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Substitution of Cysteine for Selenocysteine in the Catalytic Center of Type III Iodothyronine Deiodinase Reduces Catalytic Efficiency and Alters Substrate Preference

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Human type III iodothyronine deiodinase (D3) catalyzes the conversion of T_4 to rT_3 and of T_3 to 3, 3′-diiodothyronine (T2) by inner-ring deiodination. Like types I and II iodothyronine deiodinases, D3 protein contains selenocysteine (SeC) in the highly conserved core catalytic center at amino acid position 144. To evaluate the contribution of SeC144 to the catalytic properties of D3 enzyme, we generated mutants in which cysteine (D3Cys) or alanine (D3Ala) replaces SeC144 (D3wt). COS cells were transfected with expression vectors encoding D3wt, D3Cys, or D3Ala protein. Kinetic analysis was performed on homogenates with dithiothreitol as reducing cofactor. The Michaelis constant of T_3 was 5-fold higher for D3Cys than for D3wt protein. In contrast, the Michaelis constant of T_4 increased 100-fold. The D3Ala protein was enzymatically inactive. Semiquantitative immunoblotting of homogenates with a D3 antiserum revealed that about 50-fold higher amounts of D3Cys and D3Ala protein are expressed relative to D3wt protein. The relative substrate turnover number of D3Cys is 2-fold reduced for T_3 and 6-fold reduced for T_4 deiodination, compared with D3wt enzyme. Studies in intact COS cells expressing D3wt or D3Cys showed that the D3Cys enzyme is also active under *in situ* conditions. In conclusion, the SeC residue in the catalytic center of D3 is essential for efficient inner-ring deiodination of T_3 and in particular T_4 at physiological substrate concentrations. (*Endocrinology* 144: 2505–2513, 2003)

T_4, THE MAIN secretory product of the thyroid gland, undergoes enzymatic outer-ring deiodination in peripheral tissues to T_3 catalyzed by the deiodinases type I (D1) and type II (D2) as well as inner-ring deiodination (IRD) to rT_3 catalyzed by the deiodinase type III (D3; Refs. 1–4). In fact, D3 is the major T_4 and T_3 inactivating enzyme by catalyzing the conversion of T_4 to rT_3 and T_3 to T_2 by IRD (1–5).

In rodents and humans, D3 activity was found in brain, skin, (failing) heart, placenta, uterus, and several fetal tissues such as liver and brain (6–18). In *Xenopus laevis* tadpoles, D3 activity is present in limb buds, retina, and tail in various stages of development (19–20). Also in various other species, such as chicken and fish, D3 activity is present in brain, liver, and skin (21–23). Recently very high levels of D3 activity were described in infantile hemangiomas causing severe hypothyroidism (24).

Thyroid hormone is a major physiological regulator of brain development. The presence of D3 in organs such as the brain is thought to play a role in the local fine-regulation of intracellular T_3 levels, whereas its presence in placenta, uterus, and fetal organs may protect fetal brain tissue against premature exposure to T_3 during early stages of development (2, 10–13, 16, 17, 25–29).

The D3 protein is a selenoprotein, containing a selenocysteine residue (SeC) in the catalytic center (30–36). Complementary DNAs from several species that code for the D3 protein have been isolated, and they contain a UGA stop codon within the open reading frame that is translated as SeC (32–36). The presence of an SeC insertion sequence element in the 3′-UTR of the mRNA is essential for SeC incorporation at the UGA codon, which otherwise would function as a stop codon (37).

The catalytic mechanism of D3 seems to be different from that of D1 but similar to that of D2 enzyme. Outer-ring deiodination of T_4 or rT_3 by D1 enzyme exhibits ping-pong (bi)substrate kinetics with T_4 or rT_3 and the thiol-containing cofactor as cosubstrates (38, 39). The D2 and D3 enzymes exhibit sequential reaction kinetics, suggesting that T_4 and the thiol-containing cofactor must interact with the enzyme simultaneously before reaction takes place (7, 18, 38, 40). The SeC residue in the catalytic center is essential for maximal catalytic efficiency of deiodinases (32–33, 40–42). In D1, mutation of SeC to leucine or alanine eliminates deiodinase activity, whereas its mutation to cysteine (Cys) raises the apparent Michaelis constant (K_m) for rT_3 3-fold and reduces the substrate-turnover number about 100-fold (41). Remarkably, in D2 mutation of SeC to Cys raises the apparent K_m for T_4 about 1000-fold, whereas the substrate-turnover number is reduced only 10-fold (40, 42). For D3 it was shown that substitution of SeC with leucine eliminates deiodinase activity, whereas substitution with Cys (D3Cys) reduced enzyme activity (32–33). However, the D3Cys protein was not further characterized with regard to substrate interaction, substrate turnover number, and catalytic mechanism.

The present studies were undertaken to elucidate the functional role of the SeC residue in the catalytic center of D3

Abbreviations: BrAcT3, *N*-Bromoacetyl-T_3; BrAc[^{125}I]T_2, *N*-bromoacetyl-T_2; BrAc[^{125}I]T_3, *N*-bromoacetyl-T_3; Cys, cysteine; D1, deiodinases type I; D2, type II; D3, type III; DTT, dithiothreitol; GTG, gold thioglucose; HEK, human embryonic kidney; IAc, iodoacetate; IRD, inner-ring deiodination; K_m, Michaelis constant; SeC, selenocysteine; V_{max}, maximal velocity.

protein in more detail. We have examined effects of the SeC144 to Cys (D3Cys) or SeC144 to Ala (D3Ala) mutation in human D3 protein on substrate and cofactor interaction as well as on the substrate turnover number.

Materials and Methods

Materials

Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany) or Calbiochem (San Diego, CA). [3′-^{125}I]T_3 (2000 mCi/μmol) and [3′,5′-^{125}I]T_4 (1200 mCi/μmol) were either obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK) or prepared by radioiodination of 3,5-T_2 and 3,3′,5-T_3, respectively, using the chloramine-T method as described (43). Before each experiment, the radiolabeled iodothyronines were purified on Sephadex LH-20 columns. Radioactively labeled *N*-bromoacetyl-T_3 (BrAc[^{125}I]T_3) and *N*-bromoacetyl-T2 (BrAc[^{125}I]T_2) were synthesized from bromoacetylchloride and [3′-^{125}I]T_3 or [3′-^{125}I]T_2 as described (44). Nonradioactive *N*-bromoacetyl-T_3 (BrAcT3) was obtained from Henning. Gold thioglucose (GTG) and iodoacetate (IAc) were obtained from Sigma (St. Louis, MO). Dithiotreitol (DTT) was obtained from ICN Biochemicals Inc. (Costa Mesa, CA). *Pyrococcus furiosus* thermostable DNA polymerase and *Dpn*I restriction endonuclease were obtained from Promega Corp. (Madison, WI). XL-10 ultracompetent *Escherichia coli* cells were obtained from Stratagene (La Jolla, CA). Synthetic oligonucleotides were ordered from Invitrogen-Life Technologies, Inc. (Glasgow, UK).

Site-directed mutagenesis

A human D3 expression vector (pcDNA3-D3) containing a 1.9-kb insert including 35 nucleotides of the 5′-untranslated region (nucleotides 185-2066 in Ref. 34) was used as template for site-directed mutagenesis via the circular mutagenesis procedure, followed by selection for mutants by *Dpn*I digestion (45). Overlapping sense and antisense primers containing the nucleotide changes needed to produce the SeC144Cys D3 mutant cDNA.

The (sense 5′ TTCGGAAGCTGCACC**TGC**CCACCGTTCATGGCG) and SeC144Ala mutant cDNA (sense 5′TTCGGAAGCTGCACC**GCA**CCACCGTTCATGGCG) were used in circular mutagenesis reactions with 50 ng plasmid template and 2 U *P. furiosus* DNA polymerase (45). The cycling protocol consisted of 30 sec at 95 C, 1 min at 55 C, 14 min at 68 C for 18 cycles using a model 480 PCR machine (Perkin-Elmer, Norwalk, CT). The reaction products were incubated with 10 U *Dpn*I enzyme for 2 h at 37 C and immediately transformed to competent *E. coli* XL-10 cells according to manufacturer's instructions. Plasmid DNA isolated from up to four clones was sequenced on a model 310 DNA sequencer (ABI, Foster City, CA) to verify that the desired mutation had been generated and no spurious mutations had occurred during amplification (46). Plasmids were maintained in *E. coli* DH5α cells and purified with QIAfilter cartridges (QIAGEN, Hilden, Germany).

During sequencing of the wt D3 cDNA, we noted that codon 53 encodes glutamine instead of lysine as previously reported (Ref. 34; GenBank accession no. S79854). A more recent GenBank entry (XM007250) also describes a glutamine residue at codon 53.

Expression of D3 protein

The wt and mutant D3 enzymes were expressed in COS-1 cells or human embryonic kidney (HEK)-293 cells (65-cm^2 dishes) after diethylaminoethyl-dextran-mediated transfection of the expression vectors (40, 47). COS-1 cells were grown in DMEM-Ham's F-12 medium containing 10% fetal calf serum (Life Technologies, Inc.) and 40 nM sodium selenite. Two days after transfection, 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 −80 C.

Assay of IRD activity in cell homogenates

This assay is based on the determination of product formation (^{125}I-labeled T_2 or rT_3) by reverse-phase HPLC analysis of reaction mixtures containing outer-ring-labeled T_3 or T_4 (1, 17).

Incubations contained about 200,000 cpm labeled T_3 or T_4 with varying amounts of unlabeled substrate (T_3 or T_4) and cell homogenate (5–10 μg protein) in a final volume of 0.1 ml P100E2 buffer (0.1 M phosphate, pH 7.2; 2 mM EDTA) and varying amounts of DTT (0.1–30 mM). In some experiments a pH profile was obtained using 0.1 M phosphate, 2 mM EDTA buffers of pH 6.0–8.0. Mixtures were incubated for 60 min at 37 C, whereafter the reaction was stopped by addition of 0.1 ml ice-cold methanol. After centrifugation, 0.1 ml was mixed with 0.1 ml 0.02 M ammonium acetate (pH 4.0), and 0.1 ml (equivalent to 25-μl reaction volume) 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 15-min linear gradient of acetonitrile (28–42%) in 0.02 M ammonium acteate (pH 4) at a flow rate of 1.2 ml/min. Radioactivity in the eluate was monitored online using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Homogenate protein was adjusted to limit substrate consumption to less than 30%. Enzymatic deiodination was corrected for nonenzymatic deiodination by determining the amount of ^{125}I-labeled T_2 or rT_3 formed in blank incubations with homogenates of nontransfected cells.

Assay of IRD activity in intact transfected COS cells

COS cells were cultured in 6-well plates (10 cm^2/well) and transfected with 0.2 or 2 μg plasmid per well as described (40). One day after transfection, cell monolayers were washed with serum-free DMEM/F12 medium and cultured for an additional 24 h in serum-free DMEM/F12 supplemented with 40 nM sodium selenite, to which was added [3′,5′-^{125}I]T_4 or [3′-^{125}I]T_3 (1 × 10^6 cpm/ml) plus 1–1000 nM unlabeled T_4 or T_3. After 20–24 h the medium was harvested and extracted with ice-cold methanol (1:1). After centrifugation, the extract was mixed with 0.02 M ammoniumacetate (pH 4), and 0.1 ml of the mixture was analyzed with the same HPLC system as described. The column was 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). This gradient provides improved separation of sulfated iodothyronines from nonsulfated iodothyronines.

Polyclonal antiserum production and Western blotting

Polyclonal antisera were raised in rabbits by Eurogentec SA (Herstal, Belgium) after conjugation of the synthetic peptide (C)RYDEQLHGARPRRV (human D3 amino acid residues 265–278) to keyhole limpet hemocyanin. Antiserum (designated 677) from the final bleed was used without further purification.

COS cell homogenates (20–30 μg protein) were separated on 12% SDS-PAGE gels in the Mini-Protean II cel (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions. After electrophoresis the gels were positioned on nitrocellulose membranes (Hybond-C pure, Amersham Pharmacia Biotech) and placed in a mini-transblot cell (Bio-Rad Laboratories, Inc.) filled with buffer (25 mM Tris-HCl, pH 8.3; 150 mM glycine; and 20% vol/vol methanol). The transfer was performed for 1 h at 100 V.

Membranes were blocked with 5% (wt/vol) nonfat milk in PBS with 0.1% Tween 20 (PBS-Tween) for 1 h at 37 C and thereafter incubated with antiserum 677 at a 1:500 dilution in 5% (wt/vol) milk in PBS-Tween overnight at 8 C. Blots were washed 2 × 15 min with 5% (wt/vol) milk in PBS-Tween and 2 × 15 min with PBS-Tween, followed by incubation with peroxidase-conjugated secondary antibody (A0545, 1:16,000, Sigma) in 5% (wt/vol) milk in PBS-Tween for 2 h. After washing as described, the blot was developed with a homemade chemiluminescence system (48) consisting of 0.7 μM p-coumaric acid, 1.25 mM luminol, and 0.01% hydrogen peroxide in 0.1 M Tris HCl (pH 8.5), and exposed to Hyperfilm ECL film (Amersham) for 5–15 min.

To compare the relative amounts of enzyme expressed between wild-type and mutant D3, the mutant enzyme was diluted in cell lysate derived from nontransfected COS cells. The density of the D3 bands was quantified by densitometry.

Affinity labeling of D1 and D3 protein with BrAc[^{125}I]T_3

BrAc[^{125}I]T_3 (1500 mCi/μmol) was synthesized as described (44), and HPLC analysis demonstrated that the purity was at least 85% with unreacted [^{125}I]T_3 as the main contaminant. Unlabeled BrAcT3 was also

analyzed by HPLC and found to be more than 90% pure. The concentration was determined by mixing with a T_3 internal standard and online monitoring of the eluates with a photodiode array detector, taking into account that the molar extinction coefficient of BrAcT3 is very close to that of T_3 in the far UV (49).

Solutions of BrAc[^{125}I]T_3 (200,000 cpm, 0.06 pmol) in ethanol were pipetted into microcentrifuge tubes, and the solvent was evaporated by a stream of nitrogen. After addition of 25 μl P100E2D10 (0.1 M sodium phosphate; 2 mM EDTA; 10 mM DTT, pH 7.2) and vortexing, the cell homogenates (100–150 μg protein) were added in a total volume of 50 μl P100E2D10. The mixtures were incubated for 10–20 min on ice, at room temperature or 37 C. Reactions were terminated by addition of SDS-PAGE gel-loading buffer, and samples were analyzed by SDS-PAGE (12% gel) followed by autoradiography to BioMax MS film (Kodak, Rochester, NY) at −70 C with intensifying screen (50–52).

Alternatively, the reactions were terminated by the addition of ice-cold methanol. After centrifugation the protein-free supernatant was mixed with 0.1% trifluoracetic acid (pH 2) and analyzed with the same HPLC system as described. The column was eluted with a 20-min gradient of 40–60% acetonitrile in 0.1% trifluoracetic acid. Retention times for BrAc-iodothyronines were determined by incubation of BrAc[^{125}I]T_3 and BrAc[^{125}I]T_2 with homogenates of nontransfected COS cells in P100E2D10 buffer and HPLC analysis as described.

Results

Analysis of mutant D3 enzymes in vitro

To define the role of SeC144 with regard to the catalytic properties of D3 enzyme, we generated mutants in which cysteine (D3Cys) or alanine (D3Ala) replaced SeC144. The substitution of cysteine for SeC essentially replaces the selenium of SeC with sulfur. Alanine was chosen as a control because it has an inert side chain. COS cells were transfected with expression vectors encoding the D3wt, D3Cys, or D3Ala enzymes, and kinetic analysis with T_3 or T_4 was performed on homogenates in the presence of 0.3–10 mM DTT (Figs. 1 and 2, and Table 1).

For the D3wt and D3Cys enzyme as well as D3 activity of human placenta microsomes, Lineweaver-Burk plots of T_3 deiodination at varying reducing cofactor (DTT) concentrations yielded a set of intersecting lines (Fig. 1). The D3 protein exhibits sequential reaction kinetics as was shown previously (7, 18), suggesting that T_3 and the thiol-containing cofactor must interact with the enzyme simultaneously before reaction takes place. In the presence of 10 mM DTT, the K_m of T_3 was 5-fold higher for the D3Cys enzyme than for the D3wt enzyme, whereas the maximal velocity (V_{max}) increased about 25-fold (Table 1). The D3Ala enzyme was inactive. When T_4 was used as substrate, the K_m was 50–100 times higher for the D3Cys enzyme than for the D3wt enzyme at DTT concentrations of 1 mM or higher. In the case of T_4, V_{max} increased only 8-fold (Fig. 2 and Table 1). The V_{max}/K_m ratios were calculated as a measure of the efficiency by which the D3wt and D3Cys enzyme deiodinate the substrates T_3 and T_4. The D3wt enzyme deiodinates T_3 and T_4 with equal efficiency as reflected in the similar V_{max}/K_m ratios. In contrast, the D3Cys enzyme strongly prefers T_3 above T_4 because the V_{max}/K_m ratio for T_3 is 40-fold higher. In the absence of DTT, no deiodination of T_3 or T_4 could be measured by D3wt or D3Cys enzyme. Maximal enzyme activity was observed at 10 mM DTT, whereas at higher DTT concentrations (>30 mM), the activity was reduced (not shown). No outer-ring deiodination of T_3, T_4, or rT_3 by D3wt or D3Cys enzyme was detected.

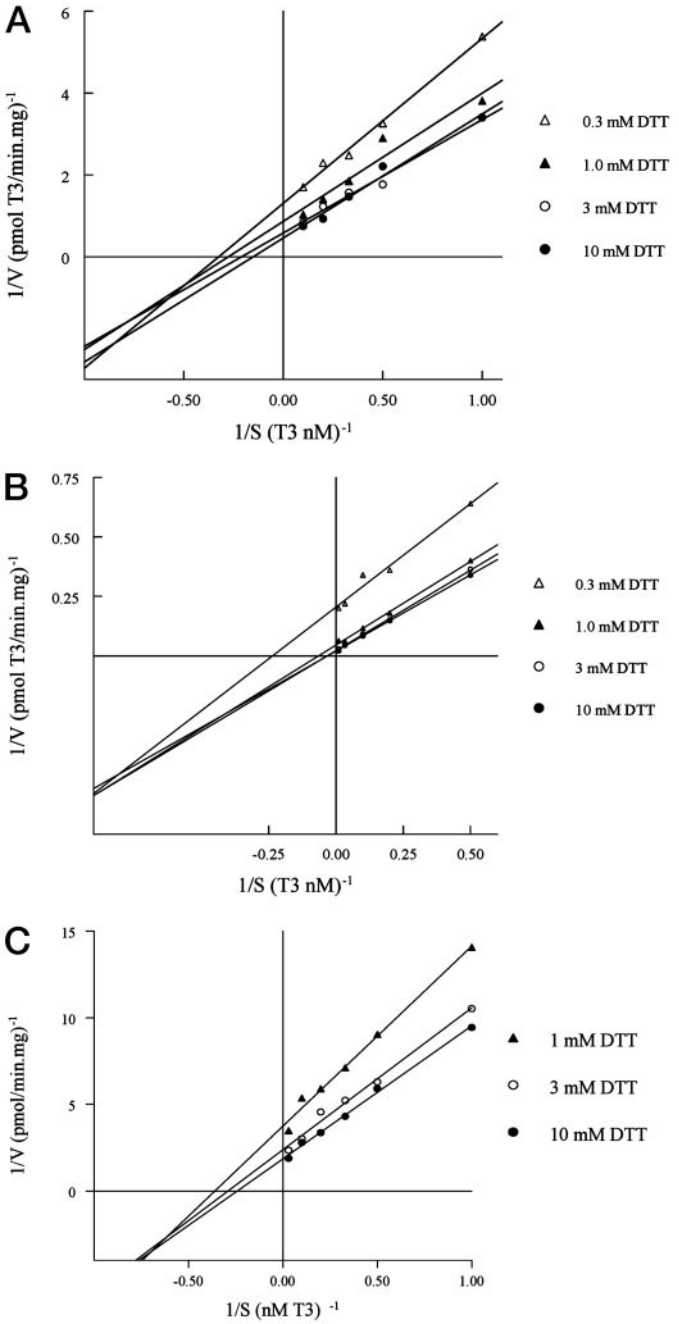

FIG. 1. Lineweaver-Burk plots of T_3 deiodination at various DTT concentrations catalyzed by D3wt (A), D3Cys (B), and D3 in human placenta microsomes (C). Each *data point* is the average of closely agreeing duplicate determinations, and the experiment was performed twice on different homogenates with similar results.

Selenoenzymes such as thioredoxin reductase, glutathione peroxidase, formate dehydrogenase, and iodothyronine deiodinases are inhibited by organic gold compounds and IAc (1–5, 53–56). GTG or aurothioglucose is a competitive inhibitor of T_3 deiodination by D3 (34). Under the conditions used (1 nM T_3, 10 mM DTT), the IC_{50} value for inhibition by GTG of D3wt enzyme was about 1 μM. Interestingly, the D3Cys enzyme is not insensitive to GTG, although the IC_{50} value increased to about 30 μM (Fig. 3). Under the same

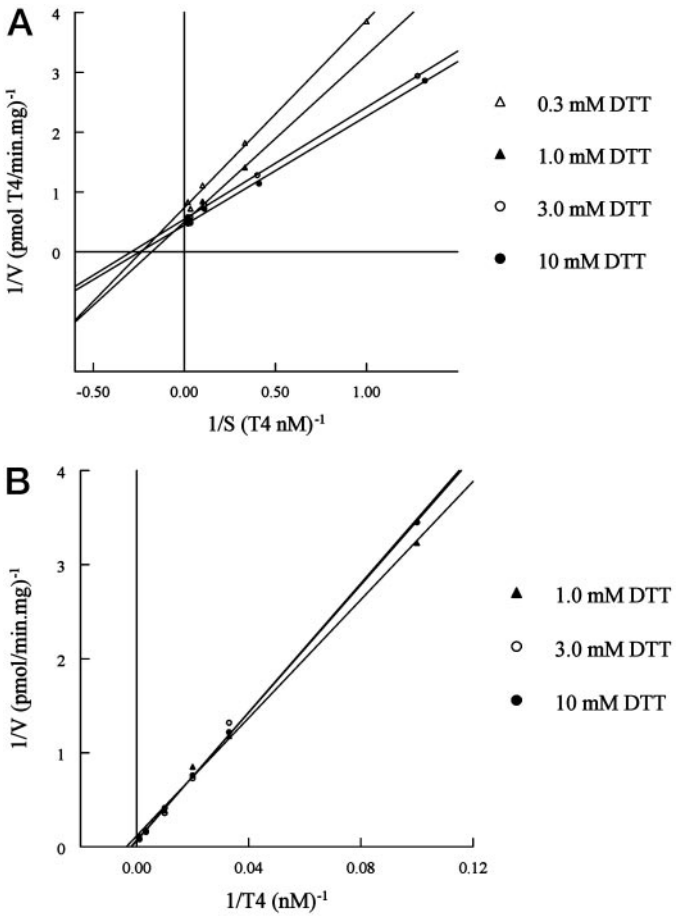

FIG. 2. Lineweaver-Burk plots of T_4 deiodination at various DTT concentrations catalyzed by D3wt (A) or D3Cys (B) enzyme. Each *data point* is the average of closely agreeing duplicate determinations, and the experiment was performed twice.

experimental conditions, both the D3wt and D3Cys enzymes were essentially insensitive to IAc ($IC_{50} > 1$ mM).

The decreased catalytic activity of D1 and D2 mutant enzymes in which Cys substitutes for SeC has been attributed to the reduced dissociation (deprotonation) of the sulfydryl moiety at physiological pH and thus to the diminished nucleophilic character of the catalytic center (41, 42). By comparing the actual pH profiles of D3wt and D3Cys enzyme (Fig. 4), no shift to more alkaline pH values for D3Cys enzyme was observed, despite the different pK values (5.2 *vs.* 9.0) of the selenol and sulfhydryl moieties in free SeC and Cys (57, 58).

Quantitation of D3 enzymes and estimation of substrate turnover number

To quantitate the effect of the substitution of Cys for SeC on the substrate turnover number, it is necessary to estimate the absolute or relative expression levels of protein. We performed semiquantitative Western blotting using a D3 antiserum on homogenates of COS cells expressing D3wt or D3Cys protein (Fig. 5). Comparison of the densities of the approximately 34-kDa D3 protein band indicated that the amount of D3Cys protein is 40- to 50-fold greater than the amount of D3wt protein. Also, the amount of D3Ala protein expressed was about 50-fold greater than D3wt protein (not shown). Using an estimate that 50-fold higher quantities of D3Cys protein are expressed relative to D3wt protein and using the V_{max} values obtained (Table 1), our results indicated that the substrate turnover number of D3Cys enzyme is 2-fold reduced for T_3 and 6-fold reduced for T_4 deiodination, compared with D3wt enzyme.

To calculate the absolute substrate turnover number of D3, it is necessary to determine the quantity of the transiently expressed protein. BrAc[^{125}I]T_3 has proven to be a very useful affinity label of D1 protein, and saturation analysis of the progressive labeling of D1 protein with increasing concentrations of BrAcT3 allowed the determination of D1 expression levels (41, 50). We therefore decided to investigate the possibility of using BrAc[^{125}I]T_3 to specifically label D3 protein in COS cell homogenates.

The deiodination of T_3 (1 nM [^{125}I]T_3, 10 mM DTT) could be completely inhibited with BrAcT3, indicating that BrAcT3 interacts with the D3 enzyme. The IC_{50} values were about 3 nM for D3wt enzyme and about 30 nM for D3Cys enzyme (not shown). After incubation of homogenates with BrAc[^{125}I]T_3 followed by SDS-PAGE, a labeled protein of 34 kDa was observed in homogenates containing D3Cys protein and a weakly labeled protein of the same molecular size was present in homogenates containing D3wt protein. Especially after prolonged autoradiography, a weakly labeled protein with slightly higher molecular mass than D3 was visible in homogenates of nontransfected COS cells (Fig. 6A). The D3Ala protein could not be labeled with BrAcT3, despite the fact that the expression level is equally high as that of D3Cys protein. This indicates that deiodinase activity is essential for labeling with BrAcT3 and probably the SeC144 or Cys144 residues are the targets for labeling by BrAcT3. The same affinity labeling experiments were done with homogenates of HEK-293 cells transfected with D3wt or D3Cys vectors. The activity of D3wt in HEK cell homogenates was 1.5-fold higher than in COS cell homogenates. The results of the labeling experiments with HEK cell homogenates were similar to those with COS cell homogenates. Both for COS and HEK cell homogenates, about 0.5% of the added BrAc[^{125}I]T_3 was incorporated into D3wt protein and about 1.5% in D3Cys protein as determined by SDS-PAGE followed by counting of radioactivity in gel slices. Altogether the labeling of D3 protein was weak, compared with that of rat D1 protein. Given the intense labeling of D1 protein, it is unlikely that COS cell proteins interfere with the labeling of D3 by BrAcT3 (Fig. 6A).

Another possible explanation for the differential labeling of D1 and D3 might be degradation or deiodination of BrAcT3 by the D3 enzyme. In a series of experiments, the labeling reactions were terminated by addition of ice-cold methanol. The protein-free supernatants were subsequently analyzed by reverse-phase HPLC (see *Materials and Methods*). The HPLC analysis revealed a shift of radioactivity to more polar components, most likely as a consequence of inner-ring deiodination of BrAc[^{125}I]T_3 to BrAc[^{125}I]T_2 and BrAc[^{125}I]T_1 by D3wt and D3Cys enzyme (Fig. 6B). Apart from D3 in COS or HEK cell homogenates, D3 in human placental microsomes also caused inner-ring deiodination of BrAcT3. Nei-

TABLE 1. Kinetic characteristics of D3 enzymes

Deiodinase	DTT (mM)	T_3			T_4		
		K_m (nM)	V_{max} (pmol/min·mg)	V_{max}/K_m	K_m (nM)	V_{max} (pmol/min·mg)	V_{max}/K_m
D3wt	0.3	3.1	0.8	0.3	4.3	1.4	0.3
	1	3.6	1.2	0.3	5.6	2.0	0.4
	3	4.8	1.7	0.4	3.5	1.9	0.5
	10	6.6	2.2	0.3	4.1	2.3	0.5
D3 placenta	1	2.8	0.3	0.1			
	3	3.4	0.4	0.1			
	10	4.1	0.5	0.1	8.0	0.6	0.07
D3Cys	0.3	4.2	5	1.2			
	1	15	22	1.5	265 ± 11	11 ± 2	0.04
	3	31	46	1.5	477 ± 67	18 ± 2	0.04
	10	33	51	1.5	570 ± 93	21 ± 5	0.04

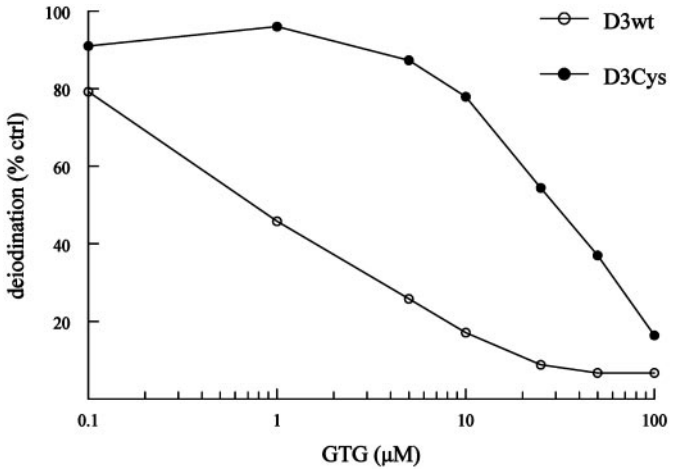

FIG. 3. Effect of GTG on T_3 deiodination (1 nM T_3, 10 mM DTT) by D3wt and D3Cys enzyme.

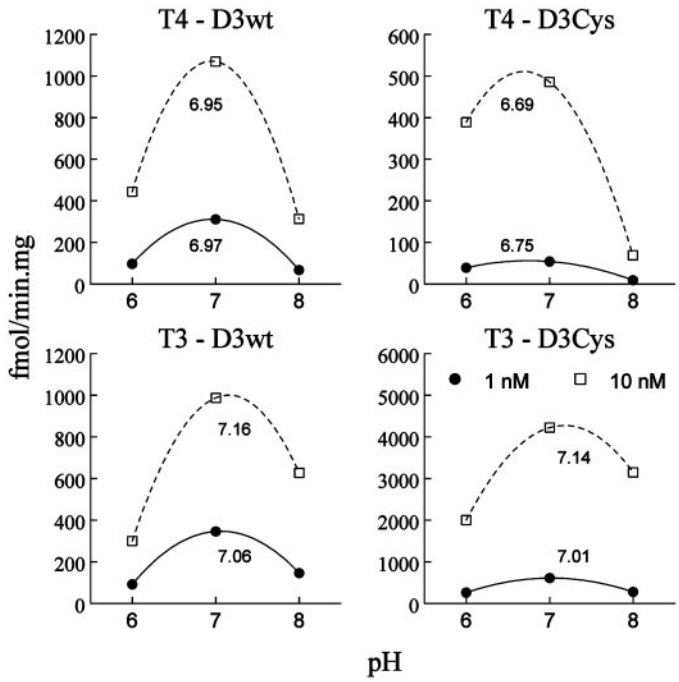

FIG. 4. Effect of pH on deiodinase activity of D3wt or D3Cys enzyme in the presence of 1 nM T_3 or T_4 (*closed symbols*) and in the presence of 10 nM T_3 or T_4 (*open symbols*). Each *point* is the mean of closely agreeing duplicate determinations, and the experiment was performed twice with similar results. The *curves* were obtained by fitting the data points to a hyperbolic function and the pH optimum was calculated.

ther inner-ring nor outer-ring deiodination of BrAc[^{125}I]T_3 by D1 or D2 enzymes could be detected (not shown). Attempts to reduce the inner-ring deiodination of BrAcT3 by lowering the DTT concentration to 0.1 mM or 1 mM instead of 10 mM increased incorporation of radioactivity in the various proteins but did not increase the relative incorporation of radioactivity in D3 protein as analyzed by SDS-PAGE (not shown). Because of the rather weak labeling of D3, a reliable estimation of D3 content by saturation analysis was not possible. Under the most favorable conditions (1 mM DTT at 37 C), 0.5% of the added BrAc[^{125}I]T_3 was incorporated into D3wt protein and about 2% in D3Cys protein. By comparison, 6% of the added BrAc[^{125}I]T_3 was incorporated into rat D1wt protein.

Deiodination by wild-type D3, D3Cys, and D3Ala protein in intact cells

The D3Cys enzyme is active *in vitro* in the presence of excess iodothyronine substrate and DTT. An important question is whether the same is true under *in situ* conditions using intact transfected COS cells. In intact cells the free substrate concentration available to the enzyme might be lower because of limited cellular uptake as well as binding of iodothyronine substrate to cytosolic iodothyronine-binding proteins or membranes (1–2).

When D3wt or D3Cys enzyme were expressed in COS cells, addition of increasing amounts of unlabeled T_3 did not result in saturation of the fractional deiodination of [^{125}I]T_3 (Fig. 7A). The D3Ala protein was inactive, whereas the percentage deiodination of T_3 by D3wt and D3Cys protein were similar. In control experiments, it was established that nontransfected COS cells sulfate [^{125}I]T_2, and therefore the sum of [^{125}I]T2 and [^{125}I]T2SO_4 was used to calculate the percentage deiodination of T_3.

When D2 enzyme was expressed in COS cells, addition of increasing amounts of unlabeled T_4 resulted in the complete saturation of [^{125}I]T_4 deiodination, as reflected by a decrease of [^{125}I]T_3 release into the medium (40) (Fig. 7B). In contrast, the fractional deiodination of [^{125}I]T_4 by D3wt and D3Cys enzyme was not saturable. The saturation of the fractional

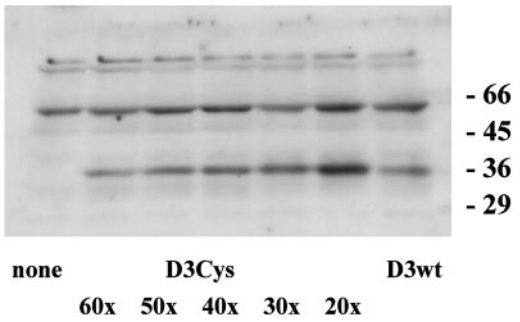

FIG. 5. Quantitation of the relative amounts of D3wt and D3Cys protein by Western blotting. COS cell homogenates were subjected to immunoblot analysis as described in *Materials and Methods*. Migration distances of molecular mass markers are indicated (kilodaltons). To compare the relative amount of protein expressed between D3wt and D3Cys, the homogenates containing D3Cys were diluted in homogenates from mock-transfected COS cells. The D3wt selenoprotein was expressed approximately 50-fold less well than the D3Cys mutant.

deiodination of T_4 by D2 at high medium T_4 concentrations indicated that cellular T_4 uptake did not limit its deiodination, whereas in the case of D3, uptake of T_4 apparently did limit deiodination. In this regard it is important to note that the *in vitro* K_m values of D2wt and D3wt for T_4 are similar (4–5 nM). The intracellular concentration of D3 might be higher than that of available T_4. Attempts to reduce the expression level of D3 enzyme by transfecting 10-fold lower amounts of plasmid DNA (0.2 μg/well instead of 2 μg/well) gave similar results, *i.e.* no saturation of the deiodination of [^{125}I]T_4 by medium T_4 concentrations of up to 1 μM.

Discussion

The aim of the present study was to investigate the role of the SeC residue in the catalytic center of D3 protein. Replacing the SeC residue with alanine inactivated the D3 enzyme, as was previously also described for D1 (41) and D2 enzymes (40, 42). Substitution of cysteine for SeC did not eliminate D3 activity but reduced the turnover number 2-fold for T_3 and 6-fold for T_4 deiodination. In addition, the K_m for T_3 increased 5-fold, whereas the K_m for T_4 increased 100-fold in comparison with D3wt enzyme.

The SeC residue in the catalytic center is essential for maximal catalytic efficiency of deiodinases and other selenoenzymes such as glutathione peroxidase, formate dehydrogenase, and thioredoxin reductase (40–42, 54–56, 58–59). Substitution of Cys for SeC has quite variable results, possibly because of different catalytic mechanisms used by D1, D2, and D3. The D1 enzyme exhibits ping-pong (bi)substrate kinetics, whereas the D2 and D3 enzymes exhibit sequential reaction kinetics (7, 18, 38, 40, 41, 60). In D1 mutation of SeC to Cys raises the K_m for rT_3 only 3-fold but reduces the substrate turnover number 100-fold (41). The SeC residue in the catalytic center of D1 is thought to function as the iodine acceptor, and the selenenyl iodide intermediate is the target for reaction with propylthiouracil (38, 39, 60, 61). The thiol group of Cys is a much weaker nucleophile than the selenol group of SeC, thus explaining the strongly reduced efficiency of formation of the sulfenyl iodide intermediate and therefore the reduced turnover of the D1Cys enzyme.

Both for D2 and D3, substitution of SeC by Cys results in a limited decrease in turnover of the substrate T_4 but a marked increase in the K_m for T_4. In D2 the K_m increased 1000-fold and the T_4 turnover was reduced 10-fold (40, 42). In this study the D3Cys enzyme has a 6-fold reduced turnover of T_4 and a 100-fold increased K_m for T_4. We proposed a catalytic model for D2 in which the SeC residue exerts a nucleophilic attack to the 2′-position of T_4, thus forming an intermediate complex between the SeC residue and T_4 (40). Subsequent steps involve the abstraction of iodonium by DTT (or another thiol group containing cofactor) providing a D2/T_3 complex that will yield T_3 and the unmodified enzyme. So 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. This model of reductive dehalogenation of T_4 by D2 might also apply to D3 enzyme. In that case it would involve a nucleophilic attack to the 2-position of T_4. In this model the lower nucleophilicity of the SH group in Cys, compared with the SeH group in SeC would result in a less favorable formation of the intermediate between T_4 and D2 or D3 enzyme, which is reflected in the 1000-fold (D2) or 100-fold (D3) increased K_m for T_4.

The decreased catalytic activity of D1 and D2 mutant enzymes in which Cys substitutes for SeC has been attributed to the reduced dissociation of the sulfhydryl group at physiological pH and thus to the diminished nucleophilic character of the catalytic center, compared with the wild-type enzymes (41, 42). By comparing the actual pH activity profiles of D3 wt and D3Cys enzyme, no shift to more alkaline pH values for D3Cys enzyme was observed, despite the different pK values (5 *vs.* 9) of the selenol and sulfhydryl moieties (57). So the pH dependence of D3 activity is not simply accounted for by the dissociation of the nucleophilic group (selenol or sulfhydryl) in the catalytic center. For thioredoxin reductase the pH optimum shifted from 7 to 9 for the mutant enzyme in which the penultimate SeC was substituted by Cys (58). For other selenoenzymes such as glutathione peroxidase and formate dehydrogenase such big pH shifts for the Cys mutant enzymes were not observed (54–56), perhaps because these enzymes display rather broad pH optima around 8.5. It is remarkable that the pH optimum for D3 is close to 7 (6.7–7.1) and D3 activity is strongly reduced at pH 8, especially with T_4 as substrate.

The selenol or sulfhydryl group might interact with side groups of other amino acid residues in the catalytic center. These interactions could stimulate deprotonation of the selenol or sulfhydryl group at physiological pH. A so-called catalytic triad involving tryptophane and glutamine residues stimulating SeC deprotonation has been described for glutathione peroxidase (55, 62). For D1 the formation of an essential imidazolium-selenolate ion pair was postulated on the basis of experiments with histidine-directed reagents, pH-dependent kinetic properties, and site-directed mutagenesis studies (1, 63, 64). The existence of a catalytic triad or an imidazolium-selenolate ion pair in D3 enzyme is speculative at the moment and needs further investigation.

The D3Cys enzyme is sensitive to GTG, and the IC_{50} value is about 30-fold higher than that of D3wt enzyme. This increase mirrors the change in sensitivity toward GTG of native *vs.* D1Cys or D2Cys enzymes, which resulted in 100-fold

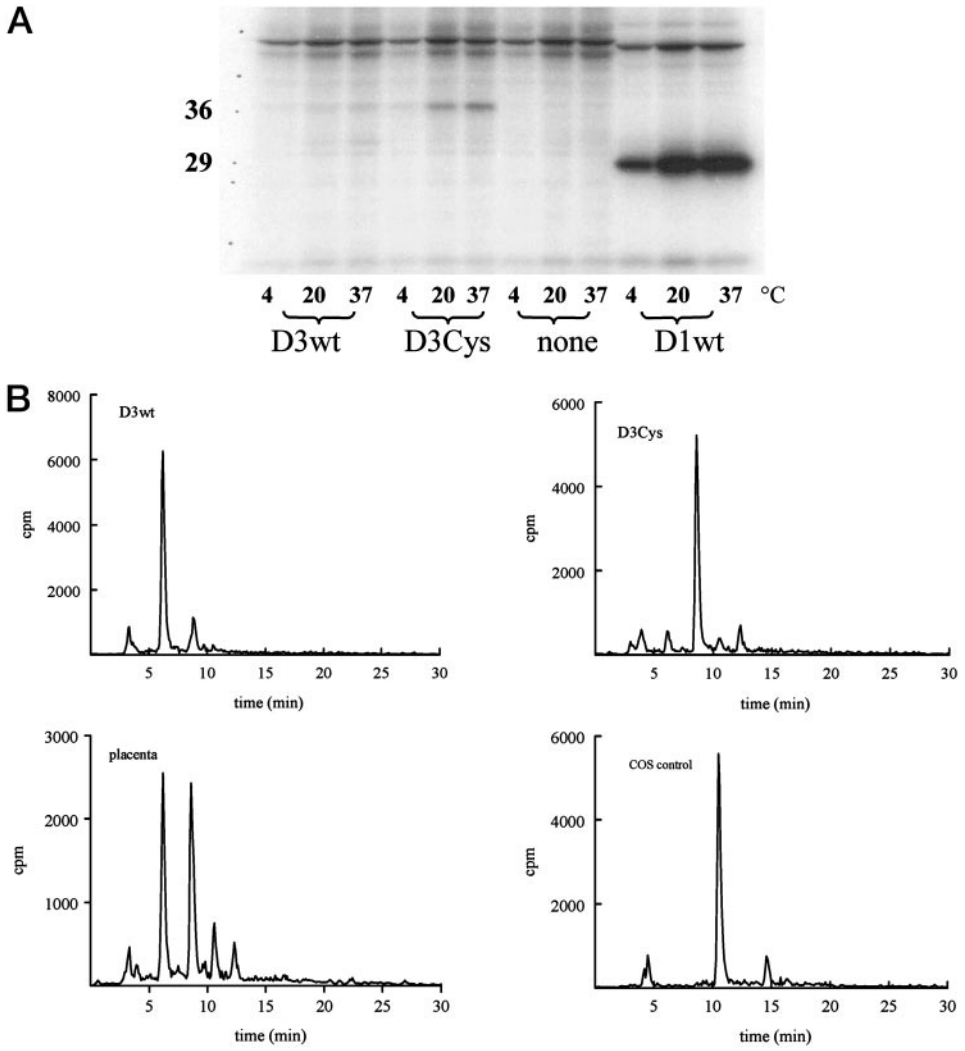

FIG. 6. A, Labeling patterns obtained after reaction of COS cell homogenates (100–150 μg protein) containing D3wt, D3Cys, or D1wt or control homogenates with BrAc [^{125}I]T_3 in the presence of 10 mM DTT at 4 C, 20 C, or 37 C. Migration distances of molecular mass markers are indicated (kilodaltons). B, HPLC analysis of metabolites recovered from incubation of BrAc[^{125}I]T_3 with D3wt enzyme, D3Cys enzyme, or human placenta microsomes in P100E2D10 at 37 C. Retention times are 11 min for BrAcT3, 9 min for BrAcT2, and 3–4 min for T_3 and T_2. The peak at 7 min most likely represents BrAc[^{125}I]T_1, although this reference compound was not synthesized. The pattern obtained with D1wt enzyme or D2wt enzyme was similar to that obtained with homogenate from nontransfected (control) COS cells (not shown).

higher inhibitory constant values (K_i) for GTG (42, 60). For both D1 and D3 enzymes, GTG is a competitive inhibitor, implying that GTG interacts directly with the conserved catalytic center (34, 60). However, this does not explain the differential sensitivity toward GTG of the deiodinases, with D1 being the most sensitive and D3 the least sensitive (65). Also, the reduced sensitivity to GTG of D3Cys enzyme does not necessarily imply that inhibition by GTG is selective for the SeC residue in the catalytic center. Other Cys residues outside the catalytic center might be involved as well.

BrAc[^{125}I]T_3 has proven to be a useful affinity label for D1, allowing specific identification of the enzyme in microsomal fractions of liver and kidney (44, 50, 66). Affinity labeling with BrAc[^{125}I]T_3 was also used for the quantitation of D1 enzyme expression levels by saturation analysis, allowing estimation of the D1 substrate turnover number in microsomal preparations and homogenates of transfected cells (41, 50). With regard to affinity labeling of D3 with BrAcT3 results have been confusing (51–52, 67). Initial experiments with brain and placenta microsomes revealed a good correlation between affinity labeling of a 32-kDa protein (p32) and inactivation of D3 by unlabeled BrAcT3 (51, 52). However, when p32 was also detected in tissues with low or undetectable D3 activity and p32 labeling was not prevented by substrates of D3, it was concluded that p32 does not represent D3 protein (51, 52). An alternative explanation for the inconsistencies might be that in one-dimensional SDS-PAGE, the low abundance D3 protein comigrates with an abundant p32 protein not related to D3 (52, 67).

Despite the ambiguous results with respect to affinity labeling of native D3 with BrAcT3, we decided to try to label recombinant D3 in COS or HEK cell homogenates. Although D3 activity was inactivated with BrAcT3, we were not able to use affinity labeling with BrAc[^{125}I]T_3 as a reliable method to quantify the D3 content of homogenates. The labeling intensity of D3wt and D3Cys protein was low, certainly when compared with D1wt protein labeled under the same experimental conditions. Possible explanations are: 1) BrAc[^{125}I]T_3 is degraded after coupling to D3, leading to a loss of radioactivity bound to D3; 2) the amount of D3 present in the homogenates is too low to allow sufficient incorporation of BrAc[^{125}I]T_3; 3) BrAcT3 blocks the interaction of T_3 with D3 but does not react covalently with D3 because of unfavorable positioning toward a nucleophilic amino acid (SeC) in the catalytic center; and 4) inner-ring deiodination of BrAcT3 by D3 and the BrAcT2 and/or BrAcT1 thus formed

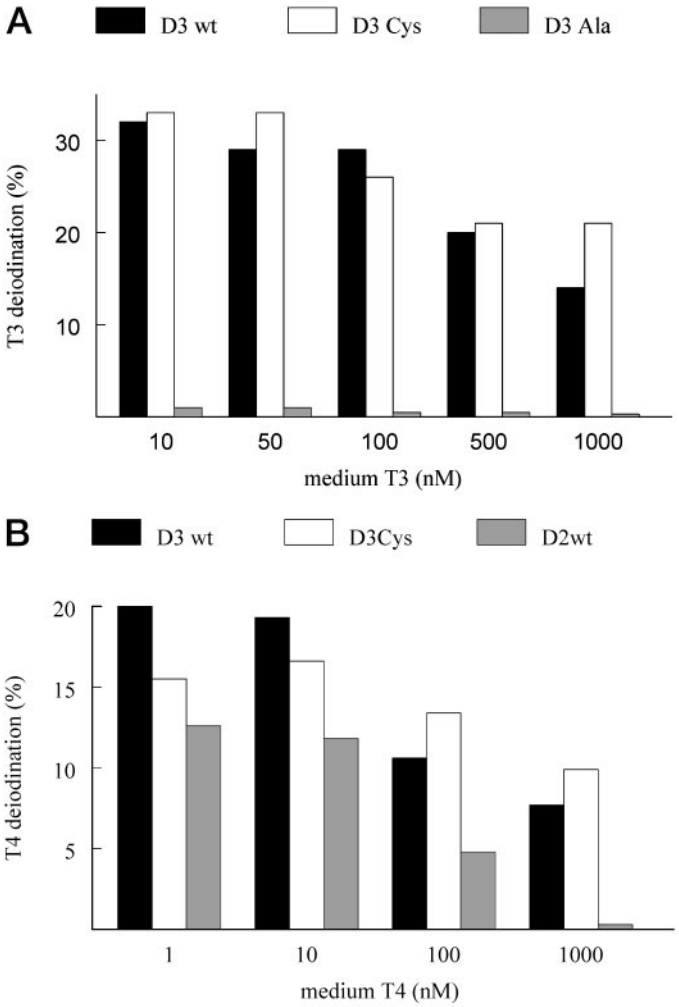

FIG. 7. *In situ* deiodination at varying T_3 (A) and T_4 (B) medium concentrations. COS cells were transfected with D3wt, D3Ala, or D3Cys (A) and D3wt, D3Cys, or D2wt (B) expression vectors, and 24 h post transfection intact cell deiodination was analyzed as described in *Materials and Methods*.

do not label D3 enzyme. We found evidence for the last possibility, although the alternatives cannot be excluded. Because of the inner-ring deiodination, it is clear that BrAcT3 is not optimal as affinity label for D3 protein, and improved affinity label(s) should be developed. In contrast to our results, Salvatore *et al.* (34) described affinity labeling with BrAc[^{125}I]T_3 and quantitation by saturation analysis of D3 in homogenates of HEK-293 cells. Inner-ring deiodination of BrAcT3 by D3 enzyme was not investigated.

From the intact cell deiodination experiments, it is clear that D3Cys enzyme is active *in situ* in the presence of endogenous cofactor, just as D3wt enzyme. The intracellular actual free substrate concentrations are unknown, so detailed kinetic studies are not possible. The lack of saturability of the fractional T_4 deiodination by D3 in contrast to D2 could be explained if the D3 expression level is higher than that of D2. The much longer half-life of D3, compared with D2 protein (68), could result in a higher D3 expression level.

As shown in the present work, the replacement of SeC by Cys results in reduced substrate turnover by D3 enzyme in particular with T_4. Moreover, when SeC was replaced by Ala the result was complete loss of catalytic activity. These results suggest a role for the SeC residue that is catalytic rather than structural. In fact, the SeC residue is essential for efficient deiodination at physiological substrate concentrations. Additional mutagenesis experiments need to be performed to identify residues (histidine?) that might be involved in interaction(s) with the SeC residue. Increased insight in the catalytic mechanism of D3 could form the basis for the design of D3 specific inhibitors and/or affinity labels.

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