122–130
- Berry MJ, Kieffer JD, Larsen PR 1991 Evidence that cysteine, not selenocysteine, is the catalytic site of type II iodothyronine deiodinase. *Endocrinology* 129:550–552
- Santini F, Chopra IJ, Hurd RE, Solomon DH, Chua Teco GN 1992 A study of the characteristics of the rat placental iodothyronine 5-monodeiodinase: evidence that it is distinct from the rat hepatic iodothyronine 5'-monodeiodinase. *Endocrinology* 130:2325–2332
- Safra M, Farwell AP, Leonard JL 1991 Evidence that type II 5' deiodinase is not a selenoprotein. *J Biol Chem* 266:13477–13480
- Meinhold H, Campos-Barros A, Walzog B, Köhler R, Müller F, Behne D 1993 Effects of selenium and iodine deficiency on type I, type II and type III iodothyronine deiodinases and circulating thyroid hormones in the rat. *Exp Clin Endocrinol* 101:87–93
- Mol K, Kaptein E, Darras VM, De Greef WJ, Kühn E, Visser TJ 1993 Different

- thyroid hormone-deiodinating enzymes in tilapia (*Oreochromis niloticus*) liver and kidney. FEBS Lett 321:140–143
22. **Moreno M, Berry MJ, Horst C, Thoma R, Goglia F, Harney JW, Larsen PR, Visser TJ** 1994 Activation and inactivation of thyroid hormone by type I iodothyronine deiodinase. FEBS Lett 344:143–146
 23. **Toyoda N, Kaptein E, Berry MJ, Harney JW, Larsen PR, Visser TJ** 1997 Structure-activity relationships for thyroid hormone deiodination by mammalian type I iodothyronine deiodinases. Endocrinology 138:213–219
 - 23a. **Sanger F, Nicklen S, Coulson AR** 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
 24. **Zuker M, Stiegler P** 1981 Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res 9:133–148
 25. **Oertel M, Gross M, Rokos H, Köhrle J** 1993 Selenium-dependent regulation of type I 5'-deiodinase expression. Am J Clin Nutr 57:313S–314S
 26. **Goldberg GS, Lau AF** 1993 Transfection of mammalian cells with PEG-purified plasmid DNA. Biotechniques 14:548–550
 27. **Berry MJ, Banu L, Harney JW, Larsen PR** 1993 Functional characterization of the eukaryotic SECIS elements with direct selenocysteine insertion at UGA codons. EMBO J 12:3315–3322
 28. **Shen Q, Leonard JL, Newburger PE** 1995 Structure and function of the selenium translation element in the 3'-untranslated region of human glutathione peroxidase mRNA. RNA 1:519–525
 29. **Van der Geyten S, Sanders JP, Kaptein E, Darras VM, Kühn ER, Leonard JL, Visser TJ** 1997 Expression of chicken hepatic type I and type III iodothyronine deiodinases during embryonic development. Endocrinology 138:5144–5152
 30. **Walczak R, Westhof E, Carbon P, Krol A** 1996 A novel structural motif in the selenocysteine insertion element of eukaryotic selenoprotein mRNAs. RNA 2:367–379
 31. **Baggio R, Shi YQ, Wu YQ, Abeles** 1996 From good substrates to good inhibitors: design of inhibitors for serine and thiol proteases. Biochemistry 35:3351–3353
 32. **Chapman HA, Munger JS, Shi GP** 1994 The role of thiol proteases in tissue injury and remodeling. Am J Respir Crit Care Med 150:S155–S159
 33. **Berry MJ** 1992 Identification of essential histidine residues in rat type I iodothyronine deiodinase. J Biol Chem 267:18055–18059
 34. **Toyoda N, Berry MJ, Harney JW, Larsen PR** 1995 Topological analysis of the integral membrane protein, type I iodothyronine deiodinase. J Biol Chem 270:12310–12318