Expression of recombinant membrane-bound type I iodothyronine deiodinase in yeast

George G J M Kuiper, Willem Klootwijk and Theo J Visser

Department of Internal Medicine, Room Ee 502, Erasmus Medical Center, PO Box 1738, 3000 DR Rotterdam, The Netherlands

(Requests for offprints should be addressed to T J Visser; Email: t.j.visser@erasmusmc.nl)

Abstract

The bioactivity of thyroid hormone is determined to a large extent by the monodeiodination of the prohormone thyroxine (T4) by the hepatic selenoenzyme type I iodothyronine deiodinase (D1), i.e. by outer ring deiodination (ORD) to the active hormone triiodothyronine (T3) or by inner ring deiodination (IRD) to the inactive metabolite reverse T3 (rT3). Since D1 is a membrane-bound protein with an N-terminal membrane-spanning domain, the enzyme is very difficult to purify in an active state. This study was undertaken in order to develop a heterologous (over)-expression system that would eventually allow the production of large amounts of purified active D1 protein. We have expressed a mutant rat D1 protein, in which the selenocysteine residue in the core catalytic center was replaced by cysteine (D1 Cys) in yeast cells (*Saccharomyces cerevisiae*). After yeast cell fractionation, kinetic analysis was performed with dithiothreitol as reducing cofactor. ORD activity was associated with membrane fractions, while no activity could be detected in the cytosolic fraction. The D1 Cys protein displayed a tenfold increase in K_m (2 μ M) for rT3 as compared with native D1 protein in rat liver microsomes. The D1 protein content is about 65 pmol/mg microsomal protein, as compared with about 3 pmol/mg in rat liver microsomal fraction. SDS-PAGE analysis of *N*-bromoacetyl-[¹²⁵I]T3 affinity-labeled D1 protein showed several labeled protein isoforms with apparent molecular masses between 27 and 32 kDa. Immunoblot analysis with a specific D1 antiserum confirmed the observed D1 protein heterogeneity. Site-directed mutagenesis of several potential N-linked glycosylation sites, phosphorylation sites and a unique myristoylation site established that D1 heterogeneity is not caused by N-linked glycosylation, but probably by a combination of O-linked glycosylation and phosphorylation. Deletion of the endoplasmic reticulum (ER)-signal sequence and the membrane-spanning domain (amino acid residue 2–35), did not result in the production of a soluble D1 enzyme. Although this mutated D1 protein is inactive, the fact that it is still membrane bound indicates the existence of additional membrane attachment site(s) or membrane-spanning domains. Overall, our studies indicate that yeast cells provide a useful system for the expression of relatively high levels of D1 protein which could be used for further structure–function analysis.

Journal of Molecular Endocrinology (2005) **34**, 865–878

Introduction

The type I iodothyronine deiodinase (D1) selenoprotein is found in mammals predominantly in the microsomal fractions of liver, kidney and thyroid gland (Bianco *et al.* 2002, Köhrle 2002, Kuiper *et al.* 2005). This enzyme is responsible for a large part of the peripheral production of 3,3',5-triiodothyronine (T3) from thyroxine (T4) in euthyroid animals (Doorn *et al.* 1983, Nguyen *et al.* 1998, Bianco *et al.* 2002). Remarkably, D1 is capable of both outer ring deiodination (ORD) and inner ring deiodination (IRD) of T4, and shows preference for reverse T3 (rT3; 3,3',5'-triiodothyronine) as the substrate (Berry *et al.* 1991a, Moreno *et al.* 1994). D1 activity *in vitro* is stimulated by thiol compounds such as dithiothreitol (DTT) and is uncompetitively inhibited by 6-propyl-2-thiouracil (Visser 1980).

N-bromoacetyl-[¹²⁵I]T3 has proven to be a useful affinity label of D1, allowing the specific labeling of the enzyme in rat liver microsomal fractions (Schoenmakers *et al.* 1992), despite the fact that it only constitutes about 0.01% of protein content (Mol *et al.* 1988). Progress in the structure–function analysis of D1 protein is hampered by the very low expression level. Due to the low expression level only small amounts of purified D1 protein (approximately 50% pure) were obtained by affinity chromatography from detergent-solubilized microsomal fractions of rat liver (Mol *et al.* 1988). Molecular sieve chromatography of the detergent solubilized D1 from rat liver or kidney cells yielded a ~ 50 kDa active enzyme preparation, suggesting that the D1 protein is composed of a homodimer of 27 kDa subunits (Mol *et al.* 1988, Köhrle *et al.* 1990, Leonard *et al.* 2001). After the D1 cDNA cloning (Berry *et al.*

1991a), site-directed mutagenesis and deletion analysis have identified several functional regions of the D1 protein (Berry *et al.* 1991b, 1992, Toyoda *et al.* 1995a,b, 1997, Leonard *et al.* 2001, Kuiper *et al.* 2003). The rat D1 protein contains an uncleaved endoplasmic reticulum (ER) transfer sequence and transmembrane domain (amino acid residue 2–35), while iodothyronine substrate selectivity is influenced by the domain between amino acid residues 40 and 70. The D1 core active center consists of a region of about 20 amino acid residues long, surrounding the single essential selenocysteine (SeC) residue.

At the same time it should be realized that analyzing the effects of individual mutations in impure D1 enzymes only reveals that the changed amino acid residue directly or indirectly affects activity, but does not necessarily deliver reliable information on the molecular mechanism(s) involved. In order to obtain pure D1 protein for more detailed structure–function analysis it is necessary to develop a heterologous (over)-expression system. The fact that D1 is an integral membrane protein limits the choice of expression systems to eukaryotic cells, for instance yeast or insect cells, since expression in bacteria of D1 protein results in the production of inactive D1 that accumulates in inclusion bodies (G G J M Kuiper, W Klootwijk & T J Visser, unpublished observations). A heterologous expression system that combines several advantages of both prokaryotic and eukaryotic expression systems are yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* (Guengerich *et al.* 1991, Bill 2001, Griffith *et al.* 2003). Yeast cells can perform proper post-translational modifications, and contain identical secretory pathways as higher eukaryotic cells. Yeast cells can be grown inexpensively in large quantities and several well-characterized inducible promoter systems that allow regulated expression of specific genes have been established. Several cytochrome P450 enzymes and short-chain dehydrogenase/reductase enzymes, inserted in membranes in much the same manner as deiodinases, have been successfully overexpressed in yeast (Guengerich *et al.* 1991, Blum *et al.* 2001, Hult *et al.* 2001).

The D1 protein contains a single SeC residue in the catalytic center, encoded by a UGA stop codon. Successful insertion of SeC at UGA codons in eukaryotes requires the presence of a specific stem-loop structure in the 3'-untranslated region of the mRNA (Berry *et al.* 1993). Although yeast cells are eukaryotic cells they are unable to synthesize selenoproteins. In fact, the yeast homologs of several higher eukaryotic proteins (glutathione peroxidase, SelX) contains cysteine residues instead of SeC residues (Lescure *et al.* 1999). In order to overcome this potential problem, we have made D1 expression vectors in which the UGA stop codon encoding SeC incorporation was replaced by UGC (Cys)

or UCA (Ser). In the present paper we describe the heterologous expression and characterization of type I deiodinase activity in *Saccharomyces cerevisiae*.

Materials and methods

Nonradioactive iodothyronines were obtained from Henning Berlin R&D (Berlin, Germany) and [3',5'-¹²⁵I]rT3 was prepared by radioiodination of 3,3'-diiodothyronine (T2) as described (Visser *et al.* 1978). [3'-¹²⁵I]T3 (1500–2000 mCi/μmol) was obtained from Amersham Biotech (Little Chalfont, Bucks, UK). Unlabeled *N*-bromoacetyl-T3 (BrAcT3) and BrAc[¹²⁵I]T3 were synthesized from *N*-bromoacetylchloride and T3 or [3'-¹²⁵I]T3 as described (Mol *et al.* 1984). HPLC analysis demonstrated that the purity of BrAc[¹²⁵I]T3 was at least 85%, the remainder being unreacted T3. Iodoacetate (IAc) and 6-propyl-2-thiouracil (PTU) were obtained from Sigma (St Louis, MO, USA). Rat liver microsomes were prepared as previously described (Fekkes *et al.* 1979).

The yeast expression vector pYES6/CT and the diploid *Saccharomyces cerevisiae* strain INVSc1 (genotype his3Δ1; leu2; trp1–289; ura3–52) were obtained from Invitrogen (Carlsbad, CA, USA). The antibiotic blasticidin S was also obtained from Invitrogen. Peptone, bacto-yeast extract and the yeast nitrogen base without amino acids were from DIFCO Laboratories (Detroit, MI, USA). Yeast lytic enzyme (80 000 U/g) was obtained from ICN Biochemicals (Costa Mesa, CA, USA). Tunicamycin was obtained from Sigma Chemicals. N-glycosidase F was obtained from New England BioLabs (Beverly, MA, USA). *Pfu* thermostable DNA-polymerase and DpnI restriction endonuclease were obtained from Promega (Madison, WI, USA). XL-10 competent *Escherichia coli* cells were obtained from Stratagene (La Jolla, CA, USA).

The rat D1 antiserum 3049/JL (Leonard *et al.* 2001), directed against the C-terminus (epitope YEEVRVLE-KLCIPPGHMPQF) was a kind gift of Drs Jack Leonard and Alan Farwell (University of Massachusetts Medical School, Worcester, MA, USA).

Construction of yeast expression vectors

In order to construct the D1 Cys (SeC126 Cys) and D1 Ser (SeC126 Ser) expression vectors site-directed mutagenesis was used to substitute the SeC (TGA codon) of rat D1 cDNA with Ser (AGC) or Cys (TGC) codons as described (Leonard *et al.* 2001). The oligonucleotide primers G21A 5'-GCGGGATCCATGG GGCTGTCCCAGCTA and G21B 5'-GCGGGATCC CTAGAACTGAGGCATGTGTCC (BamHI sites in italics and start/stop codons underlined) were used to PCR amplify the D1 cDNA. The PCR products were

digested with BamHI and cloned into the BamHI site of the pYES6/CT yeast expression vector. The D1 Cys-His tag expression vector was made by PCR amplification with the oligonucleotide primers G21A and G21BX 5'-GCGTCTAGAGAACTGAGG-CATG TGTCC (XbaI sites in italics). The PCR product was digested with BamHI/XbaI and cloned in the BamHI/XbaI site of pYES6/CT. The D1 Cys-His tag protein contains a 29 amino acid C-terminal extension (SRGPFEGKPIPNLLGLDSTRTGHHH HHH). Amino acid residues 2–35 of D1 encoding the ER signal sequence and the membrane spanning domain (Toyoda *et al.* 1995b), were deleted via PCR with an oligonucleotide primer spanning codon 1 (ATG) and codons 36–41 of D1 Cys cDNA (G21CB 5'-GCG GGATCCATGAGAGTCAAGCAGAACATC, BamHI in italics and start codon underlined) and the G21BX primer resulting in the D1 Cys-(Δ 2–35)His-tag expression vector. All constructs were verified by DNA sequencing to ensure that no unwanted mutations were introduced by PCR.

Site-directed mutagenesis of D1Cys

The D1 Cys expression vector was used as template for site-directed mutagenesis via the circular mutagenesis procedure, followed by selection for mutant plasmids by DpnI digestion (Parikh & Guengerich 1998, Sambrook & Russell 2001). Circular mutagenesis was performed with 10 ng plasmid template and 2 U *Pfu* DNA polymerase. The cycling protocol consisted of 50 s at 95 °C, 50 s at 60 °C and 14 min at 68 °C for 18 cycles using a Perkin Elmer model 480 cyclor. The reaction products were incubated with 10 U DpnI for 2 h at 37 °C, and transformed into *E. coli* XL-10 cells. Plasmid DNA isolated from several clones was sequenced to verify that the desired mutation(s) were introduced.

The putative N-linked glycosylation sites (Asn-X-Thr/Ser) Asn94 and Asn203 were mutated either alone or in combination. The D1 Cys-Asn94 Gln and the D1 Cys-Asn203 Gln expression vectors were produced using overlapping sense and antisense primers containing the nucleotide changes needed, that is for Asn94 Gln sense 5'GGGCTGGCCCCCAATGCACCGTGGTCCGC, and for Asn203 Gln sense 5'GTGGACACAATGCAG CAACAGAGCAGCCAGCTC. Mutant codons are underlined. The double mutant D1 Cys-Asn94 Gln/Asn203 Gln was made using both sets of primers. Four putative phosphorylation sites predicted by the NetPhos server (Blom *et al.* 1999), that is Ser101, Ser176, Tyr209 and Tyr217 were mutated to Ala and Phe residues respectively. The D1 Cys-Ser101 Ala and D1 Cys-Ser176 Ala expression vectors were produced using overlapping sense and antisense primers containing the nucleotide changes needed, that is for Ser101 Ala sense 5'GTGGTCCGCCTCGCAGGACAGAAGTGCAACG

and for Ser176 Ala sense 5'GGCAGCACCGAGCCCT CCAGGACCGCC. The double mutant D1 Cys Ser101 Ala/Ser176 Ala was made using both sets of primers. The D1 Cys-Tyr209 Phe/Tyr217 Phe double mutant expression vector was made using the sense primer 5'CAGCCAGCTCTTTGCAGCTCTGCCTGAGAGG CTCTTTGTGATACAG, and the corresponding anti-sense primer. The putative N-linked myristoylation site Gly2 (Simon & Aderem 1992) was mutated to Ala (D1 Cys-G2A) with the sense primer 5' GCTCGGATCCAT GGCGCTGTCCCAGCTATGG, and the corresponding antisense primer.

Yeast transformation, yeast growth conditions and subcellular fractionation

Transformation was performed by the lithium acetate procedure (Ito *et al.* 1983), and transformed cells were selected onto synthetic complete (SC) medium plates (Sherman 1991) containing 10 µg/ml blasticidin. After 5 days of incubation at 30 °C colonies were toothpicked into 5 ml SC-medium containing 10 µg/ml blasticidin and grown overnight at 30 °C. Glycerol stocks (15% vol/vol glycerol) were made and stored at –80 °C.

For large scale expression of recombinant D1 protein, 10 ml SC medium containing 10 µg/ml blasticidin were inoculated and grown overnight at 30 °C. Expression was induced by inoculating the preculture into 250–500 ml YPG-medium (2% wt/vol galactose, 1% wt/vol yeast extract, 2% wt/vol bacto-peptone) in a 2 l extra deep triple-baffled Erlenmeyer flask (Bellco Glass Inc., Vineland, NJ, USA) and grown for up to 48 h at 30 °C. The cell density obtained was approx. 0.5×10^8 viable cells/ml, and more than 80% of the cells were still blasticidin resistant. Cells were collected by centrifugation, washed with double distilled water and pellets were stored at –80 °C until use.

Subcellular fractionation of yeast cells was performed according to established procedures (Franzusoff *et al.* 1991, Kaiser *et al.* 2002) with minor modifications. Pellets (from 150 – 200 ml culture) were resuspended in 15 ml 10 mM Tris-HCl buffer (pH 7.5) containing 2 M sorbitol, 0.1 mM EDTA, 0.4 mM PMSF and 10 mM DTT. After careful resuspension, yeast lytic enzyme was added to a final concentration of 2.5 mg/ml followed by incubation for 1 h at 30 °C. Spheroplasts were collected by centrifugation for 10 min at 120 g, washed with ice-cold 10 mM Tris-HCl buffer (pH 7.5) containing 0.65 M sorbitol, 1 mM DTT, 0.1 mM EDTA and 0.4 mM PMSF, and resuspended in 15 ml of the same cold buffer. Spheroplasts were lysed by sonification using 4–6 bursts (15 s each) with a 3 mm probe of a Sanyo Soniprep 150 Ultrasonic disintegrator at an amplitude setting of 10 microns. The sonicated suspension was centrifuged for 30 min at 1000 g in the cold and the supernatant (=crude homogenate) was centrifuged for

30 min at 10 000 *g* and 8 °C. The 10 000 *g* supernatant was centrifuged for 90 min at 100 000 *g* and 8 °C. The 10 000 *g* and 100 000 *g* pellets were resuspended in 100 mM phosphate pH 7.2, 2 mM EDTA, 1 mM DTT buffer and stored in aliquots at – 80 °C.

For equilibrium sedimentation experiments, yeast cells were broken by agitation with glass beads in 10 mM Tris–HCl (pH 7.5) buffer containing 10% wt/wt sucrose and 10 mM EDTA (Kaiser *et al.* 2002). The homogenates were centrifuged for 5 min at 500 *g*, and the supernatant was loaded on 20% wt/wt – 60% wt/wt sucrose-density gradients in 10 mM Tris–HCl (pH 7.5), 10 mM EDTA buffer. The gradients were run for 16 h at 32 000 r.p.m. (100 000 *g*) in an SW60 rotor at 8 °C. Fractions of 0.3 ml were collected from the top and stored at – 80 °C.

Deiodinase enzyme activity assay

The principle of this assay is the production of radioiodide by ORD of [3',5'-¹²⁵I]rT3. Incubation mixtures contained about 100 000 c.p.m. ¹²⁵I-rT3 with or without varying concentrations of unlabeled substrate (rT3, T4 or T3) and varying amounts of yeast subcellular fractions or rat liver microsomes in 200 µl P100E2D10 (100 mM phosphate, 2 mM EDTA, 10 mM DTT, pH 7.2). In some experiments deiodinase inhibitors (PTU, IAc) were included. Mixtures were incubated in duplicate for 10 – 60 min at 37 °C, and the reactions were stopped by addition of 100 µl 5% BSA on ice. Protein-bound iodothyronines were precipitated by addition of 500 µl 10% trichloroacetic acid, and the radioiodide in the supernatant was separated from remaining iodothyronines by chromatography on Sephadex LH-20 columns as described by Eelkman *et al.* (1987). Blank incubations without enzyme were used to correct for nonenzymatic deiodination (<3% of total deiodination). Protein was adjusted in order to consume less than 30% of the substrate, and it was determined that the deiodination rate was linear up to 60 min incubation. The ¹²⁵I[–] production was multiplied by 2 to account for the random labeling and deiodination of the 3' and 5' positions in rT3.

Affinity-labeling with N-bromoacetyl-[¹²⁵I]T3

Solutions of BrAc[¹²⁵I]T3 (100 000 c.p.m.) in ethanol were pipetted into microcentrifuge tubes and the solvent was evaporated under a stream of nitrogen. After the addition of 25 µl P100E2D10 buffer and vortexing for 10 s, protein fractions (50 – 100 µg) were added in a total volume of 50 µl P100E2D10 buffer. Again the mixtures were vortexed, and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 25 µl 4× concentrated SDS-PAGE sample buffer containing 40 mM DTT. After incubation for 5 min at 80 °C, the

samples were loaded on a 12% SDS-PAGE gel and run overnight. Gels were stained with Coomassie brilliant blue R-250, dried under vacuum and autoradiographed for 16 – 24 h.

The D1 Cys protein content of yeast microsomal fractions was determined as described (Berry *et al.* 1992, Schoenmakers *et al.* 1992) by saturation analysis of the D1 protein labeling in the presence of a fixed amount of BrAc[¹²⁵I]T3 and increasing concentrations of non-radioactive BrAcT3, assuming that one molecule of D1 protein binds one molecule of BrAcT3.

Western blot of D1 protein

Homogenates or subcellular fractions (50 – 100 µg protein) were mixed with sample buffer (final concentration 40 mM Tris HCl, pH 6.8, 5% vol/vol glycerol, 2% wt/vol SDS and 10 mM DTT), heated for 5 min at 80 °C and centrifuged (10 000 *g*). The supernatants were separated on 12% SDS-PAGE gels in the Protean II mini-gel system (Bio-Rad), according to the manufacturer's instructions. After electrophoresis, the gels were positioned on nitrocellulose paper (Hybond C-pure, Amersham Pharmacia Biotech) and placed in a Bio-Rad mini Trans blot cell filled with buffer (25 mM Tris HCl, pH 8.3, 160 mM glycine and 20% vol/vol methanol). The transfer was performed for 60 min at 100 V. After blotting, the paper was blocked overnight in 10% wt/vol non-fat milk powder in PBS/0.1% vol/vol Tween 20 (PBS-Tween). Thereafter, the blot was incubated with a 1:1000 dilution of the D1 antiserum in 10% milk-PBS-Tween for 2 h up to overnight. After washing (2 × 15 min in 10% milk-PBS-Tween and 2 × 15 min in PBS-Tween), the blot was incubated with peroxidase-conjugated anti-rabbit IgG diluted 1:25 000 in 10% milk-PBS-Tween. After washing in the same way as described above, the reaction products were visualized with a home-made chemiluminescence system (Schneppenheim *et al.* 1991) consisting of 0.7 µM p-coumaric acid, 1.25 mM luminol, 0.01% hydrogen peroxide in 0.1 M Tris HCl, pH 8.5 and exposure to Kodak Biomax MS film.

Analysis of D1Cys post-translational modification

The antibiotic tunicamycin prevents the N-linked glycosylation and/or S-linked palmitoylation of proteins in eukaryotic cells (Orlean *et al.* 1991, Patterson & Pate Skene 1995). Tunicamycin was dissolved in a minimum amount of 1 M NaOH and diluted to a final concentration of 1 mg/ml with bidest. This stock solution was stored at –20 °C. Yeast cells were grown overnight at 30 °C in YPG medium or SC medium with up to 5 µg/ml tunicamycin. After homogenization, the 1000 *g* supernatant fraction was prepared and D1 protein labeled with BrAc[¹²⁵I]T3 as described.

N-glycosidase F cleaves between the innermost N-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (Orlean *et al.* 1991). For deglycosylation experiments, the D1 Cys protein in microsomal fractions was labeled with BrAc[¹²⁵I]T3 and immunoprecipitated with the 3049 D1 antiserum as described (Leonard *et al.* 2001). The purified D1 protein was incubated with 1000 U N-glycosidase F at 37 °C overnight in 50 mM sodium phosphate buffer (pH 7.5), 0.5% SDS, 1% mercaptoethanol and 1% NP-40.

Palmitoylated proteins are S-acetylated by attachment of palmitate via a thioester linkage to the sulfhydryl group of cysteine, and this linkage is cleaved by treatment with hydroxylamine (Liang *et al.* 2002). The D1 Cys protein in microsomal fractions was labeled with BrAc[¹²⁵I]T3 and thereafter incubated for 4 h with 1 M hydroxylamine (pH 8).

Results

Kinetic analysis of D1 activity in yeast subcellular fractions

The D1 Cys and D1 Ser cDNAs were cloned in the yeast expression vector pYES6. In this system proteins are expressed under the control of the *GAL1* promoter, allowing propagation of transformed cells in medium with a noninducing carbon source (glucose) and subsequent induction by the addition of galactose (Guengerich *et al.* 1991). An important feature of this system is the possibility to use different galactose induction times in order to avoid saturation of the cell's protein folding capacity (Griffith *et al.* 2003).

After induction (16 h), microsomal fractions (100 000 *g* pellet) were isolated from yeast cells harboring pYES6 (control yeast), pYES6-D1 Cys or pYES6-D1 Ser. The kinetic characteristics of the D1 Cys protein in yeast microsomal fractions were compared with those of the native D1 protein in rat liver microsomal fractions. Incubations in the presence of increasing concentrations (0.1 – 10 μ M) of rT3, T3 and T4 resulted in the progressive inhibition of the ORD of 10 nM [¹²⁵I]rT3 by D1 Cys and native D1 protein (Fig. 1A). The potencies by which the different iodothyronines inhibited the ORD of [¹²⁵I]rT3 decreased in the order rT3>T4>T3 for native D1 protein and rT3=T4>T3 for D1 Cys protein. The IC₅₀ values were 0.5 μ M rT3, 3 μ M T4 and >10 μ M T3 for native D1 protein and approximately 3 μ M rT3, 3 μ M T4 and >10 μ M T3 for D1 Cys protein. The apparent K_m (Michaelis-Menten constant) for rT3 ORD was 2.6 μ M ($n=3$) for the D1 Cys protein and 0.2 μ M ($n=2$) for the native D1 protein (Fig. 1B), while the V_{max} of the D1 Cys protein fraction was about half of that for the rat liver microsomal

fraction (400 vs 850 pmol rT3/min/mg). No significant deiodinase activity was present in microsomal fractions from yeast cells expressing D1 Ser protein or from control yeast cells.

ORD of rT3 or T4 by the D1 selenoprotein exhibits ping-pong kinetics with the iodothyronine and thiol-containing cofactor as cosubstrates (Visser 1980, Hennemann & Visser 1997). The selenol group (SeH) of the native enzyme is thought to be converted by reaction with rT3 into a selenenyl iodide (SeI) intermediate, which is converted back to the native enzyme by reaction with cofactor (DTT *in vitro*). The D1 inhibitors PTU (6-propyl-2-thiouracil) and IAc (iodoacetate) interact with the SeC residue, either with the SeI intermediate or the native SeH respectively. Keeping this in mind, it is not surprising that replacing the SeC residue by Cys (SeH to SH) reduces the sensitivity of D1 for PTU and IAc (Fig. 1C). In fact, the D1 Cys protein was essentially insensitive to the addition of PTU (IC₅₀>1000 μ M) and IAc (IC₅₀>100 μ M), in contrast to the marked inhibition of the D1 protein in rat liver microsomes (PTU IC₅₀=1 μ M and IAc IC₅₀=2 μ M).

IRD activity of D1 Cys protein was minimal. No significant deiodination of T3 could be detected, whereas with T3 sulfate (T3S) as substrate some IRD activity could be measured (K_m 2 μ M, V_{max} 8 pmol/min/mg, V_{max}/K_m 4). In comparison, the IRD of T3S by D1 protein in rat liver microsomes is more efficient (K_m 4 μ M and V_{max} 1000 pmol/min/mg, V_{max}/K_m 250) (Visser *et al.* 1983).

The thyroid hormone derivative *N*-bromoacetyl-T3 (BrAcT3) acts as an irreversible active site-directed inhibitor of D1 protein (Mol *et al.* 1984). Despite the SeC to Cys substitution, BrAcT3 strongly inhibited the ORD of rT3 by both the D1 Cys protein and the native D1 (D1 SeC) protein (Fig. 1C). The IC₅₀ value was about tenfold higher for the D1 Cys protein than for the native D1 protein (50 vs 5 nM).

In order to enable future purification by metal-chelation affinity-chromatography, the D1 Cys protein was also expressed with a C-terminal poly-His-tag (D1 Cys-His-tag). This affinity tag consists of 6 His residues and a linker of 23 amino acid residues (see Materials and methods). The kinetic characteristics of the D1 Cys-His-tag protein were essentially the same as those of the D1 Cys protein (K_m rT3 2.1 μ M and V_{max} 250 pmol/min/mg).

For evaluation of the subcellular localization of the D1 Cys protein in yeast cells, fractionation experiments were performed using sequential centrifugations (Fekkes *et al.* 1979, Kaiser *et al.* 2002). Both the 10 000 *g* and the 100 000 *g* membrane pellets were enriched in deiodinase activity compared with the 1000 *g* supernatant and the cytosolic fraction, indicating that the D1 Cys and D1 Cys-His-tag proteins are membrane associated in yeast cells (Fig. 1D). It should be realized that during yeast

Figure 1 Kinetic characterization and subcellular distribution of D1 Cys protein. (A) ORD of rT3 (10 nM ^{125}I -rT3) by rat D1 protein (open symbols) and yeast D1 Cys protein (closed symbols). Rat liver microsomes (5 μg protein/ml) and yeast microsomes (40 μg /ml) were incubated for 60 min at 37 °C in the presence of the indicated iodothyronine concentrations. (B) Double reciprocal plot of ORD of rT3 by microsomal fractions prepared from rat liver (D1 enzyme, open symbols) or from yeast (D1 Cys, closed symbols). The K_m and V_{max} values are 0.21 μM and 850 pmol/min/mg respectively for rat D1, and 2.6 μM and 400 pmol/min/mg respectively for D1 Cys protein. (C) Inhibition of rT3 ORD (100 nM ^{125}I -rT3) of rat liver D1 (open symbols) or D1 Cys (closed symbols) by PTU, IAc or BrAcT3. (D) Subcellular fractionation of yeast cells expressing D1 Cys protein (solid bars) or D1 Cys-His-tag protein (open bars). The rT3 ORD activity (100 nM ^{125}I -rT3) was measured in fractions A (1000 $\times g$ supernatant), B (10 000 $\times g$ pellet), C (100 000 $\times g$ pellet) and D (100 000 $\times g$ supernatant). Ctrl, control.

cell fractionation the plasma membranes are recovered almost entirely in the 10 000 g pellet, while the endoplasmic reticulum (ER) and Golgi complex are both divided between the 10 000 g and 100 000 g membrane fractions (Franzusoff *et al.* 1991). The possible location of D1 Cys protein in the plasma membrane was

investigated by sucrose-density gradient centrifugation (see next section under affinity labeling).

In an attempt to produce a soluble form of the D1 Cys protein, an expression vector was made in which the transmembrane domain (amino acid residues 2–35) was deleted. However, no deiodinase activity could be

Figure 2 Affinity-labeling of D1 Cys protein with BrAc[¹²⁵I]T3. Labeling patterns obtained by SDS-PAGE and autoradiography after reaction of yeast homogenates (H), 10 000 g pellets (P1) or 100 000 g pellets (P2) containing no recombinant protein (A), D1 Cys (B), D1 Ser (C), D1 Cys-His-tag (D), D1 Cys(Δ2–35)His-tag (E) or rat liver D1 protein (F) with BrAc[¹²⁵I]T3. Migration distances of molecular mass markers (kilodaltons) are indicated.

measured in any subcellular fraction prepared from yeast cells expressing the D1 Cys(Δ2–35)His-tag protein.

Affinity-labeling of D1 protein with BrAc[¹²⁵I]T3

As already described, BrAcT3 inhibited the ORD of rT3 by D1 Cys protein, indicating interaction of BrAcT3 with the D1 Cys protein. It was therefore decided to produce BrAc[¹²⁵I]T3 and to analyze the affinity-labeling of yeast subcellular fractions.

In Fig. 2 a typical example is shown of the labeling patterns obtained after reaction of BrAc[¹²⁵I]T3 with rat liver microsomes or yeast subcellular fractions. Using rat liver microsomes the protein band with an apparent molecular mass of 27 kDa represents D1 protein (Schoenmakers *et al.* 1992), while the more weakly labeled protein band of 56 kDa represents protein disulfide isomerase (Schoenmakers *et al.* 1989). Using D1 Cys-containing fractions several isoforms (at least 3) with apparent molecular masses between 27 and 32 kDa are

present, of which the 32 kDa band is the most strongly labeled. The increased apparent molecular mass of the D1 Cys protein in yeast fractions compared with the D1 protein in rat liver microsomes might reflect unique posttranslational modifications of D1 protein in yeast. It is known that post-translational modifications such as phosphorylation, glycosylation and acylation of proteins can result in so called ‘upshifts’ in apparent molecular mass during SDS-PAGE (Halligan *et al.* 2004, Wong *et al.* 2004). The D1 Cys-His-tag protein was detected as several isoforms after affinity-labeling (at least 3) with apparent molecular masses between 27–34 kDa (Fig. 2). In subcellular fractions of yeast expressing the D1 Ser or D1 Cys(Δ2–35)His-tag protein, either no or only a faintly labeled protein around 27 kDa was detected. The fact that in microsomal fractions (100 000 g pellet) from yeast cells expressing these proteins there was also no deiodinase activity measured could be explained by improper folding/processing or the absence of an essential domain.

Immunoblots (Fig. 3) were made using a rabbit antiserum directed against the C-terminus of the rat D1 protein (see Materials and methods). The D1 antibody recognized a single protein with an apparent molecular mass of 27 kDa in rat liver microsomes. In contrast, the D1 Cys protein from yeast microsomes migrated as four protein isoforms between 27 and 32 kDa. Microsomes containing D1 Cys-His-tag revealed protein isoforms between 27 and 34 kDa. For native and recombinant D1 similar isoform patterns were obtained after affinity-labeling and immunoblotting (Figs 2 and 3). Immunoblot analysis clearly proved the expression of the D1 Cys(Δ2–35)His-tag protein (single 27 kDa protein) and D1 Ser as several protein isoforms between 27 and 32 kDa (Fig. 3). Immunoblot analysis with an anti-His-tag antiserum proved the presence of the His-tag in D1 Cys(Δ2–35)His-tag protein (not shown). Obviously, the post-translational modification(s) of the D1 Cys(Δ2–35)His-tag and D1 Ser proteins are different from those of the D1 Cys protein (Fig. 3). Immunoblot analysis of

Figure 3 Immunoblot of D1 Cys protein. (A) Immunoblots of microsome fractions (100 000 g pellets) made from rat liver (lane 1 and 2) or yeast cells expressing D1 Cys(Δ2–35)His-tag (lane 3), D1 Cys-His-tag (lanes 4 and 5), D1 Ser (lane 6), and D1 Cys (lane 7) protein. Control yeast cells (lane 8) and *E. coli*-expressed D1 Cys-His-tag protein (lane 9) are also included. (B) Immunoblots of 10 000 g pellet (P1), 100 000 g pellet (P2) and cytosolic fractions (C) containing D1 Cys(Δ2–35)His-tag protein (E) or D1 Cys protein (B).

Figure 4 Sucrose-density gradient centrifugation of yeast homogenate containing D1 Cys protein. (A) ORD activity from 10% of each fraction (fraction 1=top and fraction 16=bottom) with 100 nM ^{125}I -rT3 in P100E2D10 buffer. (B) Labeling pattern obtained after incubation of 15% of each fraction with $\text{BrAc}[^{125}\text{I}]\text{T3}$ and SDS-PAGE.

the subcellular fractions revealed that the D1 Cys($\Delta 2$ –35)His-tag protein is predominantly present in the 10 000 *g* pellet, whereas the D1 Cys protein is more equally distributed over the 10 000 *g* and 100 000 *g* membrane fractions (Fig. 3B). In the cytosolic extracts no D1 Cys nor D1 Cys($\Delta 2$ –35)His-tag could be detected. For comparison, the D1 Cys-His-tag protein expressed in *E. coli* (inclusion bodies) is included on the Western blot (Fig. 3A lane 9).

The D1 content of D1 Cys and D1 Cys-His-tag microsomal fractions was determined by saturation analysis of progressive labeling of the proteins in the 27–34 kDa bands with increasing concentrations of unlabeled BrAcT3 as described previously (Berry *et al.* 1992, Schoenmakers *et al.* 1992). The D1 content thus calculated is 65 pmol/mg protein and together with the V_{max} for ORD of rT3 of this preparation (250 pmol/min/mg), this results in a substrate turnover number of about 4/min.

Fractionation by equilibrium sedimentation is a suitable method for identifying proteins that are located in either the ER or the plasma membranes. The plasma membranes are of relatively high density and under appropriate conditions they can be resolved from the relatively less dense membranes derived from the ER and Golgi complex (Kaiser *et al.* 2002). Sucrose-density gradients were run (see Materials and methods) and the fractions were analyzed by rT3 deiodination assays as well as by affinity-labeling with $\text{BrAc}[^{125}\text{I}]\text{T3}$ (Fig. 4).

Relatively high levels of activity were found in fractions 12 and 13 (fraction 1=top, fraction 16=bottom), while in fractions 6–10 intermediate levels of activity were found (Fig. 4A). The plasma membranes are present in fractions 12–14, while the ER and Golgi complex membranes are divided over fractions 6–10 (Kaiser *et al.* 2002). With regard to plasma membranes this was confirmed since SDS-PAGE showed the presence of the 100 kDa Pmalp plasma membrane H^+ -ATPase protein (Hasper *et al.* 1999) in fractions 12–14 (not shown). The presence of D1 Cys protein (27–32 kDa isoforms) in plasma membrane as well as in the ER and Golgi complex was confirmed by $\text{BrAc}[^{125}\text{I}]\text{T3}$ labeling of the fractions (Fig. 4B).

Post-translational modification of D1 protein in yeast

Galactose-induced D1 Cys protein-expressing yeast cells were harvested at different time points (4, 6, 8, 24, 32 and 52 h), and lysates were analyzed by ^{125}I -BrAcT3 labeling. From 6 h onwards the D1 Cys protein was detectable, and reached optimum levels at 24–32 h. At each time point the described three isoforms with apparent molecular masses from 27 to 32 kDa were present and the 32 kDa isoform was the most prominent (results not shown).

Many membrane and secretory proteins are glycoproteins, and yeast adds N-linked and/or O-linked oligosaccharides to glycoproteins (Orlean *et al.* 1991, Gemmill & Trimble 1999). The N-linked oligosaccharides are added co-translationally in the ER to asparagine residues within the consensus sequence Asn-X-Ser/Thr. The rat D1 protein contains two potential N-linked glycosylation sites at Asn residues 94 and 203. Therefore, we initially focussed on the possibility of N-linked glycosylation as an explanation for the observed heterogeneity of D1 protein. Tunicamycin is an inhibitor of N-linked glycosylation (Orlean *et al.* 1991). Yeast cells expressing the D1 Cys protein were grown overnight in the presence of tunicamycin, and after homogenization the D1 protein was labeled with $\text{BrAc}[^{125}\text{I}]\text{T3}$. No change in the 27–32 kDa isoform pattern was observed (not shown). However, incubation with tunicamycin dose-dependently reduced the growth of yeast cells in YPD medium (about 15-fold reduction in OD600 at 5 μg tunicamycin/ml). Due to the toxicity of tunicamycin it was not possible to increase the concentration further. Incubation of the D1 Cys protein with N-glycosidase F (see Materials and methods) did not result in changes in the 27–32 kDa isoform pattern either (not shown).

Since these experiments were inconclusive it was decided to mutate the potential N-linked glycosylation sites at asparagine residues 94 and 203. At this point, the possibility of alternative post-translational modifications was taken into consideration by site-directed

mutagenesis of potential phosphorylation sites (Blom *et al.* 1999) at serine residues 176 (casein kinase II site), serine residue 101 (protein kinase A), tyrosine residues 209 and 217 (tyrosine kinase sites) and a potential N-myristoylation site (Utsumi *et al.* 2001) at glycine residue 2.

None of these mutations caused complete reversal of the 27 – 32 kDa isoform pattern, indicating that N-linked glycosylation, myristoylation or phosphorylation at the indicated sites alone are not the source of the observed heterogeneity (Fig. 5). Some minor, but reproducible changes were observed. In the case of the D1 Cys-Tyr209 Phe/Tyr217 Phe double mutant protein the isoform pattern as a whole was downshifted to lower apparent molecular masses. This suggests phosphorylation of Tyr residues 209 and/or 217 in all D1 Cys isoforms, including the 27 kDa isoform. Remarkably, for the Gly2 Ala mutant protein (removing a potential N-myristoylation site) the isoform pattern as such was preserved but the labeling pattern was reversed in the sense that the lower 27 kDa protein isoform was now most strongly labeled. This change in isoform pattern (confirmed by Western blotting, see Fig. 5C) suggests (partial) myristoylation of D1 Cys protein at glycine residue 2 or altered targeting of the D1 CysGly2 Ala protein, thereby influencing another post-translational modification(s). N-myristoylation and/or S-palmitoylation are required for plasma membrane targeting of various proteins, for instance heterotrimeric G-protein subunits (Manahan *et al.* 2000, Chen & Manning 2001). In the case of palmitoylation, the 16-carbon fatty acid chain is attached via a thioester linkage to cysteine residues, while in the case of myristoylation the 14-carbon fatty acid chain is attached via an amide linkage to an amino-terminal glycine residue. Palmitoylation is sensitive to treatment with hydroxyl amine, while myristoylation is not (Manahan *et al.* 2000). Treatment with hydroxyl amine (see Materials and methods) did not change the isoform pattern during SDS-PAGE, thereby excluding palmitoylation of D1 Cys protein as the cause of the observed heterogeneity (not shown).

The kinetic characteristics of the mutant D1 Cys proteins were determined (Table 1 and Fig. 6A and B). Especially, the double mutant proteins, i.e. D1 Cys-Asn94 Gln/Asn203 Gln, D1 Cys-Ser101 Ala/Ser176 Ala and D1 Cys-Tyr209 Phe/Tyr217 Phe had significantly reduced enzyme activity as reflected in increased K_m values and/or decreased V_{max} values. The kinetic characteristics of the D1 Cys-Gly2 Ala protein are similar to those of the D1 Cys and D1 Cys-His tag protein. As already stated, myristoylation at glycine residue 2 is required for plasma membrane targeting of various proteins, for instance heterotrimeric G-protein subunits (Manahan *et al.* 2000). Upon subcellular fractionation of yeast cells the distribution of the D1 Cys

Figure 5 Affinity-labeling with BrAc^[125I]T3 and Western blotting of D1 Cys protein and mutant proteins. (A) Labeling patterns obtained by SDS-PAGE and autoradiography after reaction of control yeast homogenate (lane 1) or homogenates containing D1 Cys (lane 2), D1 CysSer176 Ala (lanes 3 and 4), D1 Cys-Tyr209 Phe/Tyr217 Phe (lanes 5 and 6), D1 CysSer101 Ala (lanes 7 and 8), D1 Cys-Gly2 Ala (lanes 9 and 10), D1 Cys-Ser101 Ala/Ser176 Ala (lanes 11 and 12) or rat liver D1 protein (lane 13) with BrAc^[125I]T3. The homogenates contained 20 – 25 µg protein. Migration distances of molecular mass markers (kilodaltons) are indicated. (B) Labeling patterns obtained by SDS-PAGE and autoradiography after reaction of control yeast homogenate (lane 1) or homogenates containing D1 Cys (lane 2), D1 CysAsn94 Gln (lanes 3 and 4), D1 Cys-Asn203 Gln (lanes 5 and 6), D1 Cys-Asn94 Gln/Asn203 Gln (lanes 7 and 8) or rat liver D1 protein (lane 9) with BrAc^[125I]T3. The homogenates contained 20 µg protein. Migration distances of molecular mass markers (kilodaltons) are indicated. (C) Western blot of homogenates (10 µg protein) containing D1 Cys (lane 1), D1 CysSer101 Ala (lane 2), D1 Cys-Ser176 Ala (lane 3), D1 Cys-Ser101 Ala/Ser176 Ala (lane 4), D1 Cys-Tyr209 Phe/Tyr217 Phe (lane 5), D1 Cys-Gly2 Ala (lane 6), D1 Cys-Asn94 Gln (lane 7), D1 Cys-Asn203 Gln (lane 8) and D1 Cys-Asn94 Gln/Asn203 Gln protein (lane 9).

and D1 Cys-Gly2 Ala protein over the membrane fractions was not different (Fig. 6C). So, there is no evidence for the involvement of N-myristoylation in the membrane targeting of D1 Cys protein.

Table 1 Kinetic characteristics of D1Cys mutant proteins

Protein	K_m (μ M)	V_{max} (pmol/min/mg)
D1Cys	2.8/2.4	162/120
D1Cys-His tag	1.7	87
D1Cys-Asn94Gln	1.8	56
D1Cys-Asn203Gln	4.5	61
D1Cys-Asn94Gln/Asn203Gln	2.9	19
D1Cys-Ser101Ala	2.2/3.2	84/94
D1Cys-Ser176Ala	2.0/2.4	96/84
D1Cys-Ser101Ala/Ser176Ala	>5/>5	112/57
D1Cys-Tyr209Phe/Tyr217Phe	>5/>5	61/–
D1Cys-Gly2Ala	1.5/2.4	100/91

Two values in the same column are the results of duplicate determinations.

Discussion

Establishment of an overexpression system aimed at the production of active D1 protein is important in order to enable studies on cellular targeting and processing, as well as structure determination via either electron or X-ray crystallography (Schmidt-Krey *et al.* 1998, Caffrey 2003). The overexpression of D1 protein represents a challenge because of several of its intrinsic properties: (1) the presence of an SeC residue in the catalytic center, (2) being an integral membrane protein, and (3) the requirement of a suitable native-like lipid environment essential for stability and catalytic activity upon purification (Mol *et al.* 1988). Although it is possible to overexpress certain SeC-containing proteins in *E. coli* (Müller *et al.* 1994), the expression of integral membrane proteins is difficult due to the lack of intracellular membranes into which proteins can sequester and fold. Initial attempts to overexpress D1 Cys protein in *E. coli* yielded large amounts of insoluble proteins which could not be renatured (not shown). Eukaryotic cells, such as insect and COS cells, can produce SeC-containing proteins but only in limited amounts (Berry *et al.* 1992, 1993, Kim *et al.* 1997, Kuiper *et al.* 2003) due to the inefficient synthesis of SeC-(Ser)-tRNA.

We believe that our system, involving expression of D1 Cys protein in yeast, is a good compromise. Substitution of Cys for SeC (i.e. sulfur for selenium) causes a 10-fold increase in the apparent K_m value of the enzyme rT3 ORD, compared with native D1 in rat liver microsomes. The substrate turnover number is about 200-fold reduced compared with native D1, i.e. 4/min vs 800/min (our results and Schoenmakers *et al.* 1992). Similar results, i.e. a 10-fold increased K_m value and a 100-fold decreased substrate turnover number were reported for the D1 Cys protein expressed in COS cells (Berry *et al.* 1991b, 1992). After affinity-labeling with BrAc[¹²⁵I]T3 of D1 Cys protein in COS cell homogenates, a single protein with apparent molecular mass of 27 kDa was detected after SDS-PAGE (Berry

et al. 1992). The fact that D1 Cys protein in COS cell homogenates (27 kDa) and yeast cell homogenates (27 – 32 kDa isoforms) have similar kinetic characteristics suggests that the isoform heterogeneity of the yeast-expressed protein does not change the enzyme kinetic properties. The ORD of rT3 by D1 Cys was essentially insensitive to PTU, showing that inhibition by PTU is highly dependent on the selenium atom in D1. Most likely, PTU inhibition is due to the formation of an enzyme-Se-S-PTU adduct (Visser 1980). In D1 Cys protein the formation of a mixed disulfide (enzyme-S-S-PTU) is either unlikely or the mixed disulfide is unstable. In comparison, the reduction in sensitivity for the covalent affinity-label BrAcT3 was only minor. The content of D1 Cys present in yeast microsomes is about 20-fold higher than the D1 content in rat liver microsomes, i.e. 65 pmol/mg vs 3 pmol/mg. The amount of D1 Cys protein produced (about 0.1 mg/1 yeast culture) is in principle enough for the identification of radiolabeled amino acid residue(s) after affinity-labeling with BrAc-[¹²⁵I]iodothyronine derivatives (BrAcT3, BrAcT4, BrAcT3) and sequencing of purified radioactive peptides. Site-directed mutagenesis of this identified substrate interacting residue(s) in the context of D1 Cys and D1 SeC protein could provide more insight in the molecular details of iodothyronine-deiodinase recognition. As already stated, the expression of D1 Cys in yeast is a compromise, as the substitution of SeC by Cys in the catalytic center leads to significant changes in substrate specificity and inhibitor profile. It remains to be determined whether it will be possible to extract and subsequently purify the D1 Cys-His-tag protein in the amounts needed for further structural analysis. No systems are available to overexpress the native (SeC containing) enzyme and therefore structural characterisation of the D1 Cys protein might be a good starting point. However, at this moment it remains an open question whether it will be possible to model the structure of the native D1 enzyme on that of the D1 Cys protein.

Subcellular fractionation experiments showed that in yeast the D1 Cys protein is membrane-bound. The 10 000 *g* and 100 000 *g* membrane pellets contained about equal amounts of D1 Cys protein, and sucrose-density gradient analysis confirmed that the D1 Cys protein is divided over the ER, Golgi complex and plasma membrane. This is in line with data generated by the P-Sort (Nakai & Horton 1999) and TargetP

(Emanuelsson *et al.* 2000) predictive models for subcellular protein localization. Both models predict that the D1 protein contains an uncleaved signal peptide and is divided over ER, Golgi complex and plasma membrane. There is general agreement that D1 is an integral membrane protein but different cellular localizations have been found. In rat kidney, D1 is present in the plasma membrane (Leonard & Rosenberg 1978, Leonard *et al.* 1991, 2001), while in rat liver D1 may be present in the ER with its active site oriented to the cytoplasm (Auf dem Brinke *et al.* 1979, Fekkes *et al.* 1979, Toyoda *et al.* 1995b). By immunofluorescence confocal microscopy the D1 protein was localized to the plasma membrane of transiently transfected COS cells (Baqui *et al.* 2000). Perhaps the D1 protein is incorporated in two topologies in the ER membrane, that is a type I orientation (N-terminus luminal/C-terminus cytosolic) and a type II orientation (N-terminus cytosolic/C-terminus luminal). In this model, the type I orientation would result in ER-retention, while the type II orientation is associated with transport to the Golgi complex and plasma membrane. The extent to which the D1 is divided over the type I and type II orientations could be cell-type dependent thus explaining the different localizations found. Cytochrome P450 enzymes are also localized in the ER and plasma membrane. For CYP 2E1 it was shown that the N-terminal transmembrane domain plays a critical role in directing the protein to the plasma membrane. The topological inversion (type II orientation) of a part of the CYP 2E1 protein directs it to the plasma membrane while the remainder is retained in the ER in a type I orientation (Neve & Ingelman-Sundberg 2000). Yeast could be a useful model system for studying the targeting of D1 and especially the role of the hydrophobic N-terminal transmembrane domain, much in the same way as has been done for CYP 2E1 (Neve *et al.* 2003).

Surprisingly, the D1 Cys(Δ 2–35)His-tag protein was still membrane associated and absent from the cytosolic fraction. Hydropathy analysis and *in vitro* translation studies using pancreatic microsomes have identified a single transmembrane sequence between amino acid residues 13 to 34 of rat D1 (Toyoda *et al.* 1995b). The

Figure 6 Kinetic characterisation of D1 Cys mutant proteins. (A) Double reciprocal plots of 125 I-rT3 ORD by homogenates containing D1 Cys, D1 Cys-His-tag and D1 Cys protein with mutations of potential N-linked glycosylation sites (Asp94 and Asp203). (B) Double reciprocal plots of 125 I-rT3 ORD by homogenates containing D1 Cys and D1 Cys protein with mutations of potential phosphorylation sites (Ser101, Ser176, Tyr209, Tyr217) or N-linked myristoylation site Gly2. (C) Subcellular fractionation of yeast cells expressing D1 Cys (solid bars) or D1 Cys-Gly2 Ala (open bars) protein. The rT3 ORD activity (100 nM 125 I-rT3) was measured in fractions A (1000 *g* supernatant), B (10 000 *g* pellet), C (100 000 *g* pellet) and D (100 000 *g* supernatant).

fact that D1 Cys(Δ 2–35)His-tag is not cytosolic might point to the existence of alternative transmembrane domain(s). The HMMTOP-model, which is based on the hypothesis that topology of membrane proteins is determined by maximal divergence of the various structural parts, predicts two transmembrane domains between amino acid residues 13–33 and 56–75 for rat D1 (Tusnády & Simon 1998). The first transmembrane domain (13–33) has a fivefold higher probability than the second one. It should also be noted that the D1 Cys(Δ 2–35)His-tag protein is inactive, probably due to improper folding. Very similar results have been obtained after overexpression of 11 β -hydroxysteroid dehydrogenase and CYP 2E1 in yeast, i.e. membrane attachment despite deletion of the hydrophobic N-terminal membrane anchor sequence (Blum *et al.* 2001, Neve *et al.* 2003). Whether these results are related to the overexpression in yeast or truly reflect the existence of additional transmembrane domains remains to be investigated.

From the immunoblotting and affinity-labeling data it is clear that while native D1 protein (rat liver) migrates as a single 27 kDa protein on SDS-PAGE the D1 Cys protein is present as several isoforms with molecular masses between 27 and 32 kDa. This difference is most likely caused by differential post-translational modification. There is no experimental evidence available for any post-translational modification of native D1 protein or D1 Cys expressed in COS cells. So, the D1 Cys heterogeneity is a special feature connected to the expression in yeast cells. The heterogeneity might be caused by a combination of several common modifications of proteins expressed in yeast such as phosphorylation, N-linked glycosylation, O-linked glycosylation and fatty acid acylation (Tanner & Lehle 1987, Simon & Aderem 1992, Li *et al.* 2001). By site-directed mutagenesis of consensus N-linked glycosylation sites and treatment with N-glycosidases, we found no evidence for N-linked glycosylation and it appears to be excluded. O-linked glycosylation begins in yeast in the ER by addition of a single mannose to either Ser or Thr residues and once transported to the Golgi complex sugar transferases add one or more mannose units (Tanner & Lehle 1987, Gemmill & Trimble 1999). It is difficult to prove possible O-linked glycosylation of D1 Cys protein since no glycosidases aimed at mannose sugars are available and chemical deglycosylation with hydrogen fluoride of O-linked sugars is often not complete or destroys the polypeptide chain (Sojar & Bahl 1987). In various instances Ser or Thr phosphorylation sites are also targets for O-linked glycosylation. We mutated several potential phosphorylation sites (Ser 101 and 176) but this did not change D1 Cys heterogeneity. Many more (21 in total) Ser or Thr residues are present in D1 Cys protein and they could all be targets for O-linked glycosylation. All in all, we have no evidence

for O-linked glycosylation but we also cannot exclude it as a cause of D1 Cys heterogeneity. Site-directed mutagenesis of several consensus phosphorylation sites provided evidence for phosphorylation at Tyr residues 209 and/or 217 (downshift of complete isoform pattern). Phosphorylation at other site(s) by kinases with unknown specificity cannot be excluded.

N-Myristoylation is a common modification of eukaryotic membrane proteins, and there is ample evidence that myristoylation is involved in membrane attachment and targeting of these proteins (Simon & Aderem 1992, Manahan *et al.* 2000, Chen & Manning 2001). The rat D1 protein fulfils the minimal requirement for N-linked myristoylation, that is an N-terminal Met-Gly sequence (Utsumi *et al.* 2001). On the other hand the MYR predictor program (Maurer-Stroh *et al.* 2002), which is based upon the substrate specificity and structure of the eukaryotic enzyme myristoyl-CoA-protein N-myristoyltransferase (NMT) does not predict myristoylation of the D1 protein. In various cases it has been shown by site-directed mutagenesis (Gly2 Ala) that disturbed myristoylation of G-protein subunits in yeast cells causes mislocalization of protein, resulting in accumulation of inactive proteins in ER and Golgi complex (Song *et al.* 1996). In contrast, the D1 Cys-Gly2 Ala mutant protein had kinetic characteristics and membrane distribution properties which are indistinguishable from those for the D1 Cys protein. Therefore, although part of the D1 Cys protein might be myristoylated (based on the different isoform pattern on SDS-PAGE of D1 Cys-Gly2 Ala) there is no evidence for a functional role.

In summary, heterologous expression of D1 Cys protein in yeast resulted in the overproduction of a membrane-bound bioactive enzyme. Although the D1 Cys protein expressed in yeast is posttranslationally modified in a different manner to the D1 Cys protein expressed in COS cells the kinetic characteristics are similar. Future experiments will be aimed at purification of the D1 Cys protein and overexpression in other yeast strains such as *Pichia pastoris* or *Hansenula polymorpha*, which could result in even higher expression levels and more authentic post-translational modification characteristics (Bill 2001, Li *et al.* 2001).

Acknowledgements

This work was supported by The Netherlands Organization of Scientific Research (grant number 903–40–194) and the FP5 Quality of Life Research program of the European Union (contract number QL3-CT-2000–00930). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Auf Dem Brinke D, Hesch RD & Köhrle J 1979 Re-examination of the subcellular localization of thyroxine 5'-deiodination in rat liver. *Biochemical Journal* **180** 273–279.
- Baqui MMA, Gereben B, Harney JW, Larsen PR & Bianco AC 2000 Distinct subcellular localization of transiently expressed types 1 and 2 iodothyronine deiodinases determined by immunofluorescence confocal microscopy. *Endocrinology* **141** 4309–4312.
- Berry MJ, Banu L & Larsen PR 1991a Type I iodothyronine deiodinase is a seleno- cysteine containing enzyme. *Nature* **349** 438–440.
- Berry MJ, Kieffer JD, Harney JW & Larsen PR 1991b Selenocysteine confers the biochemical properties characteristic of the type I iodothyronine deiodinase. *Journal of Biological Chemistry* **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.
- Berry MJ, Banu L, Harney JW & Larsen PR 1993 Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. *EMBO Journal* **12** 3315–3322.
- Bianco AC, Salvatore D, Gereben B, Berry ML & Larsen PR 2002 Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine deiodinases. *Endocrine Reviews* **23** 38–89.
- Bill RM 2001 Yeast: a panacea for the structure-function analysis of membrane proteins? *Current Genetics* **40** 157–171.
- Blom N, Gammeltoft S & Brunak S 1999 Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology* **294** 1351–1362.
- Blum A, Raum A, Martin H-J & Maser E 2001 Human 11 β -hydroxysteroid dehydrogenase 1/carbonyl reductase: additional domains for membrane attachment? *Chemico Biological Interactions* **130–132** 749–759.
- Caffrey M 2003 Membrane protein crystallization. *Journal of Structural Biology* **142** 108–132.
- Chen CA & Manning DR 2001 Regulation of G proteins by covalent modification. *Oncogene* **20** 1643–1652.
- Doorn van J, van der Heide D & Roelfsema F 1983 Sources and quantity of 3,5,3'- triiodothyronine in several tissues of the rat. *Journal of Clinical Investigation* **72** 1778–1792.
- Eelkman Rooda SJ, van Loon MAC & Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. *Journal of Clinical Investigation* **79** 1740–1748.
- Emanuelsson O, Nielsen H, Brunak S & von Heijne G 2000 Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300** 1005–1016 (see <http://www.cbs.dtu.dk/services/TargetP/>).
- Franzusoff A, Rothblatt J & Schekman R 1991 Analysis of polypeptide transit through yeast secretory pathway. *Methods in Enzymology* **194** 662–674.
- Fekkes D, van Overmeeren-Kaptein E, Docter R, Hennemann G & Visser TJ 1979 Location of rat liver iodothyronine deiodinating enzymes in the endoplasmic reticulum. *Biochimica et Biophysica Acta* **587** 12–19.
- Gemmell TR & Trimble RB 1999 Overview of N- and O-linked oligosaccharide structures found in various yeast species. *Biochimica et Biophysica Acta* **1426** 227–237.
- Griffith DA, Delipala C, Leadsham J, Jarvis SM & Oesterhelt D 2003 A novel yeast expression system for the overproduction of quality-controlled membrane proteins. *FEBS Letters* **553** 45–50.
- Guengerich FP, Brian WR, Sari M-A & Ross JT 1991 Expression of mammalian cytochrome P450 enzymes using yeast based vectors. *Methods in Enzymology* **206** 130–145.
- Halligan BD, Ruotti V, Jin W, Laffoon S, Twigger SN & Dratz EA 2004 ProMoST (protein modification screening tool): a web-based tool for mapping protein modifications on two-dimensional gels. *Nucleic Acid Research* **32** W638–W644.
- Hasper A, Soteropoulos P & Perlin DS 1999 Modification of the N-terminal polyserine cluster alters stability of the plasma membrane H⁺-ATPase from *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **1420** 214–222.
- Hennemann G & Visser TJ 1997 Thyroid hormone synthesis, plasma membrane transport and metabolism. *Handbook of Experimental Pharmacology* **128** 76–117.
- Hult M, Nobel CSI, Abrahmsen L, Nicoll-Griffith DA, Jornvall H & Oppermann UCT 2001 Novel enzymological profiles of human 11 β -hydroxysteroid dehydrogenase type 1. *Chemico Biological Interactions* **130–132** 805–814.
- Ito H, Fukuda Y, Murata K & Kimura A 1983 Transformation of intact yeast cells treated with alkali cations. *Journal of Bacteriology* **153** 163–168.
- Kaiser CA, Chen EJ & Losko S 2002 Subcellular fractionation of secretory organelles. *Methods in Enzymology* **351** 325–338.
- Kim IY, Guimarães MJ, Zlotnik A, Bazan JF & Stadtman TC 1997 Fetal mouse selenophosphate synthetase 2: characterization of the cysteine mutant form over- produced in a baculovirus-insect cell system. *PNAS* **94** 418–421.
- Köhrle J 2002 Iodothyronine deiodinases. *Methods in Enzymology* **347** 125–167.
- Köhrle J, Rasmussen UB, Ekenbarger DM, Alex S, Rokos H, Hesch RD & Leonard JL 1990 Affinity labeling of rat liver and kidney type I deiodinase. *Journal of Biological Chemistry* **265** 6155–6163.
- Kuiper GGJM, Wassen F, Klootwijk W, van Toor H, Kaptein E & Visser TJ 2003 Molecular basis for the substrate selectivity of cat type I iodothyronine deiodinase. *Endocrinology* **144** 5411–5421.
- Kuiper GGJM, Kester MHA, Peeters RP & Visser TJ 2005 Biochemical mechanisms of thyroid hormone deiodination. *Thyroid* In Press.
- Leonard JL & Rosenberg IN 1978 Subcellular distribution of thyroxine 5'-deiodinase in the rat kidney: a plasma membrane location. *Endocrinology* **103** 274–280.
- Leonard JL, Ekenbarger DM, Frank SJ, Farwell AP & Köhrle J 1991 Localization of type I iodothyronine 5'-deiodinase to the basolateral plasma membrane in renal cortical epithelial cells. *Journal of Biological Chemistry* **266** 11262–11269.
- Leonard JL, Visser TJ & Leonard DB 2001 Characterization of the subunit structure of catalytically active type I iodothyronine deiodinase. *Journal of Biological Chemistry* **276** 2600–2607.
- Lescure A, Gautheret D, Carbon P & Krol A 1999 Novel selenoproteins identified *in silico* and *in vivo* by using a conserved RNA structural motif. *Journal of Biological Chemistry* **274** 38147–38154.
- Li P, Gao X-G, Arellano RO & Renugopalakrishnan V 2001 Glycosylated and phosphorylated proteins – expression in yeast and oocytes of *Xenopus*: prospects and challenges. *Protein Expression and Purification* **22** 369–380.
- Liang X, Lu Y, Neubert TA & Resh MD 2002 Mass spectrometric analysis of GAP-43/neuromodulin reveals the presence of a variety of fatty acetylated species. *Journal of Biological Chemistry* **277** 33032–33040.
- Manahan CL, Patnana M, Blumer KJ & Linder ME 2000 Dual lipid modification motifs in G α and G γ subunits are required for full activity of the pheromone response pathway in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **11** 957–968.
- Maurer-Stroh S, Eisenhaber E & Eisenhaber F 2002 N-terminal N-myristoylation of proteins: prediction of substrate proteins from amino acid sequences. *Journal of Molecular Biology* **317** 541–557.
- 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. *Biochemical and Biophysical Research Communications* **124** 475–483.
- Mol JA, van den Berg TP & Visser TJ 1988 Partial purification of the microsomal rat liver iodothyronine deiodinase. *Molecular and Cellular Endocrinology* **55** 149–166.
- Moreno M, Berry MJ, Horst C, Thoma R, Coglia F, Harney JW, Larsen PR & Visser TJ 1994 Activation and inactivation of thyroid hormone by type I iodothyronine deiodinase. *FEBS Letters* **344** 143–146.
- Müller S, Senn H, Gsell B, Vetter W, Baron C & Böck A 1994 The formation of diselenide bridges in proteins by incorporation of selenocysteine residues: biosynthesis and characterisation of (Se)2-thioredoxin. *Biochemistry* **33** 3404–3412.
- Nakai K & Horton P 1999 PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends in Biochemical Sciences* **24** 34–36 (see <http://www.hgc.ims.u-tokyo.ac.jp/knakai/>).
- Neve EPA & Ingelman-Sundberg M 2000 Molecular basis for the transport of cytochrome P450 2E1 to the plasma membrane. *Journal of Biological Chemistry* **275** 17130–17135.
- Neve EPA, Hidestrand M & Ingelman-Sundberg M 2003 Identification of sequences responsible for intracellular targeting and membrane binding of rat CYP 2E1 in yeast. *Biochemistry* **42** 14566–14575.
- 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.
- Orlean P, Kuranda MJ & Albright CF 1991 Analysis of glycoproteins from *Saccharomyces cerevisiae*. *Methods in Enzymology* **194** 682–698.
- Parikh A & Guengerich FP 1998 Random mutagenesis by whole-plasmid PCR amplification. *BioTechniques* **24** 428–431.
- Patterson SI & Pate Skene JH 1995 Inhibition of dynamic protein palmitoylation in intact cells with tunicamycin. *Methods in Enzymology* **250** 284–300.
- Sambrook J & Russell DW 2001 *In vitro* mutagenesis using double-stranded DNA templates: selection of mutants with DpnI. In *Molecular Cloning, a Laboratory Manual*, pp 13:19–13:25. Cold Spring Harbor, NY: Cold Spring Harbor Press.
- Schmidt-Krey I, Lundqvist G, Morgenstern R & Hebert H 1998 Parameters for the two-dimensional crystallisation of the membrane protein microsomal glutathione transferase. *Journal of Structural Biology* **123** 87–96.
- Schneppenheim R, Budde U, Dahlmann N & Rautenberg P 1991 Luminography – a new, highly sensitive visualization method for electrophoresis. *Electrophoresis* **12** 367–372.
- Schoenmakers C, Pigman I, Hawkins H, Freedman R & Visser TJ 1989 Rat liver type I iodothyronine deiodinase is not identical to protein disulfide isomerase. *Biochemical and Biophysical Research Communications* **162** 857–868.
- Schoenmakers CHH, Pigman IGJ & Visser TJ 1992 Species differences in liver type I iodothyronine deiodinase. *Biochimica et Biophysica Acta* **1121** 160–166.
- Sherman F 1991 Getting started with yeast. *Methods in Enzymology* **194** 3–21.
- Simon SM & Aderem A 1992 Myristoylation of proteins in the yeast secretory pathway. *Journal of Biological Chemistry* **267** 3922–3931.
- Sojar HT & Bahl OP 1987 Chemical deglycosylation of glycoproteins. *Methods in Enzymology* **138** 341–350.
- Song J, Hirschman J, Gunn K & Dohlman HG 1996 Regulation of membrane and subunit interactions by N-myristoylation of a G protein alpha subunit in yeast. *Journal of Biological Chemistry* **271** 20273–20283.
- Tanner W & Lehle L 1987 Protein glycosylation in yeast. *Biochimica et Biophysica Acta* **906** 81–99.
- Toyoda N, Harney JW, Berry MJ & Larsen PR 1995a Identification of critical amino acids for 3,3',5'-triiodothyronine deiodination by human type I deiodinase based on comparative functional-structural analysis of the human, dog and rat enzymes. *Journal of Biological Chemistry* **269** 20329–20334.
- Toyoda N, Berry MJ, Harney JW & Larsen PR 1995b Topological analysis of the integral membrane protein, type I iodothyronine deiodinase. *Journal of Biological Chemistry* **270** 12310–12318.
- Toyoda N, Kaptein E, Berry MJ, Harney JW, Larsen PR & Visser TJ 1997 Structure–activity relationships for thyroid hormone deiodination by mammalian type I iodothyronine deiodinases. *Endocrinology* **138** 213–219.
- Tusnády GE & Simon I 1998 Principles governing amino acid composition of integral membrane proteins: application to topology prediction. *Journal of Molecular Biology* **283** 489–506 (see <http://www.enzim.hu/hmmtop/server/hmmtop.cgi>).
- Utsumi T, Sato M, Nakano K, Takemura D, Iwata H & Ishisaka R 2001 Amino acid residue penultimate to the amino-terminal Gly residue strongly affects two cotranslational protein modifications, N-myristoylation and N-acetylation. *Journal of Biological Chemistry* **276** 10505–10513.
- Visser TJ 1980 Mechanism of inhibition of iodothyronine 5'-deiodinase by thioureylenes and sulfite. *Biochimica et Biophysica Acta* **611** 371–378.
- Visser TJ, Fekkes D, Docter R & Hennemann G 1978 Sequential deiodination of thyroxine in rat liver homogenate. *Biochemical Journal* **174** 221–229.
- Visser TJ, Mol JA & Otten MH 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. *Endocrinology* **112** 1547–1549.
- Wong H-Y, Burghoorn J, van Leeuwen M, de Ruiter PE, Schippers E, Blok L, Li KW, Dekker H, de Jong L, Trapman J, Grootegoed J & Brinkmann AO 2004 Phosphorylation of androgen receptor isoforms. *Biochemical Journal* **383** 267–276.

Received 1 March 2005

Accepted 7 March 2005