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

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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 µM) 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.

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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, Körhle 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, Körhle 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 succesfully 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′,3′-125I]T3 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-bromoacetylccloride 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. Peptide, 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 YEEVRAVLE-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 (SeG126 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’-GGCGGATCCATGG GGCTGTCCCAGCTA and G21B 5′-GGCGGATCC CATAGAACTGAGGAGATGTGTC (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'-GGCTC-TAGG4GAATGAGG-CATG TGTTCC (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 (SRGFEGKIPPLMLGLSTRTGHHH 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'-GGCG GGATCCATGAGAGTCAAGCAAGACAC). BamHI in italics and start codon underlined) and the G21BX primer resulting in the D1 Cys-Tyr209 Phe/Tyr217 Phe double mutant 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 cycler. 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 CysAsn203 Gln expression vectors were produced using overlapping sense and antisense primers containing the nucleotide changes needed, that is for Asn94 Gln sense 5'GGGCTGTTGCCCAAGCTGAGG-CATG TGTTCC and for Asn203 Gln sense 5'GGCGGGACACATGACACAACTCAGCAGGACAGGAAGTGCACAGG.  

and for Ser176 Ala sense 5'GGCAGACCGAGGCCGGCCCT CGAGGACCCG. 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'CAGGCAAGCTTTTTGACAGCCTGGGTCGAGG CTTTGTGATTCAAG, and the corresponding antisense primer. The putative N-linked myristoylation site Gly2 (Simon & Aderem 1992) was mutated to Ala (D1 Cys-G2A) with the sense primer 5'GCTCGGATCCCAT GGCCTGTCCCCAGCTATGG, 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 pre-culture 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 × 10⁸ 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 M DTT, 0.1 mM EDTA and 0.4 M 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 where 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 radioactive iodide by ORD of \([3',5'-125I]rT3\). Incubation mixtures contained about 100 000 c.p.m. \(^{125}\text{I}\)T3 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 the 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 \(^{125}\text{I}\) 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-[\(^{125}\text{I}\)]T3

Solutions of BrAc\(^{[125}\text{I}]\)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\(^{[125}\text{I}]\)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 glycerine 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 & 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\(^{[125}\text{I}]\)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 sulfydryl group of cysteine, and this linkage is cleaved by treatment with hydroxyl amine (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 hydroxyl amine (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 μM) 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 μM rT3, 3 μM T4 and >10 μM T3 for native D1 protein and approximately 3 μM rT3, 3 μM T4 and >10 μM T3 for D1 Cys protein. The apparent Kₘ (Michaelis-Menten constant) for rT3 ORD was 2.6 μM (n=3) for the D1 Cys protein and 0.2 μM (n=2) for the native D1 protein (Fig. 1B), while the Vₘₐₙₐₛ 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 substrates (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 μM) and IAc (IC₅₀>100 μM), in contrast to the marked inhibition of the D1 protein in rat liver microsomes (PTU IC₅₀=1μM and IAc IC₅₀=2 μM).

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 (Kₘ 2 μM, Vₘₐₛ 8 pmol/min/mg, Vₘₐₛ/Kₘ 4). In comparison, the IRD of T3 by D1 protein in rat liver microsomes is more efficient (Kₘ 4 μM and Vₘₛ 1000 pmol/min/mg, Vₘₛ/Kₘ 250) (Visser et al., 1983).

The thyroid hormone derivative -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 (Kₘ rT3 2.1 μM and Vₘₛ 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 Cys D1 and Cys-His-tag proteins are membrane associated in yeast cells (Fig. 1D). It should be realized that during yeast
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 produced.
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).

**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.

**Figure 3** Immunoblot of D1 Cys protein. (A) Immunoblots of microsomal 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).
the subcellular fractions revealed that the D1 Cys(Δ2–35)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(Δ2–35)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 Vmax 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 either in 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 BrAc[125I]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 125I-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 BrAc[125I]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 isofrom 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 isofrom 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 isofrom. Remarkably, for the Gly2 Ala mutant protein (removing a potential N-myristoylation site) the isofrom pattern as such was preserved but the labeling pattern was reversed in the sense that the lower 27 kDa protein isofrom was now most strongly labeled. This change in isofrom 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 isofrom 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 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.
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Similar results, i.e. a 10-fold increased vs 800/min (our results and Schoenmakers 200-fold reduced compared with native D1, i.e. 4/min microsomes. The substrate turnover number is about enzyme rT3 ORD, compared with native D1 in rat liver cells (Berry reported for the D1 Cys protein expressed in COS 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 isofrom 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/l 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.

### Table 1 Kinetic characteristics of D1Cys mutant proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1Cys</td>
<td>2/8:2.4</td>
<td>162/120</td>
</tr>
<tr>
<td>D1Cys-His tag</td>
<td>1.7</td>
<td>87</td>
</tr>
<tr>
<td>D1Cys-Asn94Gln</td>
<td>1.8</td>
<td>56</td>
</tr>
<tr>
<td>D1Cys-Asn203Gln</td>
<td>4.5</td>
<td>61</td>
</tr>
<tr>
<td>D1Cys-Asn94Gln/Asn203Gln</td>
<td>2:9</td>
<td>19</td>
</tr>
<tr>
<td>D1Cys-Ser101Ala</td>
<td>2.2/3.2</td>
<td>84/94</td>
</tr>
<tr>
<td>D1Cys-Ser176Ala</td>
<td>2.0/2.4</td>
<td>96/84</td>
</tr>
<tr>
<td>D1Cys-Ser101Ala/Ser176Ala</td>
<td>&gt;5/&gt;5</td>
<td>61/−</td>
</tr>
<tr>
<td>D1Cys-Tyr209Phe/Tyr217Phe</td>
<td>&gt;5/&gt;5</td>
<td>61/−</td>
</tr>
<tr>
<td>D1Cys-Gly2Ala</td>
<td>1.5/2.4</td>
<td>100/91</td>
</tr>
</tbody>
</table>

Two values in the same column are the results of duplicate determinations.
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(Δ2–35)His-tag protein was still membrane associated and absent from the cytosolic fraction. Hydrophathy 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).
fact that D1 Cys($\Delta2–35$)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($\Delta2–35$)His-tag protein is inactive, probably due to improper folding. Very similar results have been obtained after overexpression of 11β-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 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 isomeric 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 postranslationally 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).

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