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

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Human type II iodothyronine deiodinase (D2) catalyzes the activation of T_4 to T_3. 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_4 (5 nM) and reverse T_3 (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_{50}) > 2 mM] in the presence of 20 mM DTT, but when tested in the presence of 0.2 mM DTT the IC_{50} 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_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. Saturation first appeared at medium T_4 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_4, THE MAIN secretory product of the thyroid gland, undergoes enzymatic outer ring deiodination (ORD) in peripheral tissues to T_3 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_m) for T_4 (~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_m for T_4 (~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_4 by D1 protein exhibits ping-pong (bi)-substrate kinetics with T_4 and the thiol-containing cofactor as cosubstrates (6, 8, 11, 12). The D2 protein exhibits sequential reaction kinetics, suggesting that T_4 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_4 or T_3 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 sulfydryl 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 ^{75}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 ^{75}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_m, Michaelis-Menten constant; ORD, outer ring deiodination; PTU, propylthiouracil; rT_3, reverse T_3; SeC, selenocysteine; SECIS, SeC insertion sequence elements; SeI, 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′-C*AAGCTT*GCCACCATGGGCATCCTCAGCGTA) or a stop 3′-outer primer (5′-C*AAGCTT*TTAACCAGCTAATCTAGT), each containing flanking *Hin*dIII 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 *Hin*dIII, 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 *Hin*dIII, 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 μg per dish), isolated by alkaline lysis and polyethyleneglycol precipitation (36), were added to 5 ml serum-free DMEM/F12 medium containing 200 μg/ml diethylaminoethyl-dextran. After 2 h, the medium was replaced by serum-free DMEM/F12 containing 100 μM 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 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 μg 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 μM), and $BrAcT_4$ (0.1–1000 nM) were included. Mixtures were incubated for 60 min at 37 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 γ-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 μg 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 Na_2SeO_3), to which was added [^{125}I]T_4 (1×10^6 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 × 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}I]T_3 produced (multiplied by two), divided by the amount of [^{125}I]T_4 added.

Incubation with N-bromoacetyl-[^{125}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}I]T_3 or BrAc[^{125}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 μl P100E2D3 [100 mM sodium phosphate; 2 mM EDTA; 3 mM DTT, pH 7.2], and vortexing, the COS cell homogenates (150 μg protein) were added in a total volume of 50 μl 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 than cultured for an additional 24 h in serum-free DMEM/F12, to which was added BrAc[^{125}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 μM selenocystine (which is converted to selenocysteine in the presence of DTT) did not further increase the deiodination rate of the D2 SeC133C enzyme (not shown).

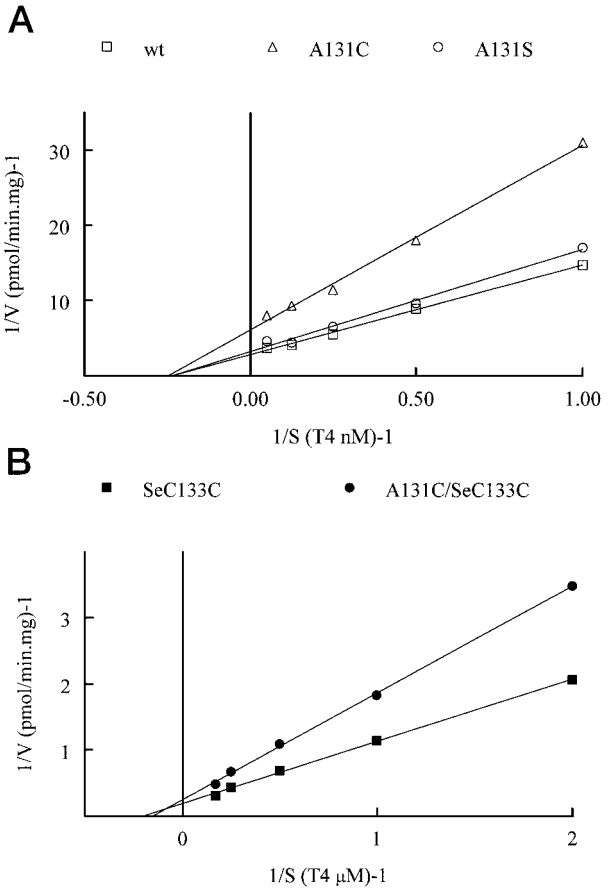

FIG. 2. Lineweaver-Burk plots of T_4 deiodination catalyzed by wt D2, D2 A131C, D2 A131S enzymes (A) and by D2 SeC133C or D2 SeC133C/A131C enzyme (B). Each data point is the average of closely agreeing duplicate determinations, and the experiment was performed twice on different homogenates with similar results.

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 (rT_3)	V_{max} (rT_3) pmol rT_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		

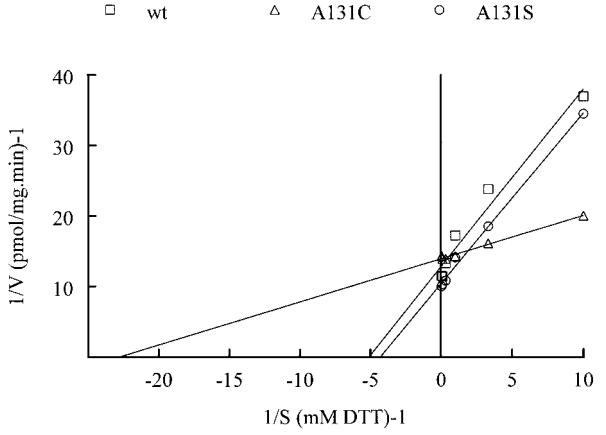

FIG. 3. Lineweaver-Burk plots of T_4 deiodination catalyzed by wt D2, D2 A131C, or D2 A131S enzymes at a single concentration of T_4 (2 nM) in the presence of varying concentrations of DTT (0.1–30 mM).

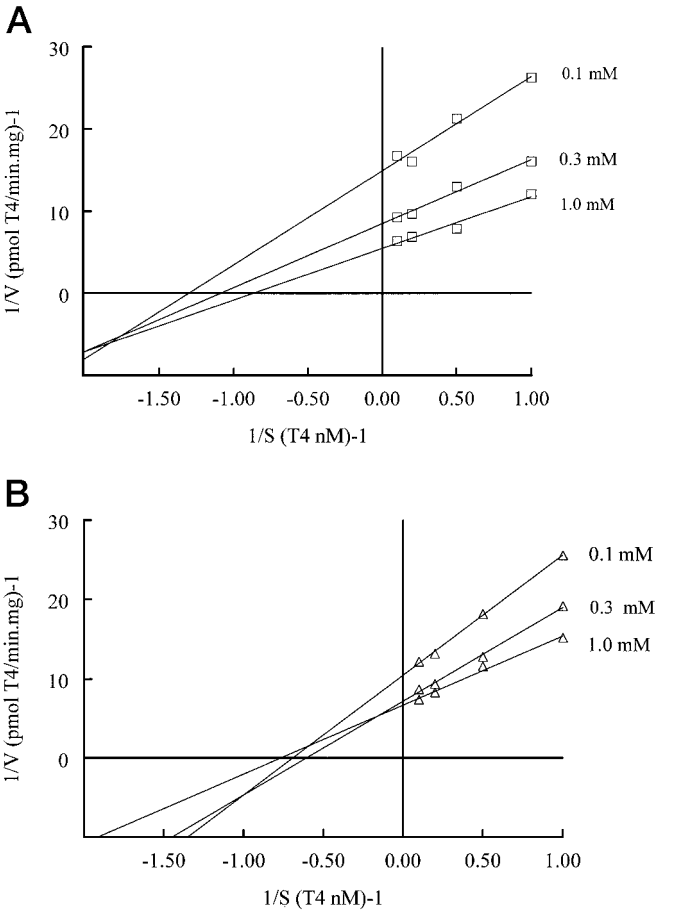

FIG. 4. Double-reciprocal plots of the rate of T_4 deiodination by wt D2 (A) or D2 A131C (B) enzyme as a function of T_4 concentration at 0.1 mM DTT, 0.3 mM DTT, or 1.0 mM DTT. Each data point is the average of closely agreeing duplicate determinations, and the experiment was performed twice with similar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

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 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

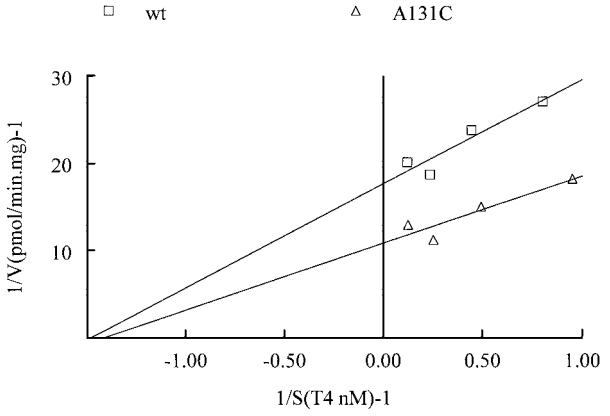

FIG. 5. Lineweaver-Burk plots of T_4 deiodination catalyzed by wt D2 or D2 A131C enzymes at a single concentration of DTT (0.1 mM) in the presence of different T_4 concentrations.

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 ($IC_{50} \sim 0.7$ μM). IAc is a weak inhibitor of native D2 ($IC_{50} = 1000$ μM) but a strong inhibitor of native D1 ($IC_{50} = 2$ μM), 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 μM (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.

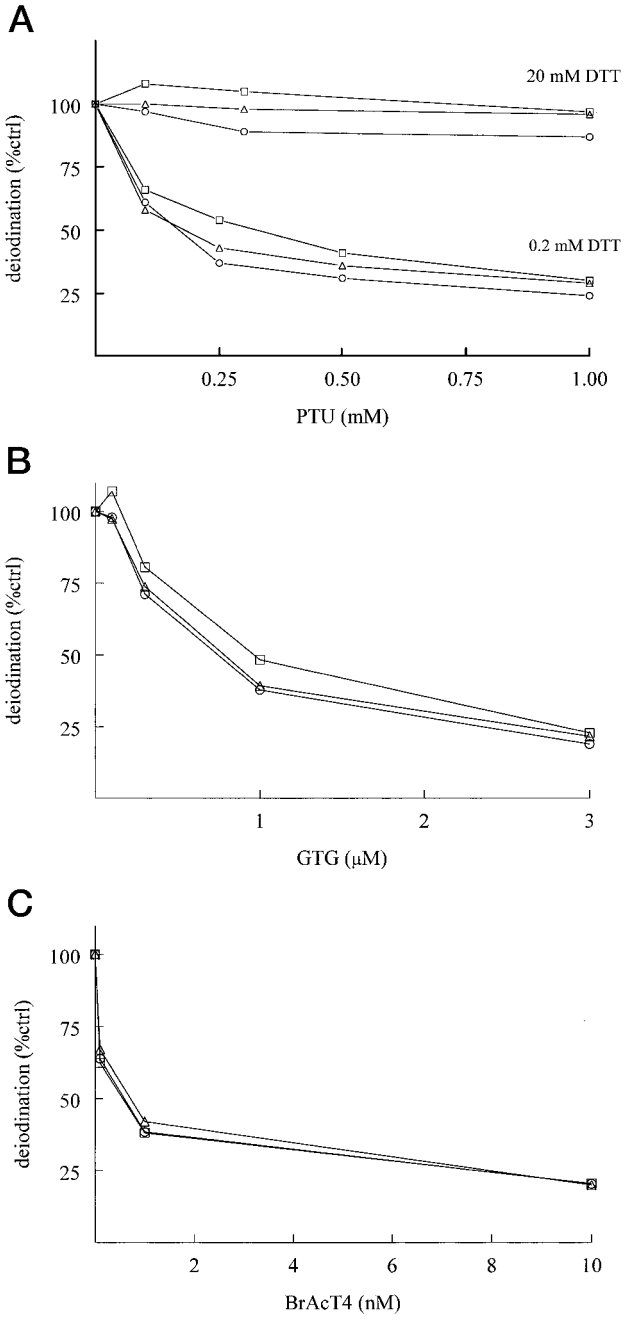

FIG. 6. Effect of PTU, GTG, or $BrAcT_4$ on T_4 deiodination by wt D2 (□), D2 A131C (△) or D2 A131S (○) enzymes. A, Inhibition of T_4 (2 nM) deiodination by wt D2, D2 A131C or D2 A131S enzymes by PTU in the presence of 0.2 mM DTT, but in the presence of 20 mM DTT the wt D2, D2 A131C or D2 A131S enzymes 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 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*.

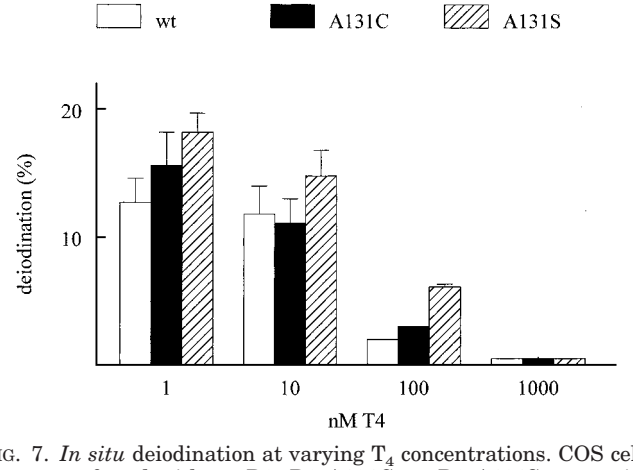

FIG. 7. *In situ* deiodination at varying T_4 concentrations. COS cells were transfected with wt D2, D2 A131C, or D2 A131S expression vectors, and 24 h posttransfection intact cell deiodination was determined as described in *Materials and Methods*.

The D1 protein is strongly inhibited by PTU (IC_{50} = 5 μM), GTG (IC_{50} = 0.05 μM), and IAc (IC_{50} = 2 μM), 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 μM), GTG (IC_{50} = 1 μM) and IAc (IC_{50} = 1,000 μM) 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 rT_3 and T_4 (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′-deoxyuridylate 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 μM (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 selenocystine did not further increase the deiodination rate of T_4 by the D2 SeC133C protein.

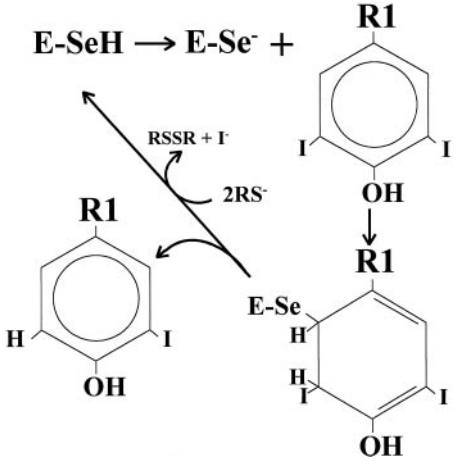

FIG. 8. Model of catalysis by D2 enzyme. We propose a model in which the SeC residue exerts a nucleophilic attack to the 2′-position of T_4, thus forming a bond between the SeC residue and T_4. This model could explain the 1,000-fold increased K_m value for T_4 of the D2 SeC133C enzyme, and the low sensitivity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

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 $BrAcT_4$ or

$BrAcT_3$. Attempts to develop an *in situ* affinity labeling system for D2 protein with BrAc-T_3 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, $BrAcT_4$ 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 $BrAcT_4$ of wt D2 and mutant D2 enzymes is in line with previous observations that $BrAcT_3$ inhibits D2 activity (52) and that alanine side chain modified analogs of T_4 inhibit D2 activity in glial cell sonicates (53).

It has been known for long that D2 activity is rapidly down-regulated by T_4 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_4 or rT_3 proposed in our catalytic model, may fulfill that role.

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