

# Substitution of Cysteine for a Conserved Alanine Residue in the Catalytic Center of Type II Iodothyronine Deiodinase Alters Interaction with Reducing Cofactor

GEORGE G. J. M. KUIPER, WILLEM KLOOTWIJK, AND THEO J. VISSER

Department of Internal Medicine, Erasmus University Medical Center, 3000 DR Rotterdam, The Netherlands

Human type II iodothyronine deiodinase (D2) catalyzes the activation of T<sub>4</sub> to T<sub>3</sub>. The D2 enzyme, like the type I (D1) and type III (D3) deiodinases, contains a selenocysteine (SeC) residue (residue 133 in D2) in the highly conserved catalytic center. Remarkably, all of the D2 proteins cloned so far have an alanine two residue-amino terminal to the SeC, whereas all D1 and D3 proteins contain a cysteine at this position. A cysteine residue in the catalytic center could assist in enzymatic action by providing a nucleophilic sulfide or by participating in redox reactions with a cofactor or enzyme residues. We have investigated whether D2 mutants with a cysteine (A131C) or serine (A131S) two-residue amino terminal to the SeC are enzymatically active and have characterized these mutants with regard to substrate affinity, reducing cofactor interaction and inhibitor profile.

COS cells were transfected with expression vectors encoding wild-type (wt) D2, D2 A131C, or D2 A131S proteins. Kinetic analysis was performed on homogenates with dithiothreitol (DTT) as reducing cofactor. The D2 A131C and A131S mutants

displayed similar Michaelis-Menten constant values for T<sub>4</sub> (5 nM) and reverse T<sub>3</sub> (9 nM) as the wt D2 enzyme. The limiting Michaelis-Menten constant for DTT of the D2 A131C enzyme was 3-fold lower than that of the wt D2 enzyme. The wt and mutant D2 enzymes are essentially insensitive to propylthiouracil [concentration inhibiting 50% of activity (IC<sub>50</sub>) > 2 mM] in the presence of 20 mM DTT, but when tested in the presence of 0.2 mM DTT the IC<sub>50</sub> value for propylthiouracil is reduced to about 0.1 mM. During incubations of intact COS cells expressing wt D2, D2 A131C, or D2 A131S, addition of increasing amounts of unlabeled T<sub>4</sub> resulted in the saturation of [<sup>125</sup>I]T<sub>4</sub> deiodination, as reflected in a decrease of [<sup>125</sup>I]T<sub>3</sub> release into the medium. Saturation first appeared at medium T<sub>4</sub> concentrations between 1 and 10 nM.

**In conclusion:** substitution of cysteine for a conserved alanine residue in the catalytic center of the D2 protein does not inactivate the enzyme *in vitro* and *in situ*, but rather improves the interaction with the reducing cofactor DTT *in vitro*. (*Endocrinology* 143: 1190–1198, 2002)

T<sub>4</sub>, THE MAIN secretory product of the thyroid gland, undergoes enzymatic outer ring deiodination (ORD) in peripheral tissues to T<sub>3</sub> catalyzed by the deiodinases type I (D1) and type II (D2) (1–3). The D1 protein (4–7) has a high Michaelis-Menten constant (K<sub>m</sub>) for T<sub>4</sub> (~5 μM), is activated by dithiothreitol (DTT) *in vitro*, and is highly sensitive to inhibition by propylthiouracil (PTU) *in vitro* and *in vivo*. The D2 protein has a low K<sub>m</sub> for T<sub>4</sub> (~5 nM), is also activated by DTT but is only minimally sensitive to PTU (8–10). The catalytic mechanism, though incompletely described, seems to be different between D1 and D2. ORD of T<sub>4</sub> by D1 protein exhibits ping-pong (bi)-substrate kinetics with T<sub>4</sub> and the thiol-containing cofactor as cosubstrates (6, 8, 11, 12). The D2 protein exhibits sequential reaction kinetics, suggesting that T<sub>4</sub> and the thiol-containing cofactor must interact with the enzyme simultaneously before reaction takes place (8, 9). Another remarkable difference between D1 and D2 is the fact that while *N*-bromoacetyl derivatives of T<sub>4</sub> or T<sub>3</sub> are excellent

covalent affinity labels of D1, they do not label the D2 selenoprotein (13–16).

The D1 protein is a selenoenzyme, containing a selenocysteine (SeC) residue in the catalytic center (4, 17, 18). This SeC residue is essential for enzymatic activity because replacement with leucine or alanine eliminates deiodinase activity, whereas replacement with cysteine reduces the turnover number about 100-fold (19, 20). The SeC in the catalytic center of D1 is thought to function as the iodine acceptor and PTU probably inhibits D1 via the formation of a SeC-PTU adduct (6, 12, 19). The inability to covalently label D2 with bromoacetyl-iodothyronine derivatives, as well as the drastically reduced sensitivity to PTU, iodoacetate, and gold thioglucose compared with D1, have been interpreted as evidence that deiodination by D2 does not require the participation of enzyme sulfhydryl groups (21) or that D2 is not a selenoprotein (19, 22). The inability to label the substrate-binding subunit of D2 (p29) in rat glial cells with <sup>75</sup>Se was also interpreted as evidence that D2 is not a selenoprotein (23). However, since the cloning of D2 cDNAs from several species and the identification of SeC insertion sequence elements (SECIS) in the 3'-UTR, it is clear that the D2 protein is a selenoenzyme containing a SeC residue in the catalytic center (10, 16, 24–29). More direct evidence for the selenoprotein character of D2 protein was recently provided by the demonstration that a <sup>75</sup>Se-labeled protein of the expected size is immunoprecipitated from mesothelioma

Abbreviations: Ala, Alanine; A131C, D2 mutants with a cysteine two-residue amino terminal to the SeC; A131S, D2 mutants with a serine two-residue amino terminal to the SeC; Cys, cysteine; D1, D2, and D3, human types I, II, and III iodothyronine deiodinase; DMEM/F12, DMEM-Ham's F-12 medium; DTT, dithiothreitol; GSH, monothiol glutathione; GTG, gold thioglucose; IAC, iodoacetate; K<sub>m</sub>, Michaelis-Menten constant; ORD, outer ring deiodination; PTU, propylthiouracil; rT<sub>3</sub>, reverse T<sub>3</sub>; SeC, selenocysteine; SECIS, SeC insertion sequence elements; Sel, selenenyl iodide; wt, wild-type.

cells with a D2 antiserum (30). Therefore, the described differences between D1 and D2 must be influenced by other factor(s) than the presence of SeC in the catalytic center.

The core catalytic center of D1 and D2 is located in a region about 15 amino acids long, surrounding the essential SeC. This region is highly conserved within and between the D1 and D2 subtype enzymes (Fig. 1). However, all of the D2 proteins cloned so far have an alanine two residues amino-terminal to the SeC, whereas all D1 proteins contain a cysteine (Cys) at this position (1–3, 29). A Cys residue in the catalytic center could assist in enzymatic action by providing a nucleophilic sulfide or by participating in redox reactions with a cofactor or enzyme residues. Replacement of Cys by alanine (Ala) in the active center of D1 caused a marked increase in the limiting  $K_m$  for DTT (11, 31). By analogy, the absence of a Cys residue at the same position (residue 131) in human D2 might reduce the turnover number compared with D1 and could perhaps explain the minimal sensitivity of D2 to PTU or the inability to react with bromoacetyl derivatives of  $T_3$  and  $T_4$ .

The present studies were performed to elucidate the functional consequences of the absence of a Cys residue in the catalytic center of human D2 protein. We have examined effects of the Ala131 to Cys (A131C) mutation either alone or in combination with a SeC133 to Cys mutation (SeC133C/A131C) on substrate affinity, reducing cofactor interaction and inhibitor profile.

## Materials and Methods

### Materials

Nonradioactive iodothyronines were obtained from Henning Berlin GmbH & Co. (Berlin, Germany) and [ $3'$ ,  $5'$ - $^{125}$ I] $T_4$  (specific activity 969 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). [ $3'$ ,  $5'$ - $^{125}$ I]reverse  $T_3$  ( $rT_3$ ) was prepared by radioiodination of  $3,3'$ - $T_2$  using the chloramine-T method as described (32). Before each experiment, the radiolabeled iodothyronines were purified on Sephadex LH-20 columns (33). Radioactively labeled *N*-bromoacetyl- $T_4$  or *N*-bromoacetyl- $T_3$  were synthesized from bromoacetylchloride and [ $^{125}$ I] $T_4$  or [ $^{125}$ I] $T_3$  as described (13).

PTU (6-*n*-propyl-2-thiouracil), gold thioglucose (GTG), and iodoacetate (IAc) were obtained from Sigma (St. Louis, MO). DTT was obtained from ICN Biochemicals, Inc. (Costa Mesa, CA). Recombinant *Taq* DNA polymerase was obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands) and synthetic oligonucleotides were ordered from Life Technologies, Inc. (Glasgow, UK).

### Site-directed mutagenesis and construction of expression vectors

A cDNA clone containing the entire coding sequence of the human D2 protein was used for mutagenesis via the overlap extension DNA-polymerase reaction procedure (34).

D1 R P L V L N F G S C T SeC P S

D2 R P L V V N F G S A T SeC P P

D3 R P L V L N F G S C T SeC P P

FIG. 1. Deduced amino acid sequences comprising the core catalytic center of the three iodothyronine deiodinases. All D2 enzymes cloned so far (human, mouse, rat, chicken, *Xenopus*, *Rana*, trout) have an Ala two residues amino-terminal to the SeC, while all D1 and D3 proteins contain a Cys at this position.

Overlapping inner sense and antisense primers containing the nucleotide changes needed to produce the SeC133Cys (SeC133C) D2 mutant cDNA, Ala131Cys (A131C) D2 mutant cDNA, Ala131Ser (A131S) D2 mutant cDNA, and SeC133Cys/Ala131Cys (double Cys) D2 mutant cDNA were used for amplification with a Kozak start consensus (*underlined*) 5'-outer primer (5'-CAAGCTTGCCACCATGGGCATCCTCAGCGTA) or a stop 3'-outer primer (5'-CAAGCTTTTAACCAGCTAATCTAGT), each containing flanking *Hind*III restriction sites (*italics*). The PCR products were agarose gel purified and used for overlap extension PCR reactions with the described outer primers. The mutated D2 cDNA fragments were subcloned into the pGEM-T vector (Promega Corp., Madison, WI) and sequenced to confirm that only the proper mutations were introduced. Because a SECIS element is required for incorporation of SeC in selenoproteins, we prepared chimeric constructs in which the D2 cDNA was inserted 5' to the SECIS element of the rat D1 gene. For this purpose, the G21-pcDNA3 rat D1 expression vector (4) was digested with *Hind*III, and the 6-kb DNA band containing vector DNA plus 0.7 kb of the rat D1 3'-UTR (including the SECIS element) was isolated from a preparative agarose gel. The pGEM vectors containing the respective mutant D2 sequences were digested with *Hind*III, and the isolated fragments were cloned into the prepared rat D1-SECIS-pcDNA3 vector. A wt human D2 expression vector was prepared in the same manner. It should be noted that the 3'-(second) TGA, which is seven codons 5' to the universal stop codon TAA in the D2 cDNA, is present in all constructs. It is not known how efficient the second UGA codon is translated as SeC.

### Expression of D2 protein

The wt D2 and mutant D2 enzymes were expressed in COS-1 cells after diethylaminoethyl-dextran-mediated transfection of the expression vectors (35). COS-1 cells were grown in DMEM-Ham's F-12 medium (DMEM/F12) containing 10% FCS (Life Technologies, Inc.) and 40 nM  $Na_2SeO_3$ . One day before transfection, cells were divided 1:10 in 90-mm diameter culture dishes. Expression constructs (8  $\mu$ g per dish), isolated by alkaline lysis and polyethyleneglycol precipitation (36), were added to 5 ml serum-free DMEM/F12 medium containing 200  $\mu$ g/ml diethylaminoethyl-dextran. After 2 h, the medium was replaced by serum-free DMEM/F12 containing 100  $\mu$ M chloroquine phosphate (Sigma). Again, 2 h later the medium was replaced with DMEM/F12 containing 10% FCS and 40 nM  $Na_2SeO_3$ . After 2 d, the cells were rinsed with PBS and collected in 0.25 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA buffer, sonicated, aliquoted, and stored at  $-75^\circ C$ .

### Assay of ORD activity in COS cell homogenates

The principle of this assay is the production of radioiodide by ORD of [ $3'$ ,  $5'$ - $^{125}$ I] $T_4$  or [ $3'$ ,  $5'$ - $^{125}$ I] $rT_3$ . Duplicate incubations contained about 100,000 cpm labeled  $T_4$  or  $rT_3$  with varying amounts of unlabeled substrate ( $T_4$  or  $rT_3$ ) and COS cell homogenates (50–100  $\mu$ g protein) in a final volume of 0.2 ml P100E2D20 buffer (0.1 M sodium phosphate; 2 mM EDTA; 20 mM DTT, pH 7.2). In some experiments, the DTT concentration was varied between 0.1 and 20 mM DTT. In various experiments, the inhibitors PTU (0.1–2 mM), GTG (0.1–3  $\mu$ M), and  $BrAcT_4$  (0.1–1000 nM) were included. Mixtures were incubated for 60 min at  $37^\circ C$ , whereafter the reaction was stopped by the addition of 0.1 ml 5% BSA on ice. Protein-bound iodothyronines were subsequently precipitated by 10% TCA on ice, and the radioiodide in the supernatant was separated from remaining iodothyronines by chromatography on Sephadex LH-20 mini columns as described (33). The fractions were counted for  $^{125}I$ -activity in a 16 channel  $\gamma$ -counter (NE1600, Nuclear Enterprises, Edinburgh, UK).

Enzymatic deiodination was corrected for nonenzymatic deiodination in blank incubations with homogenates of nontransfected COS cells (< 3% of total deiodination). Protein was adjusted to consume less than 30% of substrate, and in control experiments it was determined that the deiodination rate was linear up to 60 min incubation. The radioiodide production counted was multiplied by 2 to account for the random labeling and deiodination at the 3' and 5' positions in the substrate. By reverse-phase HPLC of reaction products, it was established that equal amounts of radioiodide and radioactive iodothyronine ( $T_3$  or  $T_2$ ) were produced. No inner-ring deiodination was detected with wt or D2 mutant enzymes.

### Assay of ORD activity in intact transfected COS cells

COS cells were cultured in 6-well plates and transfected with 2.5  $\mu$ g plasmid per well as described. One day after transfection, cell monolayers were washed with serum-free DMEM/F12 medium and then cultured for an additional 24 h in serum-free DMEM/F12 (plus 40 nM  $\text{Na}_2\text{SeO}_3$ ), to which was added [ $^{125}\text{I}$ ]T $_4$  ( $1 \times 10^6$  cpm/ml) plus 1–1000 nM unlabeled T $_4$ .

Medium was harvested and extracted with ice-cold methanol (1:1). After centrifugation, the extract was mixed with 0.02 M ammonium acetate (pH 4) and 0.1 ml of the mixture was applied to a 250  $\times$  4.6 mm Symmetry C18 column connected to an Alliance HPLC system (Waters Chromatography Division, Millipore Corp., Milford, MA), and eluted with a 20-min gradient of 24–29% acetonitrile followed by a 6-min gradient of 29–50% acetonitrile in 0.02 M ammonium acetate (pH 4) at a flow rate of 1.2 ml/min. Radioactivity in the eluate was monitored on line using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT). The percentage T $_4$  deiodination was calculated from the amount of [ $^{125}\text{I}$ ]T $_3$  produced (multiplied by two), divided by the amount of [ $^{125}\text{I}$ ]T $_4$  added.

### Incubation with *N*-bromoacetyl-[ $^{125}\text{I}$ ]T $_3$ or T $_4$

Labeling of COS cell homogenates was done according to previously published procedures (14). In short, solutions of BrAc[ $^{125}\text{I}$ ]T $_3$  or BrAc[ $^{125}\text{I}$ ]T $_4$  (150,000 cpm) in ethanol were pipetted into microcentrifuge tubes, and the solvent was evaporated by a stream of nitrogen. After addition of 25  $\mu$ l P100E2D3 [100 mM sodium phosphate; 2 mM EDTA; 3 mM DTT, pH 7.2], and vortexing, the COS cell homogenates (150  $\mu$ g protein) were added in a total volume of 50  $\mu$ l P100E2D3. Again the mixtures were vortexed and incubated at 37 C for 20 min. Reactions were terminated by the addition of gel-loading buffer, and samples were analyzed by SDS-PAGE (12% gel) followed by autoradiography (14).

For *in situ* affinity labeling experiments, COS cells were transfected with wt D2, D2 A131C, or rat D1 expression vectors, and 1 d after transfection cells were washed and then cultured for an additional 24 h in serum-free DMEM/F12, to which was added BrAc[ $^{125}\text{I}$ ]T $_4$  (200,000 cpm/ml equivalent to 0.25 nM). The medium was harvested and analyzed by HPLC as described. The cells were collected in 0.25 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA buffer, sonicated, and samples were analyzed by SDS-PAGE.

## Results

### Analysis of mutant D2 enzymes *in vitro*

To define the effects of the introduction of a Cys residue in the catalytic center of D2, expression vectors were made encoding mutant D2 enzymes with a Cys (D2 A131C) or Ser (D2 A131S) two-residue amino terminal to the SeC. A Cys at this position (as in D1 and D3) could assist in enzymatic action by providing a nucleophilic sulfide or by participating in redox reactions with cofactor or enzyme residues. Serine was chosen as a control because it is most homologous to Cys, and it is unlikely that the side chain hydroxyl group participates in redox reactions. In another set of mutants, the SeC residue was substituted by Cys (essentially exchanging Se by S), thus creating the D2 SeC133C mutant enzyme and the D2 SeC133C/A131C double mutant enzyme.

COS cells were transfected with the described expression vectors and kinetic analysis was performed on homogenates in the presence of 20 mM DTT (Fig. 2 and Table 1). The wild-type D2, D2 A131C, and D2 A131S enzymes displayed similar  $K_m$  values for T $_4$  (5 nM) as well as for rT $_3$  (9 nM). Remarkably, substituting Cys for SeCys caused a 1,000-fold higher  $K_m$  for the substrate T $_4$  and a 10-fold higher  $V_{max}$  compared with wt D2 protein. The same holds true for the double Cys mutant (D2 SeC133C/A131C) enzyme. Coincubations with up to 250  $\mu$ M selenocysteine (which is converted

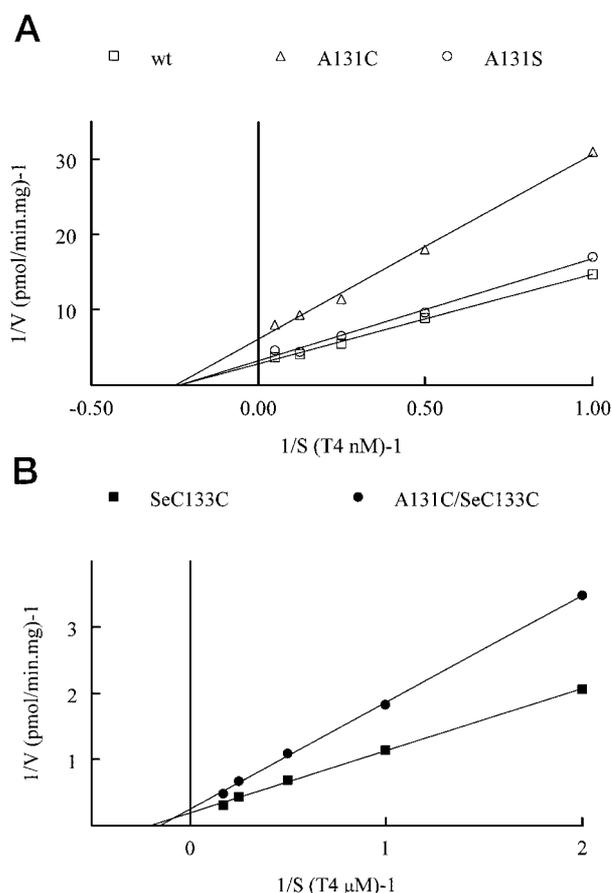


FIG. 2. Lineweaver-Burk plots of T $_4$  deiodination catalyzed by wt D2, D2 A131C, D2 A131S enzymes (A) and by D2 SeC133C or D2 SeC133C/A131C enzyme (B). Each data point is the average of closely agreeing duplicate determinations, and the experiment was performed twice on different homogenates with similar results.

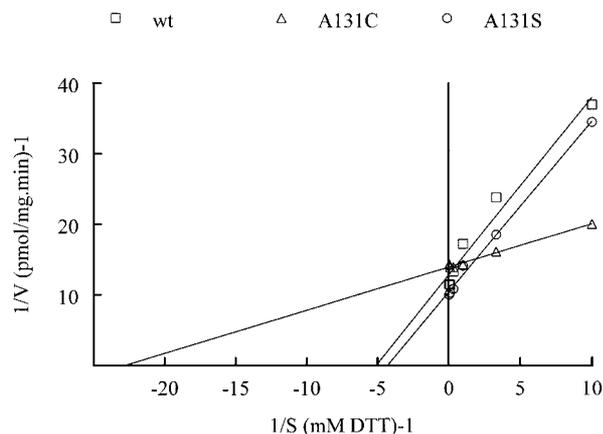
to selenocysteine in the presence of DTT) did not further increase the deiodination rate of the D2 SeC133C enzyme (not shown).

We performed Western blotting of COS cell homogenates with polyclonal antisera directed against the C terminus or the catalytic center of the rat D2 protein (37). Only in homogenates containing D2SeC133C or D2SeC133C/A131C protein, a specific band of the expected molecular mass (31 kDa) could be detected (not shown).

The D2 protein exhibits sequential reaction kinetics, and Lineweaver-Burk plots at different reducing cofactor (DTT) concentrations yield a set of intersecting lines (8, 9). To investigate the possibility that introduction of a Cys in the catalytic center influences the interaction with reducing cofactor, T $_4$  ORD was studied at several concentrations of DTT. The transfected COS cells were harvested in phosphate buffer (pH 7.2) without DTT, and without addition of DTT the net deiodination of T $_4$  was less than 0.5% in 60 min. In initial experiments, the T $_4$  concentration was fixed (2 nM) and the DTT concentration was varied over a broad range (0.1–30 mM). At the lowest DTT concentrations (0.1 and 0.3 mM), the deiodination rate of the D2 A131C enzyme was higher compared with the D2 A131S and D2 wt enzymes (Fig. 3). In more detailed studies, both DTT (0.1–1 mM) and T $_4$  (1–10 nM)

**TABLE 1.** Kinetic characteristics of D2 enzymes (incubations in presence of 20 mM DTT)

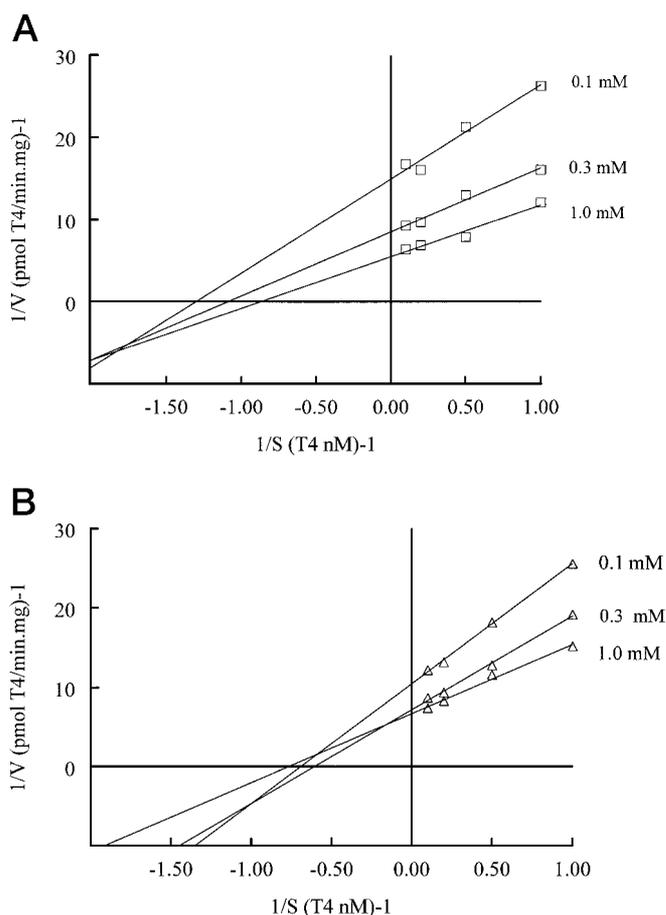
Deiodinase	$K_m$ ( $T_4$ )	$V_{max}$ ( $T_4$ ) pmol $T_4$ /min·mg	$K_m$ (r $T_3$ )	$V_{max}$ (r $T_3$ ) pmol r $T_3$ /min·mg
D2 wt	4.9 ± 0.7 nM	0.48 ± 0.12	8.9 nM	0.14
D2 A131C	4.5 ± 0.5 nM	0.25 ± 0.08	9.8 nM	0.11
D2 A131S	5.4 ± 1.1 nM	0.49 ± 0.17	10.1 nM	0.15
D2 SeC133C	5.0 μM	5.4		
D2 SeC133C/A131C	6.6 μM	4.1		

**FIG. 3.** Lineweaver-Burk plots of  $T_4$  deiodination catalyzed by wt D2, D2 A131C, or D2 A131S enzymes at a single concentration of  $T_4$  (2 nM) in the presence of varying concentrations of DTT (0.1–30 mM).

concentrations were varied. The Lineweaver-Burk plots were derived at each DTT concentration for the wt D2 and D2 A131C enzyme (Fig. 4, A and B, respectively). The plots consisted of a set of intersecting lines, implying that the sequential mechanism of deiodination is not altered by the introduction of a Cys residue in the catalytic center. A replot of the reciprocal of the apparent  $V_{max}$  values obtained against the reciprocal of the DTT concentrations yielded the limiting  $K_m$  for DTT. The limiting  $K_m$  DTT for the D2 A131C enzyme was 3-fold lower than that of the wt D2 enzyme (0.10 mM ± 0.03 *vs.* 0.37 mM ± 0.15, respectively, mean ± SEM,  $n = 3$ ). The lower limiting  $K_m$  DTT of the D2 A131C enzyme would predict a higher  $V_{max}$  compared with wt D2 enzyme at a DTT concentration of 0.1 mM, provided that the expression levels are equal (Fig. 5). Indeed, under these conditions the  $V_{max}$  of the D2 A131C enzyme was higher compared with the wt D2 enzyme (0.10 pmol/min·mg *vs.* 0.06 pmol/min·mg), whereas the  $K_m$  for  $T_4$  was similar (0.7 nM).

The cofactor dependence of the SeC133Cys D2 enzyme was also investigated. At a  $T_4$  concentration of 2 μM, the deiodination rate of the D2 SeC133C and D2 SeC133C/A131C enzymes increased with increasing DTT concentration and reached an optimum at 3 mM DTT (not shown). The deiodination rate of the D2 SeC133C/A131C enzyme was similar at the optimal DTT concentration to that of the D2 SeC133C enzyme. Therefore, the increased deiodination rate of the A131C mutant at low DTT concentrations is only detectable for D2 proteins with a SeC residue in the catalytic center.

DTT is a potent but nonphysiological reducing cofactor for D2 (8, 9). The monothiol glutathione (GSH) might be a physiological cofactor for D2. However, neither the wt D2, the D2

**FIG. 4.** Double-reciprocal plots of the rate of  $T_4$  deiodination by wt D2 (A) or D2 A131C (B) enzyme as a function of  $T_4$  concentration at 0.1 mM DTT, 0.3 mM DTT, or 1.0 mM DTT. Each data point is the average of closely agreeing duplicate determinations, and the experiment was performed twice with similar results.

A131C, nor the D2 A131S enzymes are activated by GSH (range 0.1–25 mM tested) *in vitro* (not shown).

To compare the PTU sensitivity of the wt D2, D2 A131C, and D2 A131S enzymes, the deiodination of  $T_4$  was studied in the presence of different concentrations of the reducing cofactor DTT because it has been shown that PTU inhibits D2 activity in brown adipose tissue homogenates at low DTT (<5 mM DTT) concentrations (9). In the presence of 20 mM DTT, no significant inhibition by PTU was observed (Fig. 6A). However, in the presence of 0.2 mM DTT the enzyme activity was about 70% inhibited by 1 mM PTU for the wt D2, D2 A131C, and D2 A131S enzymes (Fig. 6A). In the presence of 0.2 mM DTT, the wt D2 and D2 A131S enzymes are about 40% as active as in the presence of 20 mM

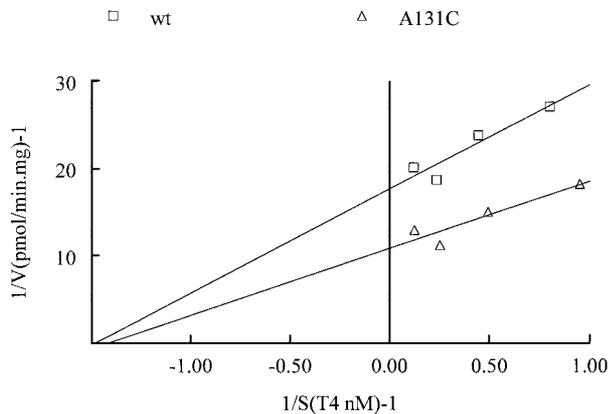


FIG. 5. Lineweaver-Burk plots of  $T_4$  deiodination catalyzed by wt D2 or D2 A131C enzymes at a single concentration of DTT (0.1 mM) in the presence of different  $T_4$  concentrations.

DTT, whereas the D2 A131C enzyme is about 70% as active as in the presence of 20 mM DTT. We have not investigated the inhibitory mechanism but it is unlikely that the inhibition is caused by a reaction of (oxidized) PTU with DTT because even at a PTU concentration of 2 mM, the inhibition was not complete.

Apart from PTU, GTG and IAc were also tested. GTG is a noncompetitive inhibitor of native D2 protein (16, 38). No significant change in sensitivity for GTG (Fig. 6B) of the D2 A131C and D2 A131S enzymes in comparison with wt D2 could be detected ( $IC_{50} \sim 0.7 \mu M$ ). IAc is a weak inhibitor of native D2 ( $IC_{50} = 1000 \mu M$ ) but a strong inhibitor of native D1 ( $IC_{50} = 2 \mu M$ ), which has a Cys in the catalytic center (21, 39). However, the sensitivity of D2 for IAc was not increased by the introduction of a Cys residue in the catalytic center. For both the wt D2 and D2 A131C enzymes, the  $IC_{50}$  was greater than 1000  $\mu M$  (not shown).

*Wild-type D2, D2 A131C, and D2 A131S proteins are not covalently labeled by N-bromoacetyl- $^{125}I$ T $_3$  or N-bromoacetyl- $^{125}I$ T $_4$*

When homogenates from COS cells expressing wt D2, D2 A131C, or D2 A131S protein were incubated with BrAc $^{125}I$ T $_3$  or BrAc $^{125}I$ T $_4$ , the labeling patterns obtained after SDS-PAGE were identical with those obtained using homogenates of nontransfected COS cells (not shown). Under identical experimental conditions, labeling patterns of homogenates from COS cells expressing rat D1 protein showed an additional labeled band of 29 kDa corresponding to the D1 protein (not shown). Analysis by SDS-PAGE of labeling patterns from *in situ* labeled COS cells revealed labeling of the rat D1 protein (>5 d autoradiography), but no labeling of wt D2 or D2 A131C protein. More than 90% of the added radioactivity was recovered in the medium, and by HPLC analysis it was found that almost all BrAc $^{125}I$ T $_3$  was converted to more polar compound(s). This conversion was not further investigated, but it may reflect reaction with free amino acids or GSH.

Despite the fact that BrAcT $_4$  does not bind covalently to D2 protein, it does inhibit  $T_4$  deiodination of wt D2, D2 A131C, and D2 A131S enzymes (Fig. 6C). The inhibition is complete at 100 nM BrAcT $_4$ , and the  $IC_{50}$  is about 0.5 nM.

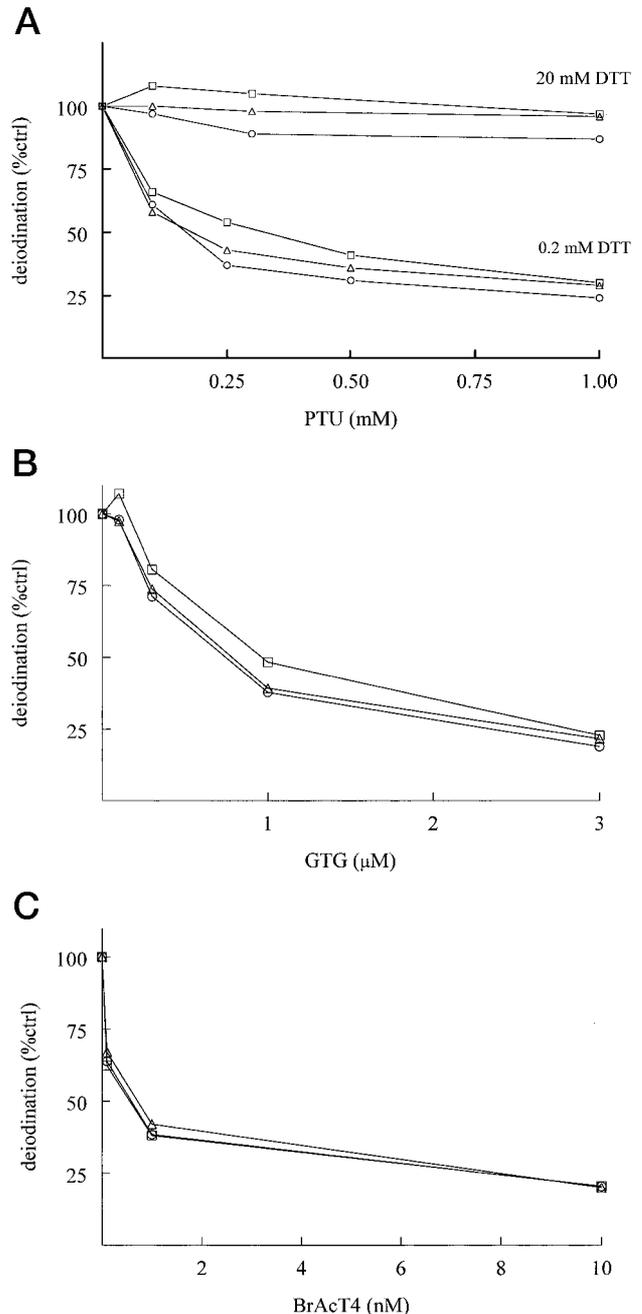


FIG. 6. Effect of PTU, GTG, or BrAcT $_4$  on  $T_4$  deiodination by wt D2 ( $\square$ ), D2 A131C ( $\triangle$ ) or D2 A131S ( $\circ$ ) enzymes. A, Inhibition of  $T_4$  (2 nM) deiodination by wt D2, D2 A131C or D2 A131S enzymes by PTU in the presence of 0.2 mM DTT, but in the presence of 20 mM DTT the wt D2, D2 A131C or D2 A131S enzymes are insensitive to PTU. B, Inhibition of  $T_4$  (2 nM) deiodination by wt D2, D2 A131C or D2 A131S enzymes by GTG. C, Inhibition of  $T_4$  (2 nM) deiodination by wt D2, D2 A131C, or D2 A131S enzymes by BrAcT $_4$ .

*Deiodination by wild-type D2, Ala131Cys, and Ala131Ser D2 mutants in intact cells*

The D2 A131C and D2 A131S enzymes are active *in vitro* in the presence of the cofactor DTT. An important question is if the same is true under *in situ* conditions using intact transfected cells, in which the availability of  $T_4$  and/or cofactor activity may be more restricted.

When wt D2 was expressed in COS cells, addition of increasing amounts of unlabeled  $T_4$  resulted in the saturation of  $[^{125}I]T_4$  deiodination, as reflected in a decrease of  $[^{125}I]T_3$  release into the medium (Fig. 7). Saturation first appeared at medium total  $T_4$  concentrations between 1 and 10 nM (the radiolabeled  $T_4$  contributes about 0.5 nM). In control experiments using  $[^{125}I]T_4$ , it was established that nontransfected COS cells contain no deiodinase activity nor sulfotransferase activity. When the D2 A131C and D2 A131S enzymes were studied in the same manner, there was a similar progressive decrease in the fractional deiodination of  $[^{125}I]T_4$ , thus indicating that the D2 mutants are active under *in situ* conditions (Fig. 7).

### Discussion

The aim of the present study was to investigate whether the absence of a Cys residue in the catalytic center of D2 protein might explain the reduced sensitivity to PTU and/or the inability to covalently label D2 with BrAc-iodothyronine derivatives. Moreover, we wanted to investigate whether the D2 A131C enzyme would still be active *in vivo* and *in vitro*.

Indeed, the D2 A131C protein is enzymatically active. The most striking finding was the reduced limiting  $K_m$  for the cosubstrate DTT of the D2 A131C enzyme, whereas the  $K_m$  values for the substrates  $T_4$  and  $rT_3$  were similar to those of wt D2. The rather low  $K_m$  for DTT of the wt D2, D2 A131C, and D2 A131S enzymes are perhaps surprising given the fact that typical D2 assays are usually done in the presence of 20 mM DTT. In many instances, D2 assays are done on microsomal fractions, and under those conditions the limiting  $K_m$  for DTT is greater than 10 mM (1, 8). Using brown adipose tissue homogenates, Goswami *et al.* (9) reported a limiting  $K_m$  DTT of 0.5 mM for D2, which is in line with our results using COS cell homogenates. The  $K_m$  values for  $T_4$  and  $rT_3$  (5 nM and 9 nM, respectively) determined in the presence of 20 mM DTT are in agreement with previously reported values using homogenates of transfected cells (10, 16, 25). In the presence of 0.1 mM DTT, the  $K_m$  for  $T_4$  is reduced to 0.7 nM, which is inherent to the sequential reaction kinetics of the D2 enzyme.

The  $V_{max}$  of D2 A131C enzyme is about 2-fold lower than

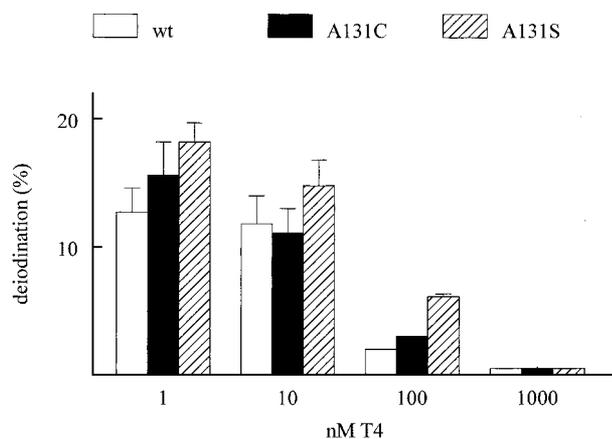


FIG. 7. *In situ* deiodination at varying  $T_4$  concentrations. COS cells were transfected with wt D2, D2 A131C, or D2 A131S expression vectors, and 24 h posttransfection intact cell deiodination was determined as described in *Materials and Methods*.

that of wt D2 and D2 A131S enzymes, when tested in the presence of 20 mM DTT (Table 1). We performed semiquantitative Western blotting of COS cell homogenates with polyclonal antisera directed against the C terminus or the catalytic center of rat D2 protein (37). Unfortunately, only in homogenates containing D2 SeC133C or D2 SeC133C/A131C protein, a specific band with the expected molecular mass (31 kDa) could be detected (not shown). It was shown before, using different antisera, that the expression level of the human D2 SeC133C protein is about 100-fold higher than that of wt D2 protein in transfected cells (38). The wt D2 and D2 A131C protein levels in COS cell homogenates are too low and/or the cross-reactivity of the rat D2 antisera with human D2 protein is not strong enough. Consequently, we cannot answer the question whether the 2-fold lower  $V_{max}$  of the D2 A131C enzyme is due to reduced protein levels or reduced catalytic efficiency in the presence of 20 mM DTT. Remarkably, when tested in the presence of 0.1 mM DTT, the  $V_{max}$  of D2 A131C enzyme is 2-fold higher compared with wt D2. It has been shown that modest changes in deiodination kinetics resulting from mutations in the catalytic center of D1 enzyme were not apparent when the same mutant constructs were tested in intact cell systems (31). On the other hand, the large (1,000-fold) increase in the  $K_m$  for  $T_4$  due to the replacement of SeC133 by Cys in D2 was reflected in assays with intact transfected cells (38). In our experiments, the modest increase in  $V_{max}$  of the D2 A131C enzyme compared with the wt D2 enzyme in the presence of 0.1 mM DTT was not reflected in the intact cell system. Limitations in the rate of cellular  $T_4$  uptake or endogenous cofactor availability could restrict the rate of deiodination and thus negate a difference in the activities of the D2 enzymes. It is clear, however, that introduction of a Cys residue in the catalytic center of D2 protein does not impair catalytic activity *in vitro* and *in situ*.

The D1 protein is strongly inhibited by PTU ( $IC_{50} = 5 \mu M$ ), GTG ( $IC_{50} = 0.05 \mu M$ ), and IAC ( $IC_{50} = 2 \mu M$ ), and this was initially explained by the presence of a reactive SH group in the enzyme-active center (6–8). When D1 protein was identified as a selenoprotein, it was proposed that the SeC residue reacts in the reduced (SeH) form with IAC and GTG, whereas the selenenyl iodide (SeI) intermediate is the target for inhibition by PTU (19, 39). The D2 enzyme also contains a SeC residue in the catalytic center but is much less sensitive to inhibition by PTU ( $IC_{50} > 1000 \mu M$ ), GTG ( $IC_{50} = 1 \mu M$ ) and IAC ( $IC_{50} = 1,000 \mu M$ ) when tested in the presence of 20 mM DTT. Only when tested in the presence of 0.2 mM DTT, the D2 enzyme becomes more sensitive to PTU. Introduction of a cysteine residue in the catalytic center (D2 A131C), as is the case in D1, did not significantly increase the sensitivity of D2 for PTU, GTG, or IAC. This suggests that the wt D2 and D2 A131C enzymes use a different mechanism of deiodination than D1. This conclusion is supported further by the fact that both the wt and D2 A131C enzymes appear to follow sequential reaction kinetics, whereas D1 follows ping-pong kinetics (8, 11, 19, 39).

Remarkably, substituting Cys for SeCys133 caused a 1,000-fold higher  $K_m$  for the substrate  $T_4$  (Table 1), in line with previously published data (38). The same holds true for the double Cys mutant (D2 SeC133C/A131C) protein. In con-

trast, substitution of Cys for SeCys in D1 caused a modest 5- to 10-fold increase in the  $K_m$  for ORD of  $T_4$  and  $T_3$  (20).

We propose a model in which the SeC residue in D2 exerts a nucleophilic attack to the 2'-position of  $T_4$ , thus forming a bond between the SeC residue and  $T_4$  (Fig. 8). Subsequent steps involve the abstraction of iodonium ( $I^+$ ) by DTT (or another thiol group containing cofactor), providing a D2· $T_3$  complex and a cofactor sulfenyl iodide. The latter will rapidly disproportionate to iodide ( $I^-$ ) and oxidized thiol, whereas the enzyme intermediate will yield  $T_3$  and the unmodified enzyme. The reaction as a whole requires two thiol groups, which could be supplied by one DTT molecule. In this model, the activated substrate is directly reduced by the cofactor, whereas in the case of D1 the SeI intermediate is reduced by cofactor (6, 8, 11, 21). In the case of the D2 A131C enzyme, an additional pathway is possible in which the iodonium is transferred to the SH group of Cys 131, followed by reduction of the SI intermediate by DTT.

This model of reductive dehalogenation has been described for the reductive dehalogenation of 5-bromo- and 5-iodo-2'-deoxyuridylylate by thymidylate synthetase (40–42) and was, among other models, discussed for the ORD of  $T_4$  (43). In thymidylate synthetase, Cys residue 198 attacks the C6 position of the pyrimidine in bromo-deoxyuridine monophosphate, and the  $K_m$  for this reductive dehalogenation reaction is about 26  $\mu M$  (42). The D2 SeC133C mutant enzyme is active, suggesting that also in D2 a correctly positioned Cys residue is able to attack the substrate. However, the SH group is a much weaker nucleophile than the SeH group, thus explaining the 1,000-fold increased  $K_m$  value and the 10-fold reduced turnover number. Also in other seleno-enzymes, as for instance thioredoxin reductase and formate dehydrogenase, exchanging Se for S reduces the catalytic turnover number up to 100-fold (44, 45). Interestingly, addition of selenocystine and thioredoxin partially restored the strongly reduced peroxidase activity of the SeC498C mutant thioredoxin reductase enzyme (44). However, addition of seleno-

cystine did not further increase the deiodination rate of  $T_4$  by the D2 SeC133C protein.

In D1, the SeI intermediate is the target for inhibition by PTU (6, 12, 19, 39, 43). According to our model, such an intermediate does not exist in D2, thus explaining the insensitivity for PTU of D2. Only under suboptimal conditions causing reduced deiodination rates, *i.e.* at low (0.2 mM DTT) reducing cofactor concentrations, the wt D2 and D2 mutant enzymes become sensitive to PTU, although the inhibition is never complete. Under these suboptimal conditions (oxidized) PTU might compete with substrate for reaction with the selenol group. Obviously, this is less favored than reaction with the selenenyl iodide intermediate in D1 explaining the much higher concentrations of PTU needed. The proposed model also explains why the D2 A131C mutant enzyme is not more sensitive to PTU because the D2 A131C enzyme has in fact a higher deiodination rate at low DTT concentrations.

The PTU sensitivity of D2 enzyme at low (and perhaps physiologically more relevant) thiol cofactor concentrations as demonstrated in this and previous studies (9, 46), poses questions with regard to the extent to which D1 and D2 are inhibited *in vivo* by PTU. Certainly because D1 inhibition by PTU is dependent on turnover (formation of SeI intermediate), which might be limited at physiological  $T_4$  concentrations. On the other hand, PTU is taken up in organs that express D1 (liver, kidney), whereas it is not or poorly taken up in several D2-expressing tissues such as brain and brown adipose tissue (46). In line with this, kinetic analysis of circulating  $T_3$  in PTU-blocked rats has suggested significant  $T_3$  production from  $T_4$  by D2 enzyme (47).

The proposed catalytic model might form the basis for the design of transition state analogs that specifically inhibit D2. For instance, brominated analogs of  $T_4$  may still bind to D2 and react with the selenol group but may be blocked in bromonium abstraction. Such analogs would permanently block the catalytic site and could in fact also be used as D2 affinity labels.

A bit puzzling still is the low susceptibility of D2 to IAc and GTG. It has been suggested that the reactivity of the selenol group in D2 is much less than that in D1 (39), implying a lower turnover number of D2 enzyme. At the moment, it is not possible to investigate this hypothesis because we have no way to determine the D2 enzyme turnover number. The inhibition by GTG of D2 is noncompetitive with substrate, whereas it is competitive with substrate for D1 (22, 38). The interaction with GTG might therefore occur with a Cys residue outside the catalytic center of D2. An alternative explanation may be that in D1 the modification of the selenol group by GTG or IAc is irreversible, whereas in D2 it is reversible, at least for IAc, because  $T_4$  also forms a covalent complex with the selenol group.

A remarkable difference between D1 and D2 protein is the fact that while *N*-bromoacetyl derivatives of  $T_4$  and  $T_3$  are excellent affinity labels of D1, they do not label D2 protein (13–16). In principle, not only SeC but also cysteine, tyrosine, histidine, lysine, and arginine residues may be modified by bromoacetyl reagents (48). After introduction of a Cys residue in the catalytic center of D2, as in D2 A131C, it is still not possible to obtain affinity labeling with BrAc $T_4$  or

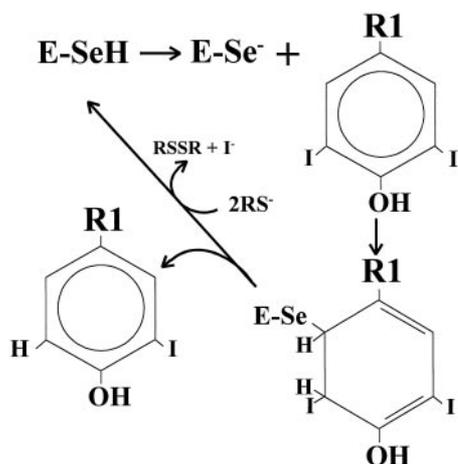


FIG. 8. Model of catalysis by D2 enzyme. We propose a model in which the SeC residue exerts a nucleophilic attack to the 2'-position of  $T_4$ , thus forming a bond between the SeC residue and  $T_4$ . This model could explain the 1,000-fold increased  $K_m$  value for  $T_4$  of the D2 SeC133C enzyme, and the low sensitivity to PTU of D2 compared with D1 enzyme.

BrAcT<sub>3</sub>. Attempts to develop an *in situ* affinity labeling system for D2 protein with BrAc-T<sub>3</sub> were also unsuccessful. These experiments were based on observations that some proteins, as for instance steroid receptors, are labeled more efficiently with affinity ligands *in situ* than in homogenates (49). Meanwhile, BrAcT<sub>4</sub> does covalently label the 29 kDa substrate-binding subunit, p29, of D2 in rat brain cells (50). The p29 protein is not a selenoprotein (51), and its relationship to the 31-kDa D2 selenodeiodinase used in this study is uncertain. The observed strong inhibition by BrAcT<sub>4</sub> of wt D2 and mutant D2 enzymes is in line with previous observations that BrAcT<sub>3</sub> inhibits D2 activity (52) and that alanine side chain modified analogs of T<sub>4</sub> inhibit D2 activity in glial cell sonicates (53).

It has been known for long that D2 activity is rapidly down-regulated by T<sub>4</sub> via a posttranscriptional mechanism that increases the rate of degradation or inactivation of the enzyme (53–58). It was recently proposed that this substrate-induced loss of D2 activity is due to proteasomal degradation of the enzyme and requires substrate interaction with the catalytic center (58). In other words, the catalytic center must be modified in some way by substrate in order for the ubiquitination machinery to be able to recognize D2 protein. The intermediate complexes formed between the SeC residue and the substrates T<sub>4</sub> or rT<sub>3</sub> proposed in our catalytic model, may fulfill that role.

### Acknowledgments

We thank Hans van Toor for synthesis of radiolabeled iodothyronines and Ronald van der Wal for DNA-sequencing. We are grateful to Drs. Jack Leonard and Alan Farwell (University of Massachusetts Medical School, Worcester, MA) for providing us with D2 antisera. The wild-type hD2 clone was kindly provided by Dr. Pascal Soularue (Laboratoire Genethon, Evry, France).

Received September 24, 2001. Accepted December 14, 2001.

Address all correspondence and requests for reprints to: Theo J. Visser, Ph.D., Department of Internal Medicine, Room Bd 234, Erasmus Medical Center, Dr. Molewaterplein 40, 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 (Grant 903-40-194) and the Quality of Life research program of the European Union (Contract QLG3-2000-00930).

### References

- Köhrle J 1999 Local activation and inactivation of thyroid hormones: the deiodinase family. *Mol Cell Endocrinol* 151:103–119
- Larsen PR 1997 Update on the human iodothyronine selenodeiodinases, the enzymes regulating the activation and inactivation of thyroid hormone. *Biochem Soc Trans* 25:588–592
- St Germain DL, Galton VA 1997 The deiodinase family of selenoproteins. *Thyroid* 7:655–668
- Berry MJ, Banu L, Larsen PR 1991 Type I iodothyronine deiodinase is a selenocysteine containing enzyme. *Nature* 349:438–440
- Moreno M, Berry MJ, Horst C, Thoma R, Caglia F, Harney JW, Larsen PR, Visser TJ 1994 Activation and inactivation of thyroid hormone by type I iodothyronine deiodinase. *FEBS Lett* 344:143–146
- Visser TJ, van Overmeeren-Kaptein E 1981 Substrate requirement for inactivation of iodothyronine 5'-deiodinase activity by thiouracil. *Biochim Biophys Acta* 658:202–208
- Leonard JL, Rosenberg IN 1980 Characterization of essential enzyme sulfhydryl groups on thyroxine 5'-deiodinase from rat kidney. *Endocrinology* 106:444–451
- Visser TJ, Leonard JL, Kaplan M, Larsen PR 1982 Kinetic evidence suggesting two mechanisms for iodothyronine 5'-deiodination in rat cerebral cortex. *Proc Natl Acad Sci USA* 79:5080–5084
- Goswami A, Rosenberg IN 1986 Iodothyronine 5'-deiodinase in brown adipose tissue: thiol activation and propylthiouracil inhibition. *Endocrinology* 119:916–923
- Croteau W, Davey JC, Galton VA, St Germain DL 1996 Cloning of the mammalian type II iodothyronine deiodinase. *J Clin Invest* 98:405–417
- Sun BC, Harney JW, Berry MJ, Larsen PR 1997 The role of the active site cysteine in catalysis by type I iodothyronine deiodinase. *Endocrinology* 138:5452–5458
- du Mont W-W, Mugesh G, Wismach C, Jones PG 2001 Reactions of organoselenenyl iodides with thiourea drugs: an enzyme mimetic study on the inhibition of iodothyronine deiodinase. *Angew Chem Int Ed Engl* 40:2486–2489
- Mol JA, Docter R, Kaptein E, Jansen G, Hennemann G, Visser TJ 1984 Inactivation and affinity labeling of rat liver iodothyronine deiodinase with N-bromoacetyl-3, 3', 5-triiodothyronine. *Biochem Biophys Res Commun* 124:475–483
- Schoenmakers CHH, Pigmans IGJ, Visser TJ 1992 Species differences in rat liver type I iodothyronine deiodinase. *Biochim Biophys Acta* 1121:160–166
- Köhrle J, Rasmussen UB, Ekenbarger DB, Alex S, Rokos H, Hesch RD, Leonard JL 1990 Affinity labeling of rat liver and kidney type I 5'-deiodinase. *J Biol Chem* 265:6155–6163
- Salvatore D, Bartha T, Harney JW, Larsen PR 1996 Molecular biological and biochemical characterization of the human type 2 selenodeiodinase. *Endocrinology* 137:3308–3315
- Arthur JR, Nicol F, Beckett GJ 1990 Hepatic iodothyronine 5'-deiodinase. The role of selenium. *Biochem J* 272:537–540
- Behne D, Kyriakopoulos A, Meinhold H, Köhrle J 1990 Identification of type I iodothyronine 5'-deiodinase as a selenoenzyme. *Biochem Biophys Res Commun* 173:1143–1149
- Berry MJ, Kieffer JD, Harney JW, Larsen PR 1991 Selenocysteine confers the biochemical properties characteristic of the type I iodothyronine deiodinase. *J Biol Chem* 266:14155–14158
- Berry MJ, Maia AL, Kieffer JD, Harney JW, Larsen PR 1992 Substitution of cysteine for selenocysteine in type I iodothyronine deiodinase reduces the catalytic efficiency of the protein but enhances its translation. *Endocrinology* 131:1848–1852
- Visser TJ, Frank S, Leonard JL 1983 Differential sensitivity of brain iodothyronine 5'-deiodinases to sulfhydryl-blocking reagents. *Mol Cell Endocrinol* 33:321–327
- Berry MJ, Kieffer JD, Larsen PR 1991 Evidence that cysteine, not selenocysteine, is in the catalytic site of type II iodothyronine deiodinase. *Endocrinology* 129:550–552
- Safran M, Farwell AP, Leonard JL 1991 Evidence that type II 5'-deiodinase is not a selenoprotein. *J Biol Chem* 266:13477–13480
- Salvatore D, Harney JW, Larsen PR 1999 Mutation of the SeCys residue 266 in human type II selenodeiodinase alters <sup>75</sup>Se incorporation without affecting its biochemical properties. *Biochimie (Paris)* 81:535–538
- Gereben B, Bartha T, Tu HM, Harney JW, Rudas P, Larsen PR 1999 Cloning and expression of the chicken type 2 iodothyronine 5'-deiodinase. *J Biol Chem* 274:13768–13776
- 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
- Buettner C, Harney JW, Larsen PR 1998 The 3'-untranslated region of human type 2 iodothyronine deiodinase mRNA contains a functional selenocysteine insertion sequence element. *J Biol Chem* 273:33374–33378
- Valverde C, Croteau W, Lafleur GJ, Orozco A, St Germain D 1997 Cloning and expression of a 5'-iodothyronine deiodinase from the liver of *Fundulus heteroclitus*. *Endocrinology* 138:642–648
- Sambroni E, Gutierrez S, Cauty C, Guiguen Y, Breton B, Lareyre J-J 2001 Type II iodothyronine deiodinase is preferentially expressed in rainbow trout (*Oncorhynchus mykiss*) liver and gonads. *Mol Reprod Dev* 60:338–350
- Curcio C, Baqui MMA, Salvatore D, Rihn BH, Mohr S, Harney JW, Larsen PR, Bianco AC 2001 The human type 2 iodothyronine deiodinase is a selenoprotein highly expressed in a mesothelioma cell line. *J Biol Chem* 276:30183–30187
- Croteau W, Bodwell JE, Richardson JM, St Germain DL 1998 Conserved cysteines in the type I deiodinase selenoprotein are not essential for catalytic activity. *J Biol Chem* 273:25230–25236
- Visser TJ, Docter R, Hennemann G 1977 Radioimmunoassay of reverse triiodothyronine. *J Endocrinol* 73:395–399
- Eelkman Rooda SJ, van Loon MAC, Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. *J Clin Invest* 79:1740–1748
- Pogulis RJ, Vallejo AN, Pease LR 1997 *In vitro* recombination and mutagenesis by overlap extension PCR. In: Trower MK, ed. *In vitro* mutagenesis protocols, methods in molecular biology. New Jersey: Humana Press; 57:167–176
- Sussman DJ, Milman G 1984 Short-term, high-efficiency expression of transfected DNA. *Mol Cell Biol* 4:1641–1643
- Goldberg GS, Lau AF 1993 Transfection of mammalian cells with PEG-purified plasmid DNA. *Biotechniques* 14:548–550
- Leonard JL, Leonard DM, Safran M, Wu R, Zapp ML, Farwell AP 1999 The mammalian homolog of the frog type II selenodeiodinase does not encode a functional enzyme in the rat. *Endocrinology* 140:2206–2215

38. **Buettner C, Harney JW, Larsen PR** 2000 The role of selenocysteine 133 in catalysis by the human type 2 iodothyronine deiodinase. *Endocrinology* 141:4606–4612
39. **Hennemann G, Visser TJ** 1997 Thyroid hormone synthesis, plasma membrane transport and metabolism. *Handb Exp Pharmacol* 128:76–117
40. **Wataya Y, Santi DV** 1975 Thymidylate synthetase catalyzed dehalogenation of 5-bromo- and 5-iodo-2'-deoxyuridylate. *Biochem Biophys Res Commun* 67:818–823
41. **Garrett C, Wataya Y, Santi DV** 1979 Thymidylate synthetase: catalysis of dehalogenation of 5-bromo- and 5-iodo-2'-deoxyuridylate. *Biochemistry* 18:2798–2804
42. **Kealey JT, Eckstein J, Santi DV** 1995 Role of the conserved tryptophan 82 of *Lactobacillus casei* thymidylate synthetase. *Chem Biol* 2:609–614
43. **Visser TJ** 1979 Mechanism of action of iodothyronine-5'-deiodinase. *Biochim Biophys Acta* 569:302–308
44. **Zhong L, Holmgren A** 2000 Essential role of selenium in the catalytic activities of mammalian thioredoxin reductase revealed by characterization of recombinant enzymes with selenocysteine mutations. *J Biol Chem* 275:18121–18128
45. **Stadtman TC** 1996 Selenocysteine. *Annu Rev Biochem* 65:83–100
46. **Goswami A, Rosenberg IN** 1990 Regulation of iodothyronine 5'-deiodinases: effects of thiol blockers and altered substrate levels *in vivo* and *in vitro*. *Endocrinology* 126:2597–2606
47. **Nguyen TT, Chapa F, DiStefano III JJ** 1998 Direct measurement of the contributions of type I and type II 5'-deiodinases to whole body steady state 3,5,3'-triiodothyronine production from thyroxine in the rat. *Endocrinology* 139:4626–4633
48. **Cheng SY, Wilchek M, Cahnmann HJ, Robbins J** 1977 Affinity labeling of human serum prealbumin with *N*-bromoacetylthyroxine. *J Biol Chem* 252:6076–6081
49. **Brinkmann AO, Kuiper GGJM, Bolt-de Vries J, Mulder E** 1988 *In situ* photolabeling of the human androgen receptor. *J Steroid Biochem* 30:257–261
50. **Farwell AP, Leonard JL** 1989 Identification of a 27 kDa protein with the properties of type II iodothyronine 5'-deiodinase in dibutylryl cyclic AMP-stimulated glial cells. *J Biol Chem* 264:20561–20567
51. **Leonard DM, Stachelek SJ, Safran M, Farwell AP, Kowalik TF, Leonard JL** 2000 Cloning, expression and functional characterization of the substrate binding subunit of rat type II iodothyronine 5'-deiodinase. *J Biol Chem* 275:25194–25201
52. **Salvatore D, Tu H, Harney JW, Larsen PR** 1996 Type II iodothyronine deiodinase is highly expressed in human thyroid. *J Clin Invest* 98:962–968
53. **Safran M, Farwell AP, Rokos H, Leonard JL** 1993 Structural requirements of iodothyronines for the rapid inactivation and internalization of type II iodothyronine 5'-deiodinase in glial cells. *J Biol Chem* 268:14224–14229
54. **Leonard JL, Silva JE, Kaplan MM, Mellen SA, Visser TJ, Larsen PR** 1984 Acute posttranslational regulation of cerebrocortical and pituitary iodothyronine 5'-deiodinases by thyroid hormone. *Endocrinology* 114:998–1004
55. **Silva JE, Leonard JL** 1985 Regulation of rat cerebrocortical and adenohipophyseal type II 5'-deiodinase by thyroxine, triiodothyronine, and reverse triiodothyronine. *Endocrinology* 116:1627–1636
56. **St Germain DL** 1988 The effects and interactions of substrates, inhibitors and the cellular thiol-disulfide balance on the regulation of type II iodothyronine 5'-deiodinase. *Endocrinology* 122:1860–1868
57. **Halperin Y, Shapiro LE, Surks MI** 1994 Down-regulation of type II 5'-deiodinase in cultured GC cells: different pathways of regulation by L-triiodothyronine and 3,3',5'-triiodothyronine. *Endocrinology* 135:1464–1469
58. **Steinsapir J, Bianco AC, Buettner C, Harney J, Larsen PR** 2000 Substrate-induced down-regulation of human type 2 deiodinase is mediated through proteasomal degradation and requires interaction with the enzyme's active center. *Endocrinology* 141:1127–1135