# ENZYMATIC DEIODINATION OF THYROID HORMONES

# (ENZYMATISCHE DEJODERING VAN SCHILDKLIERHORMONEN)

PROEFSCHRIFT

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Zomergras,
van dappere krijgsmansdromen
bleef dit slechts over.

Matsua Bashō (1644-1694) samurai

Met dank en excuses aan de proefdieren die,
zonder klagen, hun offer aan de wetenschap brachten.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ac | N-acetyl |
| BrAc | N-bromoacetyl |
| Da | Dalton |
| DIT | 3,5-diiodotyrosine |
| DOC | sodium deoxycholate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HPLC | high performance liquid chromatography |
| IRD | inner ring deiodination |
| IOP | iopanoic acid |
| $K_m$ | Michaelis constant |
| MIT | 3-monoiodotyrosine |
| $M_r$ | relative molecular mass |
| ORD | outer ring deiodination |
| PTU | 6-N-propyl-2-thiouracil |
| $rT_3$ | reverse triiodothyronine (3,3',5'-triiodothyronine) |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| $T_0$ | thyronine |
| $T_1$ | monoiodothyronine |
| $T_2$ | diiodothyronine |
| $T_3$ | 3,3',5-triiodothyronine |
| $T_4$ | thyroxine (3,3',5,5'-tetraiodothyronine) |
| TSH | thyroid-stimulating hormone; thyrotropin |
| TRH | thyrotropin-releasing hormone |
| Triac | 3,3',5-triiodothyroacetic acid |
| Tris | Tris(hydroxymethyl)aminomethane |
| $V_{max}$ | maximum velocity |

# Chapter 1

## INTRODUCTION

Thyroid hormone plays an essential role in the regulation of cell growth, differentiation and metabolism in vertebrates. These actions are initiated by binding to the nuclear thyroid hormone receptor that has been identified as the translation product of the proto-oncogene c-erbA, the cellular counterpart of the viral oncogene v-erbA (1,2). Binding of thyroid hormone to its receptor changes the interaction with *cis*-acting regulatory elements that modulate the expression of responsive target genes. There are several thyroid hormone receptor isoforms that exert regulation in an unexpected degree of complexity (3-7). Although the mechanisms of actions of thyroid hormone in various tissues are considered to be largely nucleus-mediated, several actions of this hormone are very likely extranuclear (8,9).

## 1.1 THYROID HORMONE PRODUCTION

Thyroid hormone is formed by post-translational modification of tyrosyl residues of the protein thyroglobulin that is synthesized in thyroid follicle cells (10,11). Iodide is taken up from the blood, is oxidized by $H_2O_2$ catalyzed by thyroid peroxidase and subsequently bound to tyrosyl residues of thyroglobulin, yielding monoiodotyrosyl (MIT) and diiodotyrosyl (DIT) residues. Thyroid hormone is formed from two iodotyrosyl residues by a subsequent coupling reaction that is also catalyzed by thyroid peroxidase. These steps take place at the colloidal side of the apical membrane, at the border of the lumen and thyroid cells. Thyroglobulin is taken up by the follicular cells by endocytosis and is hydrolyzed by lysosomal proteolytic enzymes, liberating the thyroid hormones that are subsequently released into the blood stream. Free MIT and DIT are deiodinated in order to reutilize the resulting iodide.

The main product of the thyroid gland is 3,3',5,5'-tetraiodothyronine (thyroxine; $T_4$) In healthy humans the average production per day is 115 nmol $T_4$ per 70 kg body weight (12). $T_4$ displays little intrinsic bioactivity and is looked upon as merely a prohormone (12,13). Besides $T_4$ the thyroid also produces 3,3',5-triiodothyronine ($T_3$), the main bioactive thyroid hormone. However, thyroidal synthesis accounts for only less than 20 % of total daily $T_3$ production, as most $T_3$ is generated by peripheral deiodination of $T_4$ (14,15; Fig. 1). Thyroidal secretion has no significant contribution to the levels of diiodothyronines in the circulation (16,17).

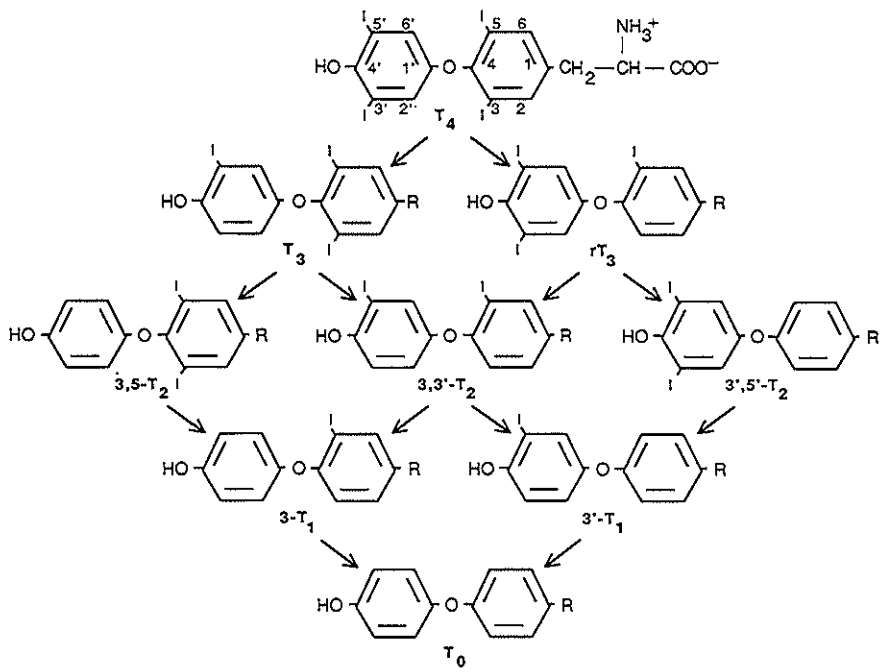


Figure 1. **Sequential deiodination of thyroxine ($T_4$)**

Thyroid action is stimulated by thyrotropin or thyroid-stimulating hormone (TSH), a glycoprotein secreted by the thyrotropic cells of the anterior pituitary gland. TSH binds to specific receptors on the thyroid plasma membrane, thereby stimulating adenylate cyclase and under certain conditions phospholipase C, which results in an increase of intracellular cyclic AMP and activation of the phosphatidylinositol pathway respectively (18). This eventually results in an increased protein synthesis in the thyroid (19). TSH secretion is inhibited by thyroid hormones (20-22), dopamine (23), glucocorticoids and somatostatin (24), whereas it is stimulated by thyrotropin-releasing hormone (TRH), a tripeptide synthesized in the medial parvocellular part of the paraventricular nucleus in the hypothalamus (25).

## 1.2 TRANSPORT OF THYROID HORMONES

Iodothyronines in the circulation are bound almost completely to plasma transport proteins. These are thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA) or transthyretin (TTR), and albumin, that carry approximately 75, 15, and 10 % of plasma $T_4$ respectively. $T_3$ is distributed in a more variable way over the transport proteins: 38-80 % bound to TBG, 9-27 % bound to TBPA, and 11-35 % bound to albumin (26-29). The normal serum free $T_4$ ($FT_4$) and $FT_3$ levels comprise only about 0.02 and 0.2 % of total $T_4$ and total $T_3$ levels, respectively. Of the iodothyronine plasma transport proteins TBPA is thought to be essential for thyroid hormone supply to tissues (30,31). TBG levels in species such as rat, dog and sheep are low, making TBPA the main plasma transporter for iodothyronines.

Because of the hydrophobic nature of iodothyronines it was assumed that these molecules enter the cell by passive diffusion (32). However, during the past 15 years evidence has accumulated that cellular uptake of thyroid hormone is an active, saturable and energy-dependent process (33-37). Furthermore, it has been demonstrated that $T_4$ and $T_3$ are transported into the cell by different carrier systems (38,39), although they are competitive inhibitors for each others uptake. Evidence was found that also the transport of $T_3$ from cytoplasm into the nucleus is mediated by a stereospecific, energy-dependent transport system (40,41). This would indicate the existence of additional means of controlling the iodothyronine concentration at the receptor.

## 1.3 THYROID HORMONE METABOLISM

Extrathyroidal metabolism of iodothyronines is one of the major processes regulating the bioavailability of $T_3$ (14). Removal of iodide from the outer ring of $T_4$ by enzymatic deiodination will increase the level of $T_3$, whereas its level can be reduced by different routes of metabolism. These routes, which will be discussed below, are not followed in a mutually exclusive way, but are interconnected in a complex manner (Fig. 2).

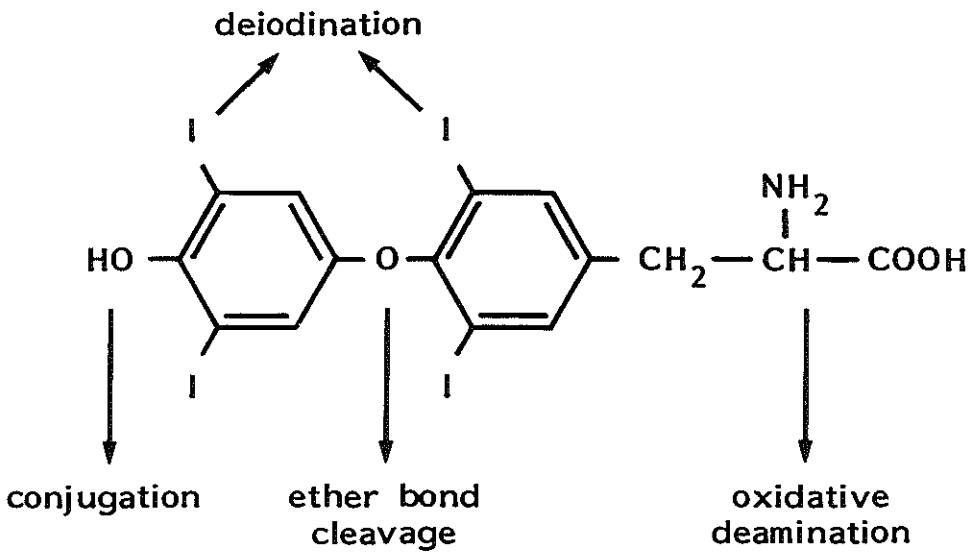


Figure 2. **Metabolism of thyroxine**

### 1.3.1 Deiodination

The quantitatively and qualitatively most important way of thyroid hormone metabolism is enzymatic deiodination. Until now three different types of deiodinases have been identified. These enzymes not only differ in characteristics, but also in their tissue distribution. Furthermore, the activity of the three deiodinases is regulated in a different manner by thyroid status, providing optimal intracellular $T_3$ levels in critical tissues such as the central nervous system (14,42-44).

Type I iodothyronine deiodinase (ID-I) is responsible for the largest part of $T_3$ supply to peripheral tissues, by catalyzing outer ring deiodination (ORD) of $T_4$. ID-I activity responds positively to thyroid status, being low in hypothyroidism and elevated in hyperthyroidism. The enzyme is capable of both inner ring deiodination (IRD) and ORD, and is mainly localized in liver, kidney and thyroid (14). ID-I is a very hydrophobic transmembrane protein, being present in liver microsomes in a concentration of approximately 0.01 % of total protein. In liver it is located in the

endoplasmic reticulum (45,46), whereas in kidney it is associated with the plasma membrane (47). In both organs the active site probably faces the cytoplasm. The enzyme requires a cytosolic cofactor of which the identity has not been resolved yet. ID-I in the microsomal fraction is strongly stimulated by low molecular weight thiols such as dithiothreitol (DTT) or reduced glutathione (GSH). Although GSH is a relatively weak stimulator, it is a possible candidate for the biological cofactor (48).

ID-I can be solubilized by a number of detergents, but only few of them are able to keep the enzyme in an active form in the absence of membrane lipids. Steric exclusion chromatography of cholate or deoxycholate (DOC) extracts of liver or kidney microsomes suggested a molecular mass for ID-I of $\approx$50-60 kDa (49,50). When rat liver microsomes were solubilized with non-ionic detergents, a pI value of $\approx$6.5 for ID-I was found in iso-electric focussing. However, upon delipidation this pI value shifted to $\approx$9.3, indicating that ID-I is a basic protein (51).

The inactive metabolite 3,3',5'-triiodothyronine (reverse $T_3$, $rT_3$) is clearly the preferred substrate of ID-I with a $V_{max}$ that is at least 500-fold higher than that of $T_4$. Modifications of the thyronine structure resulting in a higher net negative charge of the molecule increase deiodination by ID-I. It is possible that this is caused by an increased affinity for positively charged groups on the basic protein. For $T_4$ itself IRD and ORD account for almost equal fractions of its metabolism. However, sulfation of the 4'-hydroxyl group dramatically increases the IRD of $T_4$ but completely blocks its ORD, resulting in the irreversible elimination of $T_4$.

The use of N-bromoacetyl (BrAc) derivatives of iodothyronines as covalent affinity-labels has greatly advanced the identification of ID-I. First, it was found that ID-I in liver is rapidly and irreversibly inactivated by minute quantities of $BrAcT_3$ and that this inactivation could be prevented by the presence of high concentrations of ID-I substrates (52). Using BrAc[$^{125}$I]$T_3$ or BrAc[$^{125}$I]$T_4$ as affinity-labels for liver or kidney microsomes followed by SDS-PAGE analysis of labeled proteins, two major labeled protein bands were identified with relative molecular mass ($M_r$) of 27 and 56 kDa. The latter represents protein disulfide isomerase (PDI) which is a major constituent of the lumen of the endoplasmic reticulum, where it assures the proper folding of newly synthesized secretory proteins by catalyzing disulfide-bond isomerization (53,54). It has been suggested that ID-I and PDI were one and the same protein (55-58) but subsequently the 27 kDa $BrAcT_3$-labeled protein was clearly identified as ID-I (59-61). As this $M_r$ is only about half of the earlier

estimates for the molecular mass of ID-I of ≈50-60 kDa, it is likely that the enzyme is a dimer, perhaps a homo-dimer.

After it had been shown that *Xenopus laevis* oocytes were able to translate ID-I mRNA into active protein (62,63), this expression system was used to isolate the complete cDNA encoding rat liver ID-I (64). From this cDNA and from the DNA sequence of the human enzyme (65), it was deduced that in both species ID-I is a selenoenzyme containing the rare amino acid selenocysteine (SeC). This amino acid is an extra amino acid encoded by the genetic code and therefore is inserted in the enzyme cotranslationally. SeC is carried by a specific tRNA complementary to the UGA codon that normally functions as a translation stop codon (66). There are strong indications that the secondary and tertiary structure of the translated mRNA is essential for the proper cotranslational insertion of SeC. For ID-I it was shown that sequences in the 3' untranslated region of the ID-I mRNA are essential for the recognition of UGA as a SeC codon (67).

Selenium was first connected to thyroid hormone metabolism by the finding that Se deficiency in rats led to a decrease in the serum level of $T_3$ together with an increased serum $T_4$ (68,69). The amount of ID-I in liver and kidney of rats was found to be strongly related to the Se status, being markedly decreased in Se-deficient animals (68-71). Labeling studies with radioactive $^{75}Se$ intravenously administered to Se-deficient rats identified a labeled 27 kDa liver protein (72). By using peptide mapping and quantitation of both the $^{75}Se$ and BrAc[$^{125}$I]$T_4$ labeled 27 kDa proteins, it was shown that they were identical (73), providing the ultimate evidence that the 27 kDa protein represents ID-I.

A two-step model has been proposed for the reaction mechanism of deiodination catalyzed by ID-I (Fig. 3). In the first step an iodonium ($I^+$) ion is transferred from the iodothyronine to an acceptor group in ID-I, resulting in the formation of an oxidized enzyme-intermediate. In the second step this enzyme-intermediate is reduced by a cofactor, regenerating ID-I and yielding oxidized cofactor and iodide. Initially, it was thought that the iodonium-acceptor group was a protein sulfhydryl (SH) group, converted into a sulfonyl iodide in the first step of the reaction (74,75). This assumption was supported by the finding that 6-N-propyl-2-thiouracil (PTU) inhibits ID-I in a manner competitive with cofactor (DTT) but uncompetitive with substrate (74,76). It had previously been shown that thiouracil is particularly reactive towards protein sulfonyl iodides, yielding mixed disulfides (77). However, since ID-I is a

selenoenzyme (64) it is very likely that the selenocysteine residue is the actual catalytic center, alternating between the selenol (SeH) and the selenenyl iodide (E-SeI) forms during enzymatic activity. This is supported by the finding that, when using site-directed mutagenesis to replace selenium by sulfur (SeC → Cys), this leads to a strong decrease in the catalytic efficiency of ID-I (78,79). Besides SeC also histidine residues have been shown to be essential for substrate binding and function of ID-I (80,81).

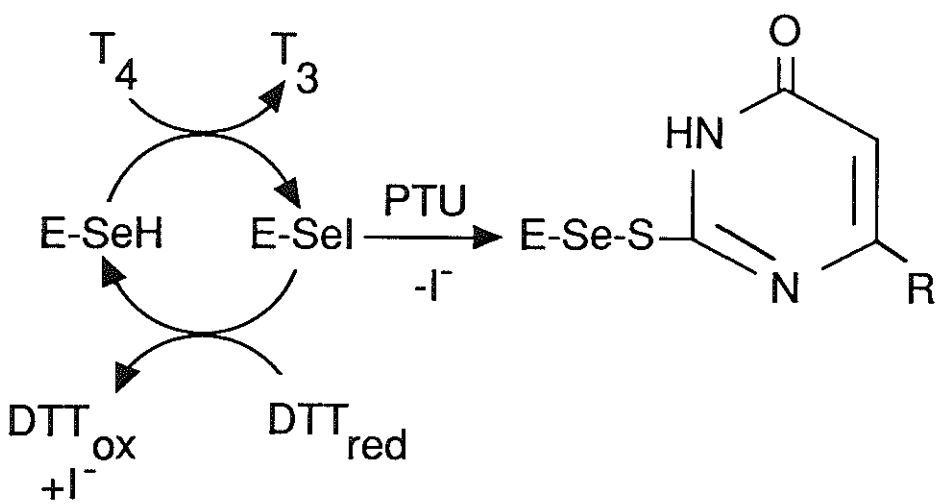


Figure 3. **Mechanism of type I iodothyronine deiodinase**

The type II iodothyronine deiodinase (ID-II) is mainly responsible for the supply of thyroid hormone to the central nervous system by local conversion of $T_4$ to $T_3$ (82). ID-II is found in the central nervous system, pituitary and brown adipose tissue (14,15). Unlike ID-I the activity of the type II enzyme is markedly increased in hypothyroidism and decreased in hyperthyroidism (43). Thus ID-II can make a significant contribution to plasma $T_3$ levels in hypothyroid subjects. In cultures of rat brain astrocytes it was found that ID-II can be induced $>10$-fold by agents that influence cellular differentiation, such as growth factors, cAMP derivatives or phorbol esters (83-86).

ID-II catalyzes only ORD of $rT_3$ and $T_4$ with the latter displaying the highest $V_{max}/K_m$. The $K_m$ values for both substrates are in the nM range and, therefore, ID-II may display significant substrate occupancy under physiological conditions. Like ID-I, the enzyme is stimulated by small thiol compounds such as DTT (14,15). Although it has been suggested that ID-II activity can be inhibited by PTU under special circumstances (87), the enzyme is generally considered to be insensitive to PTU. Iodoacetate can inhibit enzyme activity, suggesting an essential thiol group to be located in the active center domain (88).

In brain cells ID-II was shown to be an integral membrane protein (83) that can be selectively labeled with $BrAcT_4$ (89). High resolution SDS-PAGE analysis of $BrAc[^{125}I]T_4$ labeled ID-II revealed a $M_r$ of 29 kDa, slightly higher than the $M_r$ of the type I enzyme. Employing Endo-F/peptide N-glycosidase F hydrolysis it was shown that apparently neither ID-I nor ID-II is glycosylated. Peptide mapping and cyanogen bromide fragmentation further demonstrated that the labeled ID-I and ID-II proteins are different. When ID-II was labeled with $BrAc[^{125}I]T_4$ followed by subsequent solubilization with tauro-deoxycholate and analysis by sucrose density centrifugation, the enzyme was shown to have a molecular mass of 199 kDa, indicating a multimeric composition (90).

The activity of ID-II in cultures of astroglial cells is not decreased by Se-deficiency, indicating that the enzyme does not contain SeC (91). The finding that ID-II levels are decreased in Se-deficient rats is no prove for ID-II being a selenoenzyme, as $T_4$ levels are increased in Se-deficiency and this leads to down regulation of ID-II activity (92). However, increased ID-II activity resulting from hypothyroidism caused by iodine deficiency is not reversed by additional Se-deficiency in rats, indicating that ID-II is not a selenoenzyme (71). Gold thioglucose

is a potent inhibitor of type I deiodinase. However, when the SeC residue in the enzyme is replaced by Cys this leads to a 500-fold decreased sensitivity for inactivation by this substance. As ID-II is 100- to 1000-fold more resistant to gold thioglucose compared to the type I enzyme, this confirms the absence of SeC in the active site (93).

In astrocytes the organization of the cytoskeleton is changed by $T_4$ through an effect on actin polymerization. This modulates the turn-over of ID-II in a direct manner. When $T_4$ is absent almost all ID-II is associated with the plasma membrane of the astrocyte. After addition of $T_4$, the increased polymerization of actin is associated with binding of the deiodinase to actin filaments. This promotes rapid translocation of the enzyme to an internal membrane compartment, leading to a decrease in ID-II activity (94). Therefore, the increased ID-II activity found in astrocyte cultures grown in the absence of thyroid hormones is not caused by an increase in the rate of enzyme synthesis, but is the result of a decrease in enzyme degradation. This is supported by the failure of $T_4$ to induce ID-II inactivation in astrocyte cultures treated with agents that prevent actin polymerization, such as cytochalasins (95,96).

The type III iodothyronine deiodinase (ID-III) catalyzes only IRD and is considered an important enzyme for the inactivation of thyroid hormone, as it converts $T_4$ to $rT_3$ and $T_3$ to 3,3'-$T_2$ (14,15). ID-III is predominantly present in brain (97-102) and placenta (103-108). In rats, the enzyme has also been detected in retina (109), skin (110,111), fetal intestine and fetal skeletal muscle (111,112). In chicken ID-III activity was found in embryonic heart cells (113) and liver (114-116). Furthermore, the enzyme was found in certain types of human brain tumors (117). In all these tissues ID-III activity is associated with the microsomal fraction, similar to ID-I and ID-II. Furthermore, it was found that ID-III is inactivated by delipidation (118). ID-III is considered an onco-fetal protein as its ontogeny is clearly connected to embryonic development (116), its activity being generally higher in fetal than in adult tissues (100,101,119-121), whereas high activities of the enzyme are found in the human colon carcinoma Caco-2 cell line (122) and the monkey hepatocarcinoma NCLP-6E cell line (123-126). The high ID-III activities in embryonic tissues and placenta indicate that this deiodinase plays a role in the protection of differentiating tissues against excess thyroid hormone during crucial periods.

In the central nervous system of the rat high ID-III activities are found in the cerebral cortex, whereas relatively low activities are measured in cerebellum, brain stem and spinal cord (97,100,101,119,120,127). Using tissue cultures originating from fetal or neonatal rat brain, it has been established that astroglial cells contain highest levels of ID-III, whereas oligodendrocytes display lowest enzyme activity (102,128). However, growth arrest of glial cell induced by cytotoxic drugs does not diminish ID-III activity, suggesting that the enzyme is also present in neurons (129). In humans, the enzyme is found in the fetal membranes (121), but much higher activities can be detected in placental trophoblasts (124,125).

Brain ID-III activity is regulated by thyroid status in a manner similar to the type I enzyme, as it is increased in hyperthyroidism and decreased in hypothyroidism. This is supported by the reported induction of ID-III activity by thyroid hormones in astroglial cell cultures (130). However, these changes in enzyme levels are relatively small (97,101). Using cultures of hepatocarcinoma cells it was found that ID-III activity is correlated with the cell cycle, as enzyme activity is high in late G1 phase and low in the late S or G2 phase (125). In cultures of rat brain astroglial cells it was found that ID-III activity can be strongly induced by phorbol esters and both basic and acidic fibroblast growth factor. Epidermal growth factor, platelet-derived growth factor, and 8-bromo-cyclic AMP were poor inducers of ID-III activity (131).

The best substrate of ID-III is $T_3$ with a $K_m$ value about 10-fold lower than that for $T_4$, although $V_{max}$ values are comparable. Deiodination of iodothyronines by ID-III is inhibited in a competitive manner by alternative substrates such as $T_4$, $T_3$, 3,3'-$T_2$, 3,5-$T_2$ but not by very high concentrations (up to 1 $\mu$M) of the non-substrate $rT_3$ (97,101,116,124,131,132). Like ID-I and ID-II, ID-III is stimulated by small thiol compounds such as DTT, but much higher concentrations of this compound are needed to reach maximal enzyme activity. Sequential type reaction kinetics with formation of a ternary enzyme-cofactor-substrate complex was suggested by analysis of deiodination rate versus substrate concentration at fixed DTT concentrations (133). However, as these experiments were carried out under conditions uncertain to be suitable for this kind of analysis, they should be interpreted with care. ID-III activity is inhibited by iodoacetate concentrations above 10 $\mu$M. Inhibition of ID-III by PTU could only be detected when high ($\geq$ 0.1 mM) PTU concentrations were combined with low ($\leq$ 1 mM) DTT concentrations (132).

ID-III activity in rat brain, rat placenta and embryonic chicken liver is rapidly and

irreversible inhibited by $BrAcT_3$ with a $K_i$ comparable to the $K_m$ of $T_3$. This is quite different from the inactivation of the type I enzyme by $BrAcT_3$, which is half-maximal at a concentration much lower than the $K_m$ for $T_3$ or even the preferred substrate $rT_3$. Upon reaction of rat brain and placenta microsomes with $BrAc[^{125}I]T_3$, a 32 kDa protein (p32) was prominently labeled. However, p32 was also extensively labeled in microsomes of rat spleen and fetal liver that both have no ID-III activity. Furthermore, labeling of p32 could not be diminished by very high (100 $\mu$M) concentrations of inhibitors or substrate analogs of ID-III, some of which completely inhibit ID-III activity at a concentration 100-fold lower. In embryonic chicken liver microsomes no p32 or another protein possibly related to ID-III could be identified by $BrAc[^{125}I]T_3$ affinity labeling. Therefore, it is unlikely that p32 represents ID-III or a subunit thereof (134). This conclusion is in contrast with other suggestions based on similar results (135).

Se-deficiency leads to a weak but repeatedly confirmed decrease of rat brain ID-III activity, although enzyme levels in placenta are unaffected. It remains to be resolved whether this indicates a specific Se dependence of the synthesis of ID-III or is caused by the multilevel change in thyroid hormone metabolism accompanying Se-deficiency (71). The difference between ID-I and ID-III in susceptibility to inactivation by $BrAcT_3$ suggests that ID-III is not a selenoenzyme. Recently the cDNA of a tadpole protein displaying characteristics of ID-III has been cloned and sequenced. From this cDNA sequence it was deduced that the gene encodes a SeC-containing protein and displays a high degree of homology with the ID-I cDNA, in particular the region surrounding the SeC residue (136). However, until now no inner ring deiodinase displaying type I characteristics has been found in tadpole (137,138). Therefore, although this cDNA encodes a low $K_m$ deiodinase with inner ring specificity that is not inhibited by 1 mM PTU, it remains to be determined whether this enzyme represents the evolutionary divergent equivalent of ID-I or ID-III.

### 1.3.2 Conjugation

Conjugation is a general metabolic reaction that facilitates the excretion of a variety of hydrophobic endogenous and exogenous substances in bile and urine. This is achieved by covalent coupling of the hydrophobic substances with a hydrophilic molecule, yielding a more water-soluble product (139,140). For thyroid hormones conjugation represents, in importance, the second metabolic route that in most cases involves glucuronidation or sulfation of the phenolic hydroxyl group (Fig. 4).

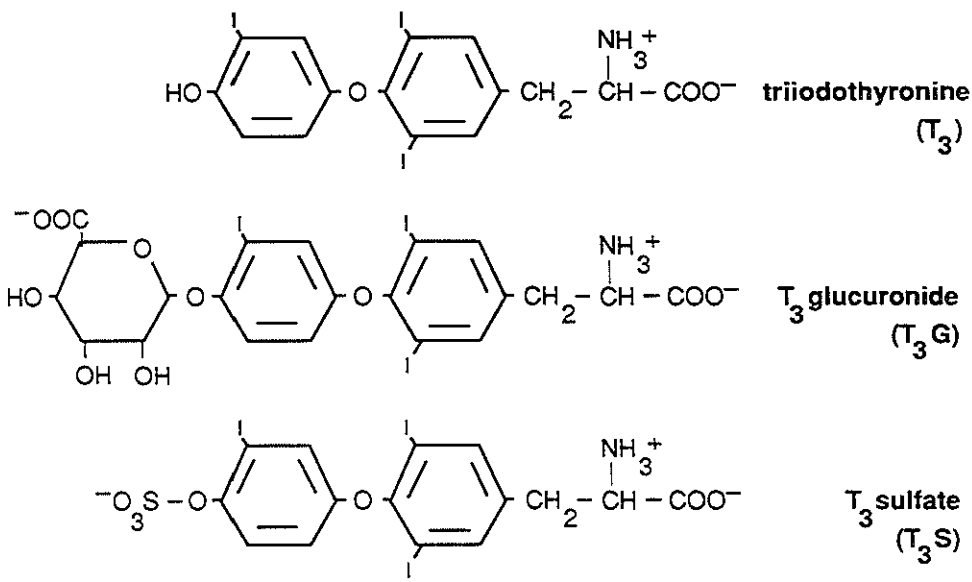


Figure 4. **Structures of $T_3$ and derivatives**

Glucuronidation represents one of the major routes of detoxification of many compounds in mammals. It is catalyzed by a group of homologous enzymes that use uridine diphosphate (UDP)-glucuronic acid as a cofactor. This cofactor is generated by reaction of uridine triphosphate (UTP) with ubiquitous glucose 1-phosphate yielding UDP-glucose, followed by oxidation of this substance. The enzymes

catalyzing glucuronidation are named UDP-glucuronyltransferases (UGTs) and are predominantly present in liver but also in kidney, intestine and other tissues. UGTs were shown to be transmembrane proteins located in the endoplasmic reticulum, with their catalytic center oriented towards the lumen of this cell organelle (141).

Using clofibrate and 3-methylcholanthrene that induce bilirubin UGT and phenol UGT, respectively, it was shown that $T_4$ and $rT_3$ are glucuronidated by these enzymes (142-144). This was confirmed by studies using Gunn rats that have a defect in the gene coding for multiple phenol and bilirubin UGT isoenzymes and accordingly show impaired glucuronidation of $T_4$ and $rT_3$ (145). Alternatively, $T_3$ is glucuronidated by androsterone UGT that is different from phenol and bilirubin UGT and is encoded by a different gene. This is confirmed by the observation that Wistar LA, Fischer and WAG rats, that all have a defect in the androsterone UGT, display impaired $T_3$ glucuronidation (142,143,146).

After $T_4$, $rT_3$ and $T_3$ glucuronides are produced in the liver, these substances are excreted in bile and subsequently hydrolysed by $\beta$-glucuronidases produced by the intestinal microflora (147). This is supported by the observation that intravenous injection of radioactive $T_3$ glucuronide ($[^{125}I]T_3G$) in intestine-decontaminated rats results in fecal excretion of radioactivity almost exclusively as $[^{125}I]T_3G$, whereas in control rats radioactivity is excreted in feces largely as $[^{125}I]T_3$ (148). The liberated iodothyronine can be reabsorbed in the circulation resulting in an enterohepatic cycle for thyroid hormone (149). Therefore, glucuronidation does not necessarily result in complete loss of thyroid hormone from the organism. The magnitude of the enterohepatic cycle has not been elucidated but seems to be higher in rat compared to man (150).

Sulfation of thyroid hormones is catalyzed by phenol sulfotransferases (PSTs) that are located in the soluble fraction of particularly liver, kidney, small intestine, brain and platelets (139). PSTs form a family of homologous enzymes and are responsible for the sulfation of various phenols. All sulfotransferases use the same universal sulfate donor, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) that is generated from inorganic sulfate and two ATP molecules (139,151).

In rat liver iodothyronines are substrates for different types of PSTs, with the efficiency of sulfation inversely correlated to the number of iodine atoms in the iodothyronine (152). Under normal conditions urinary excretion of sulfated

iodothyronines is negligible and only small amounts are excreted in the bile (153). However, if type I deiodinase activity is inhibited, for instance by administration of PTU, this results in increased levels of sulfated thyroid hormone in the circulation and in the bile (154). Under these exceptional circumstances an enterohepatic cycle exists that is comparable to the one for glucuronidated iodothyronines, since sulfatases produced by certain strains of anaerobic intestinal bacteria of human and rat are able to hydrolyze all sulfated iodothyronines (155).

Sulfation results in a 40-200 times higher IRD by ID-I of $T_4$ and $T_3$, but completely blocks the ORD of $T_4$ (156). For both iodothyronines sulfation results in a significant decrease of $K_m$ and a dramatic increase of $V_{max}$ values, resulting in rapid clearance from the circulation. Therefore, this conjugation reaction seems to induce the irreversible elimination of thyroid hormone, although, meanwhile it allows for reutilization of iodide for thyroidal hormone synthesis (157). In rats that were treated with the ID-I inhibitor PTU, a 4-fold increase of plasma $T_3S$ levels was observed (158) paralleled by a higher excretion in the bile (159). Furthermore, the clearance of injected $T_3S$ was largely inhibited in these animals, also indicating the accumulation of the intermediate $T_3S$ in the circulation upon inactivation of ID-I (158,160). These data show that deiodination is preceded by sulfation in a significant part of $T_3$ metabolism in the rat (158). PTU treatment of rats resulted in a 5-fold increase of biliary excretion of $T_4S$, demonstrating that also $T_4$ is sulfated significantly in rats *in vivo* (161).

Synthetic N-sulfonated iodothyronines, that have a sulfonated aminogroup of the alanine side chain, are deiodinated by ID-I with 4 to 17-fold higher efficiency. This mainly seems to result from lower $K_m$ values of ID-I for N-sulfonated thyroid hormones compared to native hormones. Opposed to the blocked ORD of naturally occurring 4'-O-sulfonated $T_4$ it was shown that N-sulfonated $T_4$ can be degraded by both IRD and ORD, indicating the importance of the site of sulfation for efficient inactivation of $T_4$ (162).

### 1.3.3 Oxidative deamination

Conversion of the alanine side chain of thyroid hormones to the acetic acid derivative plays a minor but distinctive role in the metabolism of iodothyronines under normal conditions. Oxidative deamination of thyroid hormone was demonstrated in homogenates of liver, kidney and brain (13,163-166). Using sonicated rat kidney mitochondria, the reaction sequence of this metabolic route was elucidated for various iodothyronines (166). In a first step the alanine side chain is converted to successively a pyruvic acid and an acetaldehyde intermediate that is processed to the final acetic acid derivative (Fig. 5).

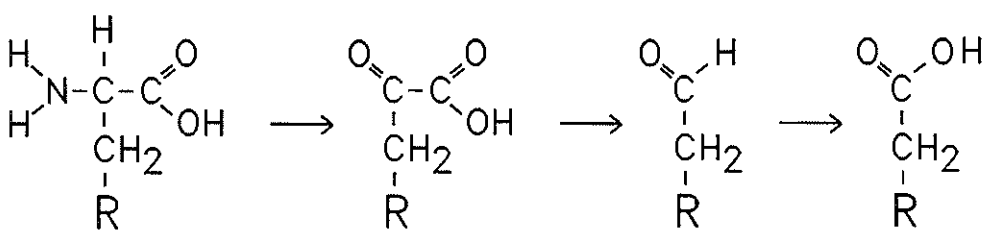


Figure 5. **Mechanism of oxidative deamination**

In healthy humans only 2 percent of total $T_4$ is metabolized by oxidative deamination resulting in the formation of 3,3',5,5'-tetraiodothyroacetic acid, with the trivial name tetrac ($TA_4$). Furthermore, about 14 percent of $T_3$ turnover in man follows this route yielding 3,3',5-triiodothyroacetic acid, with the trivial name triac ($TA_3$), indicating the physiological significance of oxidative deamination (13). Although triac binds more tightly to the nuclear receptor protein than native $T_3$, it displays only about one tenth of the thyromimetic activity of this iodothyronine (167-169). In the circulation $TA_4$ and $TA_3$ bind more firmly to the carrier protein TBPA

compared with $T_4$ and $T_3$, whereas their relative affinity for TBG is diminished (27,170).

Like their parent iodothyronines, the acetic acid analogues can be conjugated by glucuronidation or sulfation. In rats, the lesser iodinated derivatives such as 3,3'-diiodothyroacetic acid are preferentially sulfated, $TA_3$ is conjugated in both ways, whereas reverse $TA_3$ and $TA_4$ are predominantly glucuronidated (171-173). In human liver, $TA_3$ and $TA_4$ are also rapidly glucuronidated. However, in humans this involves the carboxyl group, yielding an ester derivative, whereas in rats glucuronidation involves the phenolic group, yielding an ether derivative (174). Sulfated $TA_3$ ($TA_3S$) is the best known substrate for inner ring deiodination by the type I deiodinase, as a result of its very high affinity for this enzyme (175,176).

Significantly increased levels of $TA_3S$ have been found in the circulation of hypothyroid rats, and in rats treated with PTU that inhibits ID-I activity. PTU treatment also results in an increased biliary excretion of $TA_3S$ in rats (172,176,177). These observations indicate that, like $T_3$, at least part of $TA_3$ is normally degraded by successive sulfation and deiodination in rat. However, unlike $T_3$, sulfation does not appear to be an obligatory step in $TA_3$ metabolism as it is efficiently cleared from the circulation by glucuronidation upon inhibition of both sulfation and deiodination (176). PTU treatment of rats causes a marked accumulation of the sulfate conjugate of 3,3'-$TA_2$ in the circulation, also reflecting reduced deiodinative clearance (178).

### 1.3.4 Ether link cleavage

In healthy humans, ether link cleavage (ELC) probably represents the least important metabolic route for thyroid hormones (13,179,180), although it may be of more significance in rats (181,182). However, in phagocytosing human leucocytes ELC comprises the major route for thyroid hormone breakdown. ELC is associated with the "respiratory burst" that leads to generation of superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), that can cause cleavage of the ether bridge between the two ring structures in the iodothyronine molecule. Therefore, the role of ELC may become more important in disease states accompanied by severe leucocytosis. In unstimulated leucocytes ELC is latent (182).

## 1.4 THYROID HORMONE RECEPTORS AND RESPONSE ELEMENTS

Thyroid hormone effects at the genomic level are mediated via the nuclear thyroid hormone receptor (TR) that binds $T_3$ with high affinity and specificity (3,183,184). When the TR was cloned, analysis of the coding sequence showed it to be a member of a large family of gene-regulating receptor proteins. All these receptors possess a domain, required for binding to their target genes, which contain two highly conserved clusters of cysteine residues thought to chelate zinc, forming so called zinc-fingers. Next to this DNA-binding domain the TR contains a ligand-binding domain that also plays a major role in receptor dimerisation and a hinge region that possibly also is involved with the localization in the nucleus (185).

Sequence comparison of c-erbA cDNAs originating from human placenta (1) and chicken embryo (2) revealed that they were derived from different genes nowadays designated TR$\alpha$ and TR$\beta$. Both genes have been found in the genome of all vertebrate species examined until now. In the human genome these genes are located on chromosomes 17 and 3, respectively (185). Further diversity in thyroid hormone receptor proteins is generated by alternative splicing of the primary transcript of the TR$\alpha$ gene. This yields two different mRNAs that result in translation to $\alpha$1 and $\alpha$2 proteins (186-188). The amino acid sequence of these proteins are identical up to residue 370, but differ C-terminal of this residue. As the thyroid hormone-binding domain is situated in the C-terminus this results in a marked difference: TR$\alpha$1 is a fully functional receptor, whereas the $\alpha$2 protein is defective in binding of $T_3$ and cannot by itself mediate $T_3$ responsiveness. However, it can inhibit $T_3$ responses through other thyroid hormone receptors by yet unknown mechanisms (189,190).

The TR$\beta$ gene also encodes two different receptor proteins named TR$\beta$1 and TR$\beta$2. However, for the TR$\beta$ gene diversity is not induced by alternative splicing of RNA but by use of alternative translation initiation sites. This results in the synthesis of two completely functional thyroid hormone receptors, that have identical amino acid sequences for the three functional domains but posses different N-terminal regions (191). The various TR isoforms are expressed in different levels in a tissue-specific pattern, resulting in ratios of isoforms that vary from tissue to tissue (192,193). Furthermore, the levels of TR mRNAs are possibly affected in an isoform-specific manner by hormones and other regulatory factors (194,195). These findings could thus explain, at least part of, the observed differential tissue

responsiveness to thyroid hormone.

Thyroid hormone resistance is a collection of syndromes characterized by reduced responses of target tissues to thyroid hormone. Affected individuals have increased thyroid hormone plasma levels combined with a normal or elevated TSH concentration, but usually require no treatment as they are clinically euthyroid. In most cases studied, this syndrome results from mutations in the TR$\beta$-gene resulting in an amino acid substitution in the thyroid hormone-binding domain of the receptor, or even in the partial or complete deletion of this domain. Thyroid hormone resistance is inherited in a dominant negative fashion, as the mutant receptors inhibit the function of unaffected $\beta$-receptors (in heterozygotes) and normal $\alpha$-receptors. This may result from competition for specific regulatory DNA elements and/or formation of inactive dimers (196-199).

Current models for thyroid hormone action suggest that $T_3$, the iodothyronine displaying the most potent bioactivity, enters the cell and migrates to the nucleus where it binds to its receptor. The resulting $T_3$-receptor complex regulates the activity of target genes by interacting with thyroid hormone response elements (TREs) situated in the regulatory sequences of these genes. Using molecular biology techniques, TREs have been identified and sequenced in several genes. All these TREs seem to contain two copies of a more or less conserved general motive, or consensus sequence that is referred to as a half-site. Like steroid hormone receptors, the thyroid hormone receptors appear to bind as dimers and to interact with two half-sites. The functional specificity of TREs may be exerted by the orientation of the two copies of the consensus sequence and the number of nucleotides separating them (200-202).

Binding of a thyroid hormone receptor dimer to a TRE can result in either positive or negative regulation of the transcriptional activity of the gene. In the latter case the TRE is predominantly situated near the TATA box, a DNA motive situated close to the transcription start site of most genes (203-205). Therefore, a steric mechanism was proposed for this down regulation of gene activity. However, negative TREs have also been found more upstream of the TATA box, suggesting the TRE sequence to be more important than its location (200). Another model for the regulation of genes emerges from the finding that thyroid hormone decreases binding of TR homo-dimers to specific TREs (206). In this model, unliganded, DNA-bound

TRs cause a diminished gene activity that is reversed by $T_3$-binding to the receptor and its subsequent release from the TRE.

## 1.5 SCOPE OF THE THESIS

The studies described in this thesis were undertaken to gain more knowledge in the genetic and molecular mechanisms involved in the regulation of thyroid hormone bioactivity. The investigations were focussed on the characterization of thyroid hormone deiodinating enzymes, especially ID-I and later ID-III.

Chapter 2 describes a study performed to identify the substrate-binding subunit of rat liver microsomal ID-I, using affinity-labeling with $BrAcT_3$. Furthermore, it aimed to test the hypothesis in previous literature that ID-I was identical to protein disulfide isomerase. Identification of ID-I is done by inhibition of labeling of this protein by the presence of substrates and inhibitors of ID-I. Furthermore, correlations were assessed between ID-I activity and protein-labeling under widely varying conditions such as protease treatment and progressive elimination of microsomal proteins.

In order to elucidate the genetic structure of the gene encoding ID-I, one of the strategies included the purification of the ID-I protein for subsequent determination of its amino acid sequence. As it was impossible to purify rat or human ID-I to a quality and quantity high enough to meet the demands for protein sequencing, various other species were explored as alternative sources for the purification of ID-I. In this interspecies comparison, that is described in Chapter 3, both concentrations and characteristics of ID-I in liver microsomes were investigated.

Around this time the genetic structure of rat and human ID-I cDNA was elucidated by others and was shown to contain the rare amino acid SeC that is inserted co-translationally and is encoded by a TGA codon that normally signals termination of translation. In the interspecies comparison the laboratory mouse substrain Balb/c had been used as the murine representative. It was found that ID-I varied considerably between various substrains, and that most mice of the C3H/He substrain were almost devoid of liver ID-I activity, with the other mice displaying highly variable enzyme levels. The studies in Chapter 4 describe the investigations undertaken to identify the molecular mechanisms responsible for these phenomena. ID-I activity in C3H/He

mice was compared with levels of ID-I mRNA in these animals. Furthermore, the possible role of selenium in mouse ID-I was investigated using [$^{75}$Se]-selenite injection of Se-deficient or control mice.

In the multi-species comparison of ID-I a prominently labeled band with $M_r$ of 32 kDa was seen in pig liver microsomes. Several other observations were in agreement with the idea that this band might represent ID-III. In the studies reported in Chapter 5 the possible relation between the 32 kDa band and ID-III is investigated, by affinity-labeling of this protein with $BrAcT_3$ in the presence of substrates and inhibitors of ID-III. Furthermore, this possible relation is examined in microsomes originating from fetal rat liver and embryonic chicken liver.

Chapter 6 further explores the possible link between ID-III and the 32 kDa protein, employing labeling with the non-substrate derivative $BrAcrT_3$ in the presence of substrates and inhibitors of ID-III. Prevalence of ID-III activity was compared to prevalence of the labeled 32 kDa band in various organs and their subfractions. Using peptide mapping, it was investigated if the labeled 32 kDa protein is identical in these organs.

In the General Discussion (Chapter 7) the reasons for performing the studies described in this thesis are discussed. Furthermore, the results of these studies are evaluated together with related results reported by others in literature. Finally, some possibilities for future research are reviewed.

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## Chapter 2

# RAT LIVER TYPE I IODOTHYRONINE DEIODINASE IS NOT IDENTICAL TO PROTEIN DISULFIDE ISOMERASE

# RAT LIVER TYPE I IODOTHYRONINE DEIODINASE IS NOT IDENTICAL TO PROTEIN DISULFIDE ISOMERASE

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SUMMARY: This study was done to test the recent hypothesis (Boado et al. (1988) Biochem. Biophys. Res. Commun. 155, 1297-1304) that type I iodothyronine deiodinase (ID-I) is identical to protein disulfide isomerase (PDI). Autoradiograms of rat liver microsomal proteins, labeled with N-bromoacetyl-[$^{125}$I]triiodothyronine (BrAc[$^{125}$I]$T_3$) and separated by SDS-PAGE, show predominantly 2 radioactive bands of $M_r$ 27 and 56 kDa. Substrates and inhibitors of ID-I inhibited labeling of the 27 kDa band but not that of the 56 kDa band. Treatment of microsomes with trypsin abolished labeling of the 27 kDa protein and destroyed the activity of ID-I but did not prevent labeling of the 56 kDa protein. Following treatment of microsomes at pH 8.0-9.5 or with 0.05 % deoxycholate (DOC) PDI content and labeling of the 56 kDa protein were strongly diminished but ID-I activity and labeling of the 27 kDa protein were not affected. The latter decreased in parallel after treatment at pH $\geq$ 10. Rat pancreas microsomes contain high amounts of PDI but show no ID-I activity. Reaction of these microsomes with BrAc[$^{125}$I]$T_3$ results in extensive labeling of a 56 kDa protein but no labeling of a 27 kDa protein. Pure PDI ($M_r$ 56 kDa) was readily labeled by BrAc[$^{125}$I]$T_3$ but showed no deiodinase activity. These results strongly suggest that the 27 kDa band represents (a subunit of) ID-I while the 56 kDa band represents PDI. From these and other data it is concluded that PDI and ID-I are not identical proteins. 

In humans and animals, the development and metabolic function of many tissues is under control of thyroid hormones of which 3,3',5-triiodothyronine ($T_3$) is the predominant bioactive form. This control is largely exerted by the interaction of the $T_3$-nuclear receptor complex with cis-acting DNA sequences which regulate the transcription of thyroid hormone-responsive genes. Thyroid hormone receptors have recently been identified as the products of the c-erbA proto-oncogenes (1,2). In humans and rats, most plasma $T_3$ is derived from the enzymatic deiodination of thyroxine ($T_4$), while only about 20 % of $T_3$ is secreted by the thyroid gland (3). In keeping with its low affinity for the nuclear thyroid hormone receptor (1), $T_4$ possesses little intrinsic bioactivity and is generally looked upon as a prohormone for $T_3$ (3).

Several iodothyronine-deiodinating enzymes have been identified, but the type I iodothyronine deiodinase (ID-I) of liver and kidney is most important

for peripheral production of $T_3$ (3). However, the inactive metabolite 3,3',5'-triiodothyronine (reverse $T_3$, $rT_3$) is clearly the preferred substrate of ID-I (3). In liver, ID-I is associated with the endoplasmic reticulum, where it constitutes ~0.01 % of total microsomal protein (4). It is a basic protein that in the delipidated state has a pI value of 9.3 (5). Previous studies have demonstrated the usefulness of N-bromoacetyl-[$^{125}$I]$T_3$ (BrAc[$^{125}$I]$T_3$) as a specific affinity-label of this enzyme (4).

Recently, it was claimed (6) that ID-I is identical to protein disulfide isomerase (PDI); this claim was based on characterization of a clone purporting to code for ID-I whose sequence was found (6) to be that already determined for PDI (7). PDI is a major constituent of the lumen of the endoplasmic reticulum, where it catalyses formation of disulfide bonds in newly-synthesized secretory proteins (8,9). Accordingly, it is especially abundant (1-2 % of total microsomal protein) in tissues with high rates of protein secretion such as liver and pancreas (9). PDI from several vertebrate sources has been established as an acidic protein with pI < 4.5 and subunit $M_r$ of ~57 kDa (10,11). Recent findings indicate that PDI is readily labeled with BrAc[$^{125}$I]-$T_3$ (12,13). Because of their apparent differences, the hypothesis that PDI and ID-I are identical proteins is surprising. The purpose of this study was to test this hypothesis by analysis of the BrAc[$^{125}$I]$T_3$ labeling of rat liver and pancreas microsomes and the PDI contents and ID-I activities of these fractions under different conditions. The clear dissociation between the latter as well as the complete lack of ID-I activity of pure PDI indicate that these enzymes represent completely different proteins.

## MATERIALS AND METHODS

Materials. [3',5'-$^{125}$I]$rT_3$ and [3'-$^{125}$I]$T_3$ (> 780 Ci/mmol) were obtained from Amersham (Amersham, UK); unlabeled iodothyronines from Henning (Berlin, FRG); 4-aminodiphenylamine diazonium sulfate, sodium deoxycholate (DOC), 3,5-diiodotyrosine (DIT), dithiothreitol (DTT), naphthol AS-MX phosphate, 6-n-propyl-2-thiouracil (PTU), soybean trypsin inhibitor and trypsin from Sigma (St. Louis, MO); iopanoic acid (IOP) from Sterling Winthrop (Newcastle, UK); electrophoresis grade SDS-PAGE reagents from Bio-Rad (Richmond, IL); $M_r$ markers and Sephadex LH-20 from Pharmacia (Uppsala, Sweden); Coomassie Brilliant Blue R-250 from Merck (Darmstadt, FRG); Tween 20 from Serva (Heidelberg, FRG); goat anti-rabbit IgG conjugated with alkaline phosphatase (GAR-AP) from Tago (Burlingame, CA).

Preparation of microsomes. Microsomes were prepared from perfused livers of male Wistar rats (~200 g BW) in buffer A (10 mM Tris/HCl, pH 7.4, 3 mM EDTA and 3 mM DTT) as previously described (14) and stored at -70 C. Rat pancreas microsomes were prepared similarly without perfusion of the tissue. Protein content was measured with the Bio-Rad protein assay using bovine serum albumin as the standard.

Deiodinase assay. ID-I activity was determined by incubation of the appropriate amount of microsomal protein for 20 min at 37 C with 75 nCi [$^{125}$I]$rT_3$ and 10 nM or 1 uM $rT_3$ in 200 ul buffer B (0.2 M phosphate, pH 7.2, 4 mM EDTA and 3 mM DTT). The reaction was stopped by placing the samples on ice and adding 750 ul of 1 M HCl. Released $^{125}I^-$ was separated from iodothyronines on

Sephadex LH-20 as described before (15), and the data were corrected for non-enzymatic deiodination as determined in incubations without microsomes. For calculation of ID-I activity, random labeling of the 3' and 5' positions of [$^{125}$I]$rT_3$ was taken into account.

Treatment of microsomes with DOC or high pH. Rat liver microsomes (7.2 mg protein) were suspended in 10 ml 25 mM HEPES (pH 7.4), 0.25 M sucrose and 50 mM KCl with or without 0.05 % DOC or in 0.1 M sodium carbonate (pH 8.0-11.5). The mixtures were kept on ice for 1 h with occasional stirring and subsequently layered on 2 ml 50 mM HEPES (pH 7.4), 0.5 M sucrose, 50 mM KCl and 2 mM DTT, and centrifuged for 90 min at 105,000 x g and 4 C. The resulting pellets were resuspended in 1 ml of buffer A and stored at -20 C, as were the supernatants, until further examination.

Synthesis of BrAc[$^{125}$I]$T_3$. The affinity-label was prepared essentially as previously published (4). The product was checked by HPLC, showing at least 80 % purity with unreacted [$^{125}$I]$T_3$ as the main contaminant.

Affinity-labeling. The desired amount of BrAc[$^{125}$I]$T_3$ was brought in an Eppendorf tube and the solvent was evaporated at 42 C under a stream of nitrogen. To the residue was added the desired amount of microsomal protein in 60 ul buffer A, and the mixture was vortexed for 30 s. After further incubation for 10 min at 37 C, 30 ul of SDS-sample buffer containing 5 % $\beta$-mercaptoethanol was added to stop the labeling, and proteins were separated by SDS-PAGE in a 10 % gel (16). Gels were stained with 0.25 % Coomassie Briliant Blue R-250 in 50 % methanol/10 % acetic acid and destained with 5 % methanol/7.5 % acetic acid, both at 60 C. Gels were dried under vacuum and autoradiographed at -70 C with Kodak T-MAT G film. After autoradiography, lanes were excised from the gel and cut into 1 mm fractions, which were counted for radioactivity.

Protein disulfide isomerase. PDI was purified to homogeneity from bovine liver by published procedures (17). Rabbit polyclonal antiserum was raised against the purified enzyme (18) and anti-PDI antibodies were immuno-affinity-purified from the antiserum by adsorption onto the antigen blotted onto nitrocellulose paper (S.J. Murant and R.B. Freedman, in preparation).

Immunoblotting. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter by the electroblotting method of Towbin et al. (19). Blots were subsequently blocked with skimmed milk, incubated for 2 h with 500-fold diluted anti-bovine PDI antibodies or non-immune serum, and for 2 h with $10^3$-fold diluted GAR-AP with appropriate intermittent washings (PBS/0.05 % Tween 20). The blots were stained for alkaline phosphatase using a mixture of 4-aminodiphenylamine diazonium sulfate and naphthol AS-MX phosphate (20).

Reproducibility. The data shown are from representative experiments which were repeated 2 or 3 times with closely agreeing results.

## RESULTS

Reaction of rat liver microsomes with BrAc[$^{125}$I]$T_3$ resulted in the predominant labeling of 2 protein bands with $M_r$ 27 and 56 kDa. Figure 1A shows that labeling of the 27 kDa band was strongly inhibited if the reaction with BrAc-[$^{125}$I]$T_3$ was carried out in the presence of 10 uM $rT_3$. Inhibition was also observed with 100 uM PTU and to a lesser extent with 10 uM IOP, whereas addition of 10 uM $T_4$, $T_3$, DIT or thyronine ($T_0$) had no significant effect. Inhibition of labeling of the 27 kDa band by $rT_3$ and/or PTU was accompanied by an increased BrAc[$^{125}$I]$T_3$ incorporation into the 56 kDa protein. Labeling of the latter was only slightly diminished by $T_3$ and $T_0$ and was not affected by IOP or DIT.

Labeling of the 27 kDa band was completely inhibited by coincubation with 10 uM $rT_3$ and 100 uM PTU. The reversibility of this inhibition was investiga-

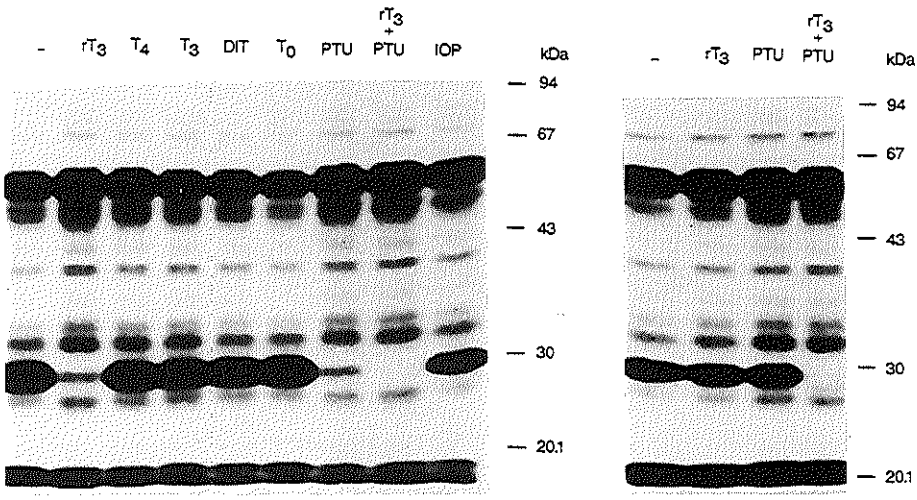


Fig.1. Effects of substrates and inhibitors of ID-I on the labeling of rat liver microsomal protein with BrAc[$^{125}$I]$T_3$.
A: Microsomes (50 ug protein) were reacted for 10 min at 37 C with 0.5 uCi BrAc[$^{125}$I]$T_3$ as described in Materials and Methods in the absence or presence of 10 uM $rT_3$, $T_4$, $T_3$, DIT, $T_0$, or IOP, or 100 uM PTU or 10 uM $rT_3$ + 100 uM PTU. After SDS-PAGE, film was exposed for 4 h.
B: Microsomes were incubated for 10 min at 37 C without or with 10 uM $rT_3$ and/or 100 uM PTU. Subsequently, microsomes were isolated by centrifugal column chromatography on Sephadex LH-20 (21), and 50 ug protein thus obtained was reacted for 5 min at 37 C with 0.5 uCi BrAc-[$^{125}$I]$T_3$. After SDS-PAGE, film was exposed for 4 h.

ted by preincubation of microsomes for 10 min with 10 uM $rT_3$ or 100 uM PTU alone or in combination. Following removal of the inhibitors on Sephadex LH-20 using the centrifugal column chromatography method of Penefsky (21), the microsomes were reacted for 5 min with BrAc[$^{125}$I]$T_3$. Figure 1B shows that preincubation of rat liver microsomes with $rT_3$ or PTU alone did not affect labeling of the 27 kDa band. However, in combination $rT_3$ and PTU completely eliminated subsequent BrAc[$^{125}$I]$T_3$ incorporation into the 27 kDa protein. Again, this was accompanied by a significant increase in the labeling of the 56 kDa band.

The effect of trypsinization on the labeling of the 27 and 56 kDa bands was studied by treatment of 120 ug microsomal protein for 1 h at 37 C with 2 ug of the protease. Digestion was terminated by addition of excess soybean trypsin inhibitor. Half of the mixture was reacted for 20 min at 37 C with 0.35 uCi BrAc[$^{125}$I]$T_3$, and proteins were separated by SDS-PAGE. Figure 2 shows that after trysinization only a faint band appears at the position of the 27 kDa protein after prolonged exposure of the film, while labeling of the 56 kDa protein was rather increased following trypsin treatment.

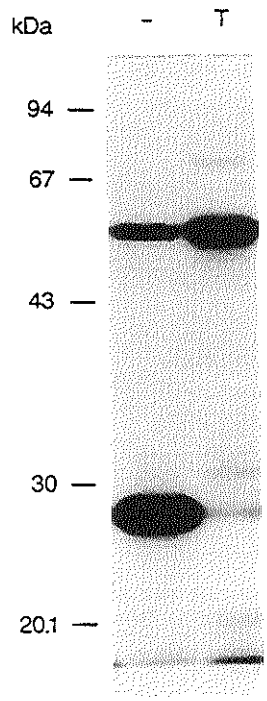


Fig.2. Effects of treatment of rat liver microsomes with trypsin on protein labeling with BrAc[$^{125}$I]$T_3$. Microsomes (120 ug protein) were incubated for 1 h at 37 C without (-) or with (T) 2 ug trypsin, followed by addition of 6 ug soybean trypsin inhibitor. Microsomal protein (60 ug) was subsequently labeled for 20 min at 37 C with 0.35 uCi BrAc[$^{125}$I]$T_3$ and analysed by SDS-PAGE. Film exposure was for 17 h.

ID-I activity was determined in the remainder of the trypsinized microsomes in parallel with untreated controls. At a microsomal protein concentration in the assay of 50 ug/ml, ID-I activity was undetectable in trypsin-treated microsomes, whereas complete deiodination of substrate was observed in control microsomes. No effects of trypsin on protein labeling or ID-I activity were seen if trypsin inhibitor was added to the microsomes before the protease (not shown). Previous work has established that, in conditions where marker enzymes of the cytosolic surface of microsomal membranes are extensively degraded by proteases, PDI is completely protected from degradation (22).

After treatment of rat liver microsomes with carbonate buffers of increasing pH, ID-I activity of the resulting membrane fractions remained constant over the pH-range 8.0 to 9.5 and strongly diminished at pH $\geq$ 10. Figure 3A shows an autoradiogram of the labeled proteins separated by SDS-PAGE after reaction of these microsomal preparations with BrAc[$^{125}$I]$T_3$. The quantitative results of these experiments are presented in Table 1. The incorporation of

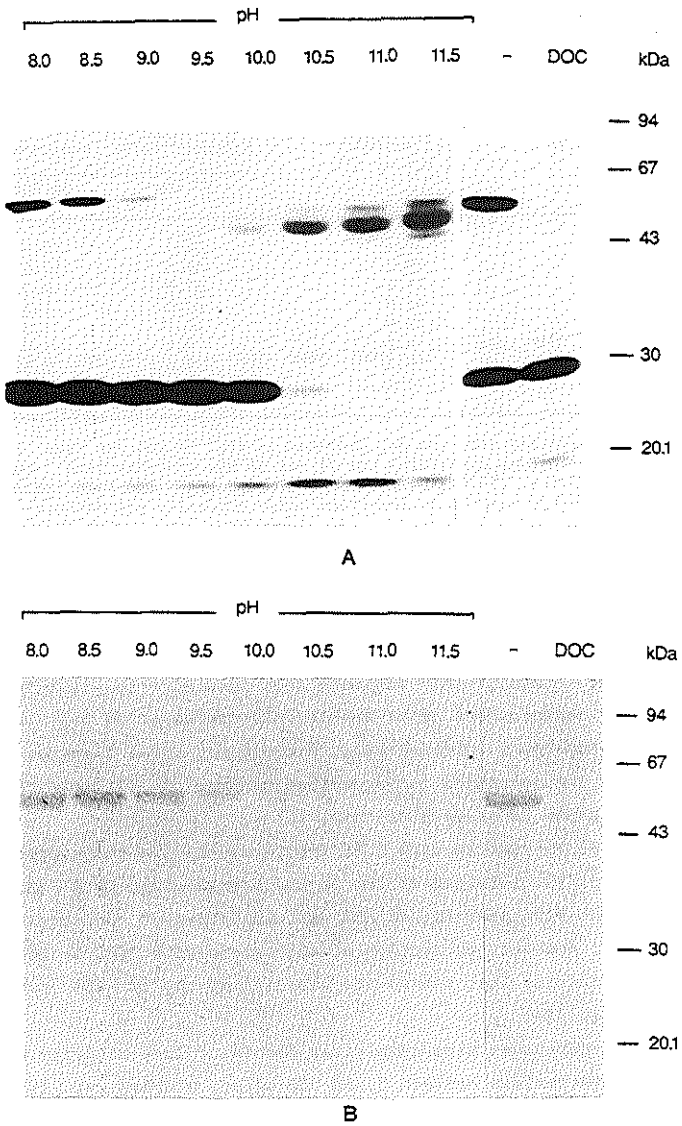


Fig.3. A: Influence of pretreatment of rat liver microsomes at pH 8.0-11.5 or with 0.05 % DOC on labeling of proteins with BrAc[$^{125}$I]$T_3$. Microsomes (7.2 mg protein) were processed as described under Materials and Methods. The resulting membrane suspensions (15 ul) were diluted 4-fold and reacted for 5 min at 37 C with 0.25 uCi BrAc[$^{125}$I]$T_3$. After SDS-PAGE, film was exposed for 2 h.
B: Appearance of PDI on Western blot after pretreatment of rat liver microsomes at pH 8.0-11.5 or with 0.05 % DOC. After pretreatment, proteins in the resulting membrane suspensions (15 ul) were diluted 4-fold, separated by SDS-PAGE and transferred to a nitrocellulose filter. PDI was visualized by rabbit anti-bovine PDI antibodies as described under Materials and Methods. No bands could be detected with non-immune serum (not shown).

TABLE 1

Effects of pretreatment of rat liver microsomes at pH 8.0-11.5 on ID-I activity and labeling of 27 and 56 kDa proteins with BrAc[$^{125}$I]$T_3$

| Pretreatment pH | ID-I activity (% $rT_3$ -> $I^-$) | % BrAc[$^{125}$I]$T_3$ incorporation | |
|---|---|---|---|
| | | 27 kDa | 56 kDa |
| 8.0 | 34.8 | 58.1 | 12.7 |
| 8.5 | 29.7 | 56.3 | 11.0 |
| 9.0 | 27.4 | 57.8 | 6.3 |
| 9.5 | 31.9 | 61.5 | 1.4 |
| 10.0 | 21.0 | 49.1 | 1.5 |
| 10.5 | 2.2 | 8.2 | 1.8 |
| 11.0 | 0.2 | 4.4 | 2.0 |
| 11.5 | 0.1 | 4.2 | 3.4 |

Lanes were excised from the gel depicted in Fig. 3A and cut into 1 mm fractions. Fractions containing the 27 and 56 kDa proteins were counted for radioactivity. ID-I activity was determined after 160-fold dilution of unfractionated samples as described under Materials and Methods.

BrAc[$^{125}$I]$T_3$ into the 27 kDa band closely paralleled the ID-I activity of the membrane fractions, showing a pronounced diminution only at pH $\geq$ 10. However, labeling of the 56 kDa protein was much more sensitive to elevation of pH, being virtually undetectable after washing microsomes at pH 9.5 and higher. Immunoblots of carbonate buffer-treated microsomes showed a decrease of PDI that paralleled the decreased incorporation of BrAc[$^{125}$I]$T_3$ into the 56 kDa band. Figure 3B shows an example of this.

Pretreatment of microsomes with 0.05 % DOC resulted in the complete elimination of BrAc[$^{125}$I]$T_3$ labeling of the 56 kDa protein band (Fig. 3A). This was paralleled by the disappearance of PDI on immunoblots of DOC-treated microsomes (Fig. 3B). However, extraction of microsomes with 0.05 % DOC affected neither ID-I activity (not shown) nor labeling of the 27 kDa protein with BrAc[$^{125}$I]$T_3$ (Fig. 3A).

Rat pancreas microsomes were completely devoid of ID-I activity when tested at protein concentrations between 5 and 500 ug/ml, whereas rat liver microsomes showed 43 % deiodination at 5 ug protein/ml. Figure 4A shows that reaction of rat pancreas microsomal proteins with BrAc[$^{125}$I]$T_3$ resulted in the labeling of predominantly 2 protein bands with $M_r$ 52 and 56 kDa. Both bands were visualized with anti-PDI antibodies on Western blots (not shown). Even after prolonged exposure of the film, no radioactive band corresponding to a 27 kDa protein could be detected.

Purified bovine PDI (1 ug) was reacted with 0.2 uCi BrAc[$^{125}$I]$T_3$. This resulted in a clearly labeled 56 kDa protein band (Fig. 4B). The homogenous PDI showed no ID-I activity when tested at concentrations of 5 or 50 ug/ml,

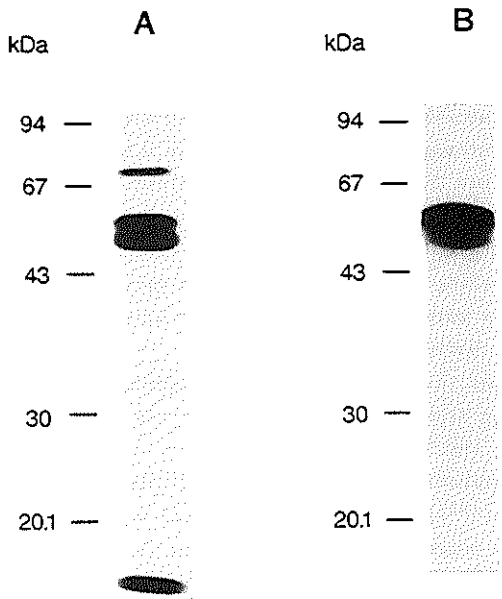


Fig.4. A: Autoradiogram of BrAc[$^{125}$I]$T_3$-labeled rat pancreas microsomes after SDS-PAGE. Rat pancreas microsomes (50 ug protein) were reacted for 5 min at 37 C with 0.5 uCi BrAc[$^{125}$I]$T_3$. Film was exposed for 2 h.
B: Autoradiogram of BrAc[$^{125}$I]$T_3$-labeled bovine PDI after SDS-PAGE. PDI (1 ug) was reacted for 10 min at 37 C with 0.2 uCi BrAc[$^{125}$I]$T_3$. Film was exposed for 19 h.

while rat liver microsomes showed 40-100 % deiodination in parallel incubations at similar total protein concentrations.

## DISCUSSION

The evidence that the 27 kDa protein which is labeled by reaction of rat liver microsomes with BrAc[$^{125}$I]$T_3$ represents ID-I or a subunit of this enzyme may be summarized as follows.

1) Labeling of the 27 kDa protein is inhibited by the preferred ID-I substrate $rT_3$ and the competitive inhibitor IOP but is little affected by the poor substrates $T_4$ and $T_3$ or the non-substrates DIT and $T_0$. Although PTU itself also inhibits BrAc[$^{125}$I]$T_3$ incorporation into the 27 kDa protein, its action is potentiated in the presence of substrate. This is especially evident in the experiments where preincubation of microsomes with either PTU or $rT_3$ alone had no effect on the labeling of the 27 kDa protein, whereas preincubation with PTU plus $rT_3$ completely eliminated subsequent labeling of this protein. These results are compatible with the view that the uncompetitive inhibition of ID-I by PTU is due to covalent binding of the inhibitor with a substrate-induced

oxidized form of the enzyme, probably a sulfenyl iodide intermediate (3).
2) Labeling of the 27 kDa protein is inhibited in parallel with a decrease in ID-I activity following treatment of rat liver microsomes with trypsin. Further, neither labeling of the 27 kDa protein nor ID-I activity are affected by treatment of microsomes with 0.05 % DOC or with buffers of pH 8.5-9.5, conditions which are known to specifically release luminal proteins (22,23). These results are in agreement with the idea that ID-I is a cytoplasm-oriented, transmembrane protein of the endoplasmic reticulum (24,25). The correlation between labeling of the 27 kDa protein and ID-I activity is further underscored by the parallel decrease in these parameters following treatment of microsomes with buffers of pH $\geqslant$ 10.
3) Previous findings in our laboratory have suggested that the kinetics of ID-I inactivation by $BrAcT_3$ are indentical with the kinetics of $BrAc[^{125}I]T_3$ incorporation into the 27 kDa protein (4).
4) The tissue distribution of the microsomal 27 kDa protein labeled with $BrAc-[^{125}I]T_3$ or $BrAc[^{125}I]T_4$ appears to correspond with the occurence of ID-I activity. This is suggested by the high levels found in liver (ref. 4; this paper) and kidney (26), and the lack of ID-I activity and of the labeled 27 kDa protein in pancreas.

There is a clear discrepancy between the $M_r$ of the 27 kDa protein labeled in rat liver and kidney microsomes with bromoacetyl-iodothyronine derivatives and the minimum molecular weight estimates for detergent-dispersed ID-I preparations, i.e. 55-60 kDa (27,28). This suggests that the 27 kDa protein indeed represents a subunit of the deiodinase, but it remains to be established if ID-I is composed of identical or different subunits.

Several lines of evidence suggest that incorporation of $BrAc[^{125}I]T_3$ into the 56 kDa microsomal protein represents labeling of PDI; these may be summarized as follows.
1) Parallel changes were observed in the PDI content of microsomes, determined on Western blots, and the extent of labeling of the 56 kDa protein by $BrAc-[^{125}I]T_3$, after various treatments of the microsomal preparations. Thus, treatment with trypsin had no effect on the labeling of the 56 kDa protein or the PDI content, as previous studies showed the protease-resistance of PDI in intact microsomes (22). Treatment of microsomes at pH $\geqslant$ 9 or with low concentrations of surfactant (0.05 % DOC) released PDI from the microsomal preparations, as judged by its disappearance from Western blots of microsomal proteins after these treatments; labeling of the 56 kDa protein by $BrAc-[^{125}I]T_3$ decreased in parallel. The behaviour of the 56 kDa reactive protein is therefore characteristic of a protein located on the luminal side of the endoplasmic reticulum membrane, such as PDI (22,29).
2) Labeling of the 56 kDa band is extensive in tissues with a high PDI con-

tent, i.e. liver and pancreas; we have not analysed tissues with low PDI activity.

3) Purified PDI is extensively labeled with BrAc[$^{125}$I]$T_3$, producing a radioactive band that comigrates with the labeled 56 kDa microsomal protein on SDS-PAGE, which is in close agreement with the molecular weight for PDI of 56.8 kDa (7,17).

4) Other investigators have purified the BrAc[$^{125}$I]$T_3$-labeled 56 kDa protein. On the basis of monoclonal antibodies produced against this protein, they have isolated and sequenced cDNA clones that were found to code for PDI (12,13).

The high susceptibility of PDI to modification with alkylating agents and its abundance in rat liver and pancreas (9) explain its extensive labeling with BrAc[$^{125}$I]$T_3$ in microsomes of these tissues. A possible contributory factor in the ready labeling of PDI by BrAc[$^{125}$I]$T_3$ is the presence within the sequence of PDI of a domain highly homologous in sequence to the steroid-binding region of the mammalian oestrogen receptor (30); this domain might have some affinity for $T_3$ analogues.

The non-identity of ID-I and PDI is further substantiated by the widely different properties reported for these enzymes. As mentioned above, PDI is known to be an acidic protein with a pI of 4.2 (10), whereas in the delipidated state ID-I is a basic protein of pI 9.3 (5). Moreover, in contrast to the abundance of PDI in liver, where it represents 1-2 % of microsomal protein (9), the level of ID-I in rat liver microsomes amounts to only ~0.01 % of total protein (4). It has recently been reported that ID-I activity is expressed in Xenopus laevis oocytes by injection of a polyadenylated rat liver RNA fraction of 1.5-2.0 kb, while mRNA fractions of greater size were ineffective (31). Since the major PDI mRNA species in rat liver is 2.8 kb in size (7), this observation also implies that PDI and ID-I are not identical.

The reason that Boado et al. (6) have cloned cDNA that codes for PDI in the course of experiments designed to elucidate the sequence of ID-I must be sought in the nonspecific screening method they have used. This was done by an indirect assay of proteins produced by clones of a liver cDNA library which competed with ID-I in CHAPS-solubilized microsomes for binding to polyclonal antibodies raised against crude liver microsomal protein (32). It is known that solubilization of rat liver microsomes with CHAPS gives rise to the formation of large complexes that apart from lipid and detergent may be composed of multiple proteins (5). It may be envisaged in such a procedure, therefore, that antibodies which apparently bind to certain microsomal proteins (e.g. ID-I) are in fact specific for epitopes on other proteins (e.g. PDI) also present in these complexes. It is even possible that artefactual association between ID-I and PDI in such complexes may arise from the functional properties of the proteins. ID-I is regularly assayed in the presence of DTT

as reductant, and the small dithiol-containing cytosolic protein thioredoxin is - to a limited extent - capable of supporting microsomal deiodination (33). The sequence of PDI contains two domains which are highly homologous to thioredoxin (7,9), and could thus interact with the active site of ID-I.

In conclusion, we have provided strong evidence that the hypothesis that PDI and ID-I are the same protein is not correct. The identity of ID-I, therefore, remains to be determined.

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## Chapter 3

# SPECIES DIFFERENCES IN LIVER TYPE I IODOTHYRONINE DEIODINASE

# Species differences in liver type I iodothyronine deiodinase

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Key words: Thyroid hormone; Deiodination; Animal; Liver; Selenium; Affinity-labeling; Bromoacetyl iodothyronine

The type I iodothyronine deiodinase (ID-I) of liver is an important enzyme for the conversion of the prohormone thyroxine ($T_4$) to the active thyroid hormone 3,3',5-triiodothyronine ($T_3$). Because it is an integral membrane protein of low abundance, purification of ID-I from rat liver has proven to be difficult. We have analyzed ID-I in liver microsomal fractions from various animals to reveal possible species differences and to explore alternative sources for the isolation of the enzyme. ID-I was characterized by enzyme assay with 3,3',5'-triiodothyronine ($rT_3$) as the preferred substrate and by affinity-labeling with *N*-bromoacetyl-[$^{125}$I]$T_3$ (BrAc[$^{125}$I]$T_3$). Labeled ID-I subunit was identified and quantified by SDS-PAGE and autoradiography. The $M_r$ of ID-I in the species investigated varied between 25.7 and 29.1 kDa. Rat and dog liver microsomes had a markedly higher enzyme content than microsomes of human, mouse, rabbit, cow, pig, sheep, goat, chicken or duck liver. Rat liver microsomes showed the highest ID-I activity of all species examined. Turnover numbers for ID-I varied between 264 and 1059 $min^{-1}$, with rabbit and goat showing the highest values. However, dog liver ID-I displayed an exceptionally low turnover number of 78 $min^{-1}$. In conclusion, ID-I has similar properties in all species examined with the notable exception of dog.

## Introduction

The thyroid gland of healthy humans produces predominantly $T_4$, which shows little intrinsic bioactivity and is therefore regarded as a prohormone. By deiodination, $T_4$ is converted to the bioactive thyroid hormone $T_3$ or to the biologically inactive metabolite $rT_3$. About 80% of circulating $T_3$ and more than 95% of circulating $rT_3$ are generated by peripheral deiodination of $T_4$. Deiodination is also an important pathway for the metabolism of $T_3$ and $rT_3$, yielding in both cases 3,3'-diiodothyronine (3,3'-$T_2$) as the product [1].

The enzyme ID-I, predominantly located in liver and kidney, is responsible for the major part of peripheral $T_3$ production [1]. In human and rat liver, ID-I is associated with the endoplasmic reticulum and displays both 5- and 5'-deiodinase activities [1]. The enzyme shows preference for $rT_3$ as substrate and is specifically inhibited by 6-propyl-2-thiouracil (PTU) [1]. Affinity-labeling of ID-I in rat liver microsomes with *N*-bromoacetyl iodothyronine derivatives has identified an enzyme subunit of $M_r \approx 27$ kDa [2–5].

As ID-I plays a key-role in thyroid hormone metabolism, its characterization has been subject of extensive investigation. Several attempts have been made to isolate ID-I from rat liver or kidney microsomes, but purification has at best been modest [6–8], which is explained by the findings that ID-I is a very hydrophobic [5], low-abundance [9,10], integral membrane protein that is probably composed of two subunits [8]. Cloning strategies using antibodies against solubilized ID-I have led to the incorrect identification of ID-I as protein disulfide isomerase (PDI) [11], a protein completely different from ID-I [3].

The purpose of the present study was 2-fold, i.e. to investigate the homology between ID-I of different species and to explore alternative sources for the isolation of the enzyme. This was done by determination of ID-I activity and content in liver microsomal fractions from various animals by enzyme assays and affinity-labeling with BrAc[$^{125}$I]$T_3$.

## Materials and Methods

### *Materials*

[3',5'-$^{125}$I]$rT_3$ and [3'-$^{125}$I]$T_3$ (≈ 1700 Ci/mmol) were obtained from Amersham (Amersham, UK); unlabeled

Abbreviations: ID-I, type I iodothyronine deiodinase; $T_4$, prohormone thyroxin; $T_3$, 3,3',5-triiodothyronine; $rT_3$, reverse 3,3',5'-triiodothyronine; PTU, 6-propyl-2-thiouracil; PDI, protein disulfide isomerase; BrAc[$^{125}$I]$T_3$, *N*-bromoacetyl-[$^{125}$I]$T_3$; 3,3'-$T_2$, 3,3'-diiodothyronine.

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iodothyronines from Henning (Berlin, Germany); dithiothreitol (DTT) and PTU from Sigma (St. Louis, MO, USA); electrophoresis grade SDS-PAGE reagents from Bio-Rad (Richmond, IL, USA); BCA protein assay reagent from Pierce Europe (Oud Beijerland, Netherlands); $M_r$ markers and Sephadex LH-20 from Pharmacia (Uppsala, Sweden); and Coomassie brilliant blue R-250 from Merck (Darmstadt, Germany).

*Preparation of microsomes*

Liver microsomes were prepared in buffer A (10 mM Tris-HCl, pH 7.4, 3 mM EDTA and 3 mM DTT) as previously described [12] and stored at −70°C. Protein content was measured by Pierce BCA protein assay, using bovine serum albumin as the standard.

*Deiodinase assay*

ID-I activity was determined by incubation of the appropriate amount of microsomal protein for 20 min at 37°C with 10 $\mu$M $rT_3$ and 75 nCi [$^{125}$I]$rT_3$ in 200 $\mu$l buffer B (0.2 M phosphate, pH 7.2, 4 mM EDTA and 10 mM DTT). The reaction was stopped by placing the samples on ice and adding 750 $\mu$l of 1 M HCl. Released $^{125}I^-$ was separated from iodothyronines on Sephadex LH-20 as described before [13], and the data were corrected for non-enzymatic deiodination as determined in incubations without microsomes. For calculation of ID-I activity, random labeling of the 3′ and 5′ positions of [$^{125}$I]$rT_3$ was taken into account.

*Synthesis of BrAc[$^{125}$I]$T_3$ and nonradioactive $BrAcT_3$*

The affinity labels were prepared essentially as previously published [2]. HPLC analysis demonstrated that the purity of BrAc[$^{125}$I]$T_3$ was at least 85% with unreacted [$^{125}$I]$T_3$ as the main contaminant, while nonradioactive $BrAcT_3$ was more than 95% pure.

*Affinity-labeling*

Solutions of BrAc[$^{125}$I]$T_3$ and $BrAcT_3$ in ethanol were pipetted into an Eppendorf tube and the solvent was evaporated at 42°C under a stream of nitrogen. The desired amount of microsomal protein in 100 $\mu$l buffer A was added to the residue and the mixture was vortexed for 30 s. After further incubation for 10 min at 37°C, labeling was stopped by addition of 50 $\mu$l of SDS-sample buffer containing 30% $\beta$-mercaptoethanol and treatment for 5 min at 100°C. Proteins were separated overnight by SDS-PAGE in a 14 cm 10% T, 3% C gel, overlaid by a 2 cm 3% T, 3% C stacking gel [14]. With all samples tested, over 90% of applied radioactivity had moved into the separation gel. Gels were stained with Coomassie brilliant blue R-250, dried under vacuum and autoradiographed at −70°C with Kodak T-MAT G film. After autoradiography, lanes were excised from the gel and cut into 1 mm fractions, which were counted for radioactivity. Apparent molecular mass ($M_r$) was determined by interpolation with protein markers.

*Deiodinase content*

The ID-I content of the microsomes was calculated by saturation analysis of the progressive labeling of protein in the ≈ 27 kDa band with increasing concentrations of $BrAcT_3$, assuming that one molecule of ID-I can only bind one molecule of $BrAcT_3$. Two methods were used for this purpose. First, data were analyzed by a direct linear plot of the amount of $BrAcT_3$ incorporated in the ≈ 27 kDa band as a function of the amount of $BrAcT_3$ added, similar to the

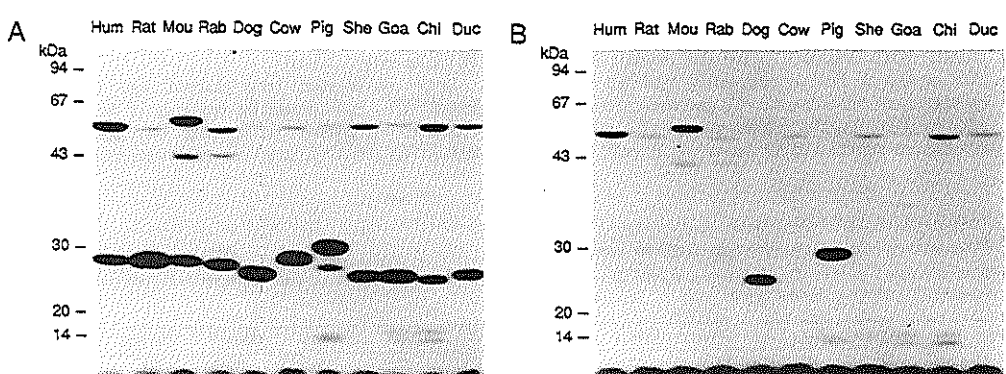


Fig. 1. Labeling of liver microsomal proteins of different species with BrAc[$^{125}$I]$T_3$. Microsomes (50 $\mu$g protein) were reacted for 10 min at 37 C with 0.1 $\mu$Ci BrAc[$^{125}$I]$T_3$ in the absence (A) or presence (B) of 10 $\mu$M $rT_3$ and 100 $\mu$M PTU as described in Materials and Methods. After SDS-PAGE, film was exposed for 17 h.

method described by Safran et al. [9]. At each $BrAcT_3$ concentration used, labeling of the ≈ 27 kDa band in the absence of $rT_3$ and PTU was corrected for the nonspecific labeling and background activity observed after labeling in the presence of $rT_3$ and PTU. The maximum value of the thus calculated specific labeling of the ≈ 27 kDa band obtained at high $BrAcT_3$ concentrations represents the ID-I content of the microsomes. Second, data were analyzed in a Scatchard-like plot of the radioactivity in the ≈ 27 kDa band divided by the radioactivity not associated with this band as a function of the amount of $BrAcT_3$ incorporated in the ≈ 27 kDa band. Non-linear plots were resolved into two linear components according to the method of Rosenthal [15]. The second component represents non-saturable and, thus, nonspecific $BrAcT_3$ incorporation in the ≈ 27 kDa band. The first component reflects saturable and, thus, specific $BrAcT_3$ labeling of the ≈ 27 kDa band, the maximum of which is determined by the intercept with the horizontal axis, representing the ID-I content of the microsomes.

*Reproducibility*

Liver samples were obtained from 2 or 3 animals of each species, but the results of the ID-I assays are representative compared with more detailed studies, e.g., human [12], rat [1] and chicken [16]. Unless stated otherwise, the data shown are from representative experiments which were repeated 2 or 3 times with closely agreeing results.

## Results

After reaction of $BrAc[^{125}I]T_3$ with liver microsomes followed by SDS-PAGE, two prominent radioactive proteins of ≈ 56 kDa and ≈ 27 kDa are observed in all 11 species examined (Fig. 1A). It has been demonstrated with rat liver microsomes that the 56 kDa band represents labeling of PDI, and that labeling of the 27 kDa subunit of ID-I is completely inhibited by coincubation with the substrate $rT_3$ and the uncompetitive inhibitor PTU [3]. The combination of $rT_3$ and PTU is much more effective in inhibiting the $BrAcT_3$ labeling

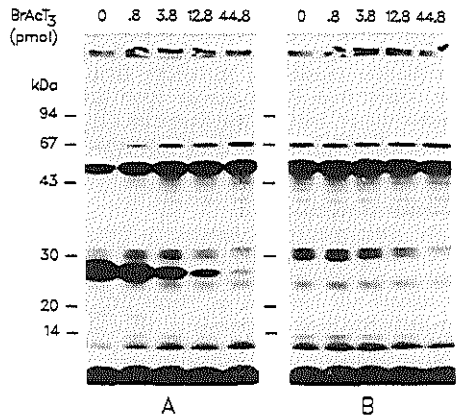


Fig. 2. Effect of increasing amounts of unlabeled $BrAcT_3$ on the labeling of rat liver microsomes by $BrAc[^{125}I]T_3$. Microsomes (100 μg protein) were reacted for 10 min at 37°C with 0.2 pmol (0.25 μCi) $BrAc[^{125}I]T_3$ in the presence of 0, 0.8, 3.8, 12.8 and 44.8 pmol of $BrAcT_3$ and in the absence (A) or presence (B) of 10 μM $rT_3$ + 100 μM PTU. After SDS-PAGE, film was exposed for 16 h.

of ID-I than either $rT_3$ or PTU alone, because of the formation of a stable, inactive PTU-enzyme complex which only occurs in the presence of substrate. ID-I was identified in other species by comparison of labeling patterns in the absence (Fig. 1A) and presence (Fig. 1B) of 10 μM $rT_3$ and 100 μM PTU. Labeling of the ≈ 56 kDa protein was not inhibited by these compounds. $BrAcT_3$ labeling of the ≈ 27 kDa protein was completely prevented by $rT_3$ plus PTU in all microsomal preparations except in dog liver microsomes where labeling was reduced by about 50%. The $M_r$ of ID-I in liver microsomes of the different species is given in Table I, showing a narrow range of 25.7 (chicken) to 29.1 kDa (cow).

Liver microsomes were reacted with 0.2 pmol $BrAc[^{125}I]T_3$ and increasing amounts of $BrAcT_3$. In order to determine nonspecific incorporation of $BrAc[^{125}I]T_3$ in ID-I, these reactions were also done in

TABLE I

*Characteristics of ID-I in liver microsomes of different species*

Molecular mass is given in kDa; ID-I content in pmol ID-I/mg protein (mean of two experiments); ID-I activity in pmol $rT_3$/min per mg protein (mean ± S.D.); turnover number in $min^{-1}$.

| | Human | Rat | Mouse | Rabbit | Dog | Cow | Pig | Sheep | Goat | Chicken | Duck |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $M_r$ | 28.0 | 28.2 | 28.3 | 27.5 | 26.2 | 29.1 | 27.6 | 26.2 | 26.6 | 25.7 | 26.5 |
| Content | 0.51 | 3.65 | 0.65 | 0.55 | 4.74 | 1.15 | 0.67 | 0.44 | 0.79 | 0.36 | 0.28 |
| Activity | 341 ± 9 | 3022 ± 81 | 233 ± 21 | 560 ± 16 | 369 ± 29 | 311 ± 27 | 267 ± 29 | 116 ± 15 | 836 ± 22 | 231 ± 28 | 130 ± 26 |
| Turnover number | 669 | 828 | 359 | 1019 | 78 | 270 | 399 | 264 | 1059 | 642 | 464 |

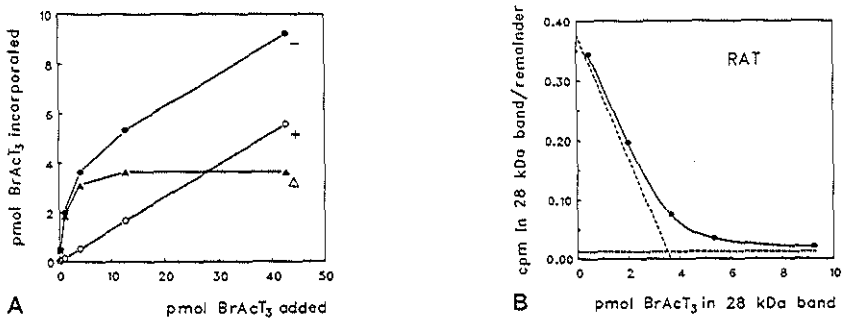


Fig. 3. Saturation analysis of the progressive incorporation of $BrAcT_3$ in rat liver ID-I with increasing amounts of added $BrAcT_3$. (A) Direct plot of $BrAcT_3$ incorporation in the ≈ 27 kDa band as a function of the amount of $BrAcT_3$ added in the absence (−) or presence (+) of 10 μM $rT_3$ and 100 μM PTU. The difference between these curves (△) represents specific $BrAcT_3$ incorporation in ID-I. (B) Scatchard-like plot of radioactivity in the ≈ 27 kDa band divided by radioactivity not associated with this band as a function of the amount of $BrAcT_3$ incorporated in the ≈ 27 kDa band. Resolution of the curve in a saturable and a non-saturable component was done as described under Materials and Methods.

the presence of 10 μM $rT_3$ and 100 μM PTU. Fig. 2 is an example of the SDS-PAGE analysis of such an experiment with rat liver microsomes. The autoradiogram in Fig. 2A demonstrates that incorporation of $BrAc[^{125}I]T_3$ in the ≈ 27 kDa band in the absence of $rT_3$ and PTU is progressively inhibited with non-radioactive $BrAcT_3$, indicating that this is largely a saturable process. The results obtained after SDS-PAGE of mixtures containing $rT_3$ and PTU (Fig. 2B) show very little nonspecific labeling of the ≈ 27 kDa band. The dose-dependent $BrAcT_3$ incorporation was determined from the radioactivity in the ≈ 27 kDa band and subjected to two methods of saturation analysis. Fig. 3A is the direct plot of $BrAcT_3$ labeling versus the amount of $BrAcT_3$ added. $BrAcT_3$ incorporation in the presence of $rT_3$ and PTU is a linear function of the amount of $BrAcT_3$ added and is subtracted from the total $BrAcT_3$ incorporation occurring in the absence of $rT_3$ and PTU. The thus determined specific $BrAcT_3$ incorporation in the ≈ 27 kDa band reaches a plateau at increasing $BrAcT_3$ concentrations, which corresponds to an ID-I content of 3.74 pmol/mg microsomal protein. Fig. 3B is the Scatchard-like plot of the $BrAcT_3$ incorporation in the ≈ 27 kDa band in the absence of $rT_3$ and PTU. The intercept of the saturable component of this plot with the horizontal axis represents the maximum specific $BrAcT_3$ incorporation, corresponding to an ID-I content of 3.56 pmol/mg microsomal protein. Therefore, the results of both analyses are in excellent agreement.

The ID-I contents of liver microsomes from other species were determined similarly. Fig. 4 shows the SDS-PAGE patterns of human liver microsomal proteins labeled with varying amounts of $BrAcT_3$ in the absence or presence of $rT_3$ and PTU. The graphical analysis of these results is depicted in Fig. 5, indicating once again the close agreement between the two methods for the determination of the maximum specific $BrAcT_3$ labeling of the ≈ 27 kDa band. The thus calculated ID-I content of human liver microsomes was found to be significantly lower than in the rat, i.e. 0.51 pmol/mg protein. Dog liver microsomes provided the single exception where ID-I content could not be determined directly. The autoradiograms in Fig. 6 demonstrate that the concentration-dependent $BrAcT_3$ labeling of the ≈ 27 kDa band was only partially

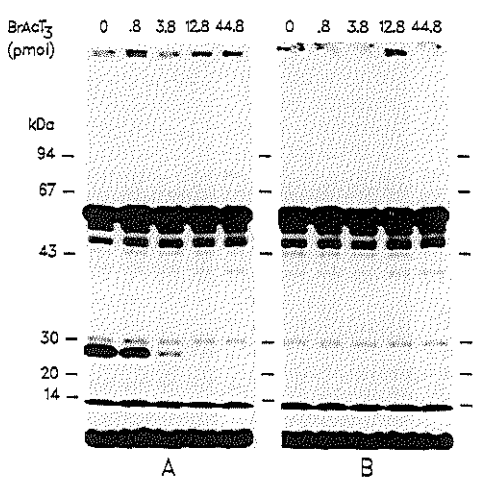


Fig. 4. Effect of increasing amounts of unlabeled $BrAcT_3$ on the labeling of human liver microsomes by $BrAc[^{125}I]T_3$ in the absence (A) or presence (B) of 10 μM $rT_3$ + 100 μM PTU. For further details, see Fig. 2.

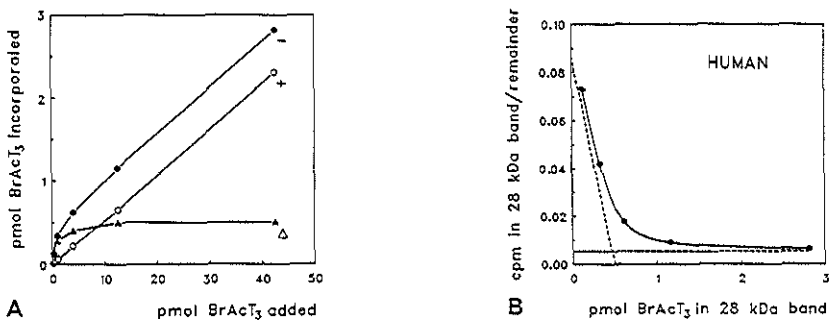


Fig. 5. Saturation analysis of the progressive labeling of human liver ID-I with increasing concentrations of $BrAcT_3$ by (A) direct plot or (B) Scatchard-like plot of the results depicted in Fig. 4. For further details, see Fig. 3.

inhibited by addition of $rT_3$ plus PTU, in agreement with Fig. 1. Therefore, the direct plot of these data as shown in Fig. 7A could not be used to determine the ID-I content of these microsomes. However, specific $BrAcT_3$ labeling of ID-I in dog liver microsomes was determined accurately by Scatchard-like analysis as shown in Fig. 7B, corresponding to an enzyme content of 4.74 pmol/mg protein. The ID-I contents of liver microsomes from the different species are summarized in Table I. It is clear that enzyme contents of rat and dog liver microsomes are markedly higher than in all other species examined.

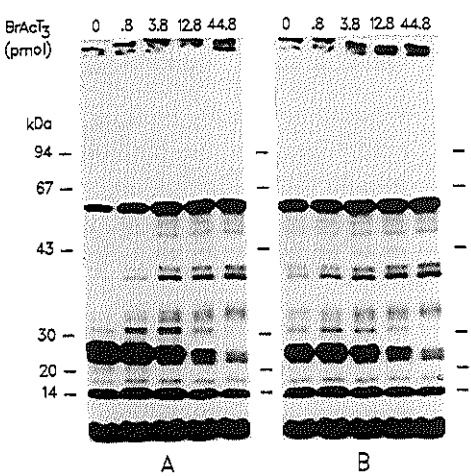


Fig. 6. Effect of increasing amounts of unlabeled $BrAcT_3$ on the labeling of dog liver microsomes by $BrAc[^{125}I]T_3$ in the absence (A) or presence (B) of 10 $\mu$M $rT_3$ + 100 $\mu$M PTU. For further details, see Fig. 2.

ID-I activity was determined at 10 $\mu$M $rT_3$, although in most species 1 $\mu$M $rT_3$ proved to be a near-saturating substrate concentration except for dog. The lower affinity of $rT_3$ for dog ID-I is supported by estimations of its $K_m$ value of $\approx 5$ $\mu$M (T.J. Visser, unpublished work) as opposed to $\approx 0.1$ $\mu$M in human [12], rat [1] and chicken [16] and is further reflected by the finding that dog ID-I is still labeled by $BrAc[^{125}I]T_3$ in the presence of $rT_3$ and PTU (Fig. 1B). The results of the ID-I assays are presented in Table I. Lowest ID-I activity is observed in sheep liver and highest activity in rat liver. Division of ID-I activity by ID-I content yields turnover numbers for the enzymatic deiodination of $rT_3$. Table I shows that turnover numbers vary between 264 and 1059 $min^{-1}$ except for dog ID-I which has an extremely low turnover number of 78 $min^{-1}$.

## Discussion

ID-I has been studied extensively in liver of humans [12], rats [1], dogs [17] and chickens [16]. Less information is available about the enzyme in pig [18], cow [19], mouse [20], duck [21] and sheep [22] liver, while very little is known about the deiodinase in rabbit and goat. Available evidence indicates a high degree of similarity between liver ID-I enzymes from different species, with the following characteristics: (a) localization in microsomal fraction; (b) catalysis of both 5- and 5'-deiodinations; (c) stimulation of deiodinase activity by small thiol-containing compounds such as DTT; (d) uncompetitive inhibition by PTU; (e) preference for $rT_3$ as the substrate; and (f) facilitated deiodination of sulfated iodothyronines [1]. The present findings extend these previous observations by demonstrating that $BrAcT_3$ is an equally effective affinity-label for ID-I from different species with a narrow range of subunit $M_r$. Recently a cDNA encoding rat ID-I has been

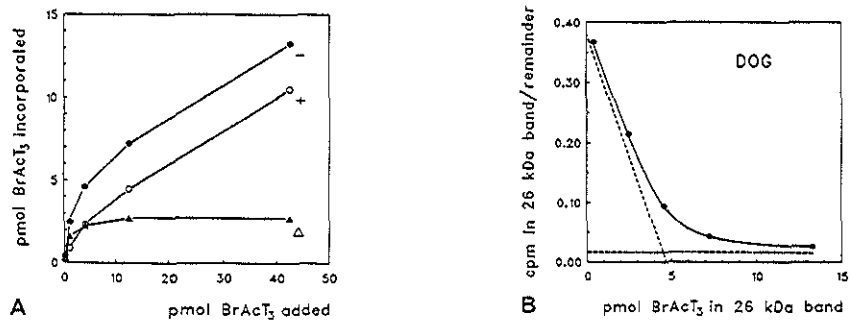


Fig. 7. Saturation analysis of the progressive labeling of dog liver ID-I with increasing concentrations of $BrAcT_3$ by (A) direct plot or (B) Scatchard-like plot of the results depicted in Fig. 6. For further details, see Fig. 3.

cloned using the *Xenopus* oocyte expression system [23]. From the DNA sequence it is deduced that ID-I is a hydrophobic protein with a molecular mass of 29.7 kDa and contains a selenocysteine residue.

It has been demonstrated that ID-I of rat liver and kidney is highly susceptible to inhibition by iodoacetate, showing that $\mu$M concentrations of this reagent are sufficient to completely inactivate the enzyme [10]. This inactivation has previously been thought to result from the covalent modification of a catalytically active sulfhydryl group, but it is now realized that this is probably due to carboxymethylation of the selenocysteine residue [23]. It remains to be seen if the convenient labeling of ID-I with $BrAcT_3$ is based on reaction with the same functional group in all species examined. The finding that $BrAcT_3$ labeling, which is prevented by $rT_3$ and PTU [3], results in a loss of enzyme activity suggests that the enzyme active site is modified upon labeling.

Two observations in the present study deserve further comment. Firstly, in addition to the $\approx$ 27 kDa and $\approx$ 56 kDa proteins, another prominent band ($M_r$ 32 kDa) was observed after labeling of pig liver microsomes (Fig. 1), which also showed saturation with increasing $BrAcT_3$ concentrations (not shown). A similar faint band was observed in liver microsomes of other species (Figs. 2, 4 and 6). Preliminary evidence suggests that this may represent labeling of a type III iodothyronine deiodinase (ID-III), a low $K_m$ 5-deiodinase with preference for $T_3$, since (a) pig liver microsomes possess significant ID-III activity, and (b) a similar $\approx$ 32 kDa band was observed in rat brain and placenta, tissues with high ID-III activities [1]. However, the relationship of the $\approx$ 32 kDa protein to ID-III remains to be determined. Secondly, the labeling of dog liver ID-I with $BrAcT_3$ is inhibited much less effectively by the presence of $rT_3$ and PTU than the ID-I labeling in other species. This is compatible with the findings that the substrate specificity of dog liver ID-I differs from the enzymes of human [12], rat [1] and chicken [16] liver, indicating a lesser preference for $rT_3$ over other iodothyronines as the substrate (Ref 17; T.J. Visser, unpublished work).

In conclusion, affinity-labeling with $BrAcT_3$ suggests a high degree of homology between different species with respect to subunit structure of liver type I iodothyronine deiodinase.

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## Chapter 4

# IMPAIRMENT OF THE SELENOENZYME TYPE I IODOTHYRONINE DEIODINASE IN C3H/He MICE

# Impairment of the Selenoenzyme Type I Iodothyronine Deiodinase in C3H/He Mice*

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**ABSTRACT**

Type I iodothyronine deiodinase (ID-I) activity is impaired in C3H/He (C3H) mice compared with BALB/c and C57BL/6N (C57) mice. In this study we compared ID-I activity and protein labeling with *N*-bromoacetyl-[$^{125}$I]$T_3$ (BrAc[$^{125}$I]$T_3$) or $^{75}$Se in liver microsomes of C3H and C57 mice. Hepatic ID-I activity in C3H mice was highly variable with a median of only 18% of that in C57 mice. However, C3H mice had normal serum $T_4$ and $T_3$ levels, although serum reverse $T_3$ was increased. The 28-kilodalton (kDa) ID-I protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of BrAc[$^{125}$I] $T_3$-labeled microsomes. Labeling of this protein was virtually undetectable in C3H samples with low enzyme activity. ID-I activity in liver microsomes was strongly decreased in Se-deficient mice, which was paralleled by a drastic decrease in BrAc[$^{125}$I]T3-labeling of the 28-kDa band compared with control mice. Labeling of ID-I with $^{75}$Se was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of liver microsomes of [$^{75}$Se]selenite-injected mice. $^{75}$Se labeling of the 28-kDa band was markedly higher in Se-deficient than in control mice and was also markedly higher in C57 than in C3H mice. Finally, liver ID-I messenger RNA (mRNA) was measured on Northern blots using a rat ID-I complementary DNA probe. Messenger RNA levels correlated strongly with ID-I activity, showing a significant decrease in C3H mice. We conclude that in mice, like in rats and humans, ID-I is a selenoprotein. ID-I activity is impaired in C3H mice because of decreased transcription of the ID-I gene or reduced stability of the mRNA. (*Endocrinology* **132:** 357–361, 1993)

MANY processes involved with growth, development, and metabolism in vertebrates are controlled directly or indirectly by the thyroid hormone $T_3$ (1, 2). These actions are initiated by binding of $T_3$ to nuclear receptors, which interact with *cis*-acting elements, modulating the expression of target genes (1, 2). Most $T_3$ in the plasma of humans and rats originates from the peripheral 5′-deiodination of the prohormone $T_4$, which is the major secretory product of the thyroid (3, 4). Alternatively, $T_4$ is inactivated by 5-deiodination to reverse $T_3$ ($rT_3$) (3, 4).

Three types of enzymes catalyzing the deiodination of thyroid hormone have been identified (3, 4). Type I iodothyronine deiodinase (ID-I) is found in liver, kidney, and thyroid, and is responsible for the major part of peripheral $T_3$ production. However, the enzyme catalyzes both 5- and 5′-deiodinations and shows preference for $rT_3$ as the substrate. ID-I is located in the endoplasmic reticulum of liver cells and requires thiol compounds as cofactor (3, 4). It is specifically inhibited by 6-propyl-2-thiouracil (PTU) and by *N*-bromoacetyl-$T_3$ (Br$AcT_3$), which has proven to be a very useful affinity label of this enzyme (4, 5). Type II iodothyronine deiodinase (ID-II) catalyzes exclusively 5′-deiodination and is found in brown adipose tissue, pituitary, and brain (3, 4). Type III iodothyronine deiodinase (ID-III) catalyzes the 5-deiodination of thyroid hormone and is present predominantly in brain and placenta (3, 4).

Recently, the complete nucleotide sequence of rat liver ID-I (6) and part of the sequence of the human enzyme (7) have been reported, showing that in both species ID-I contains a selenocysteine residue. In rats, the amount of ID-I in liver, kidney, and thyroid is greatly influenced by the Se status, ID-I activity being markedly decreased in Se-deficient animals (8, 9). We have previously demonstrated that the structural and enzymatic properties of liver ID-I are very similar between different species with exception of the dog (10). Hepatic ID-I content was found to be much lower in BALB/c mice than in rats (10). However, large differences have also been observed between different mouse strains, with markedly impaired ID-I activity in C3H/He (C3H) mice compared with BALB/c and C57BL/6N (C57) mice (11). In the present study ID-I activity was compared with protein labeling with BrAc[$^{125}$I]$T_3$ or $^{75}$Se in liver and kidney microsomes of C3H and C57 mice. It was further investigated if the impaired ID-I activity in C3H mice could be due to alterations in ID-I gene expression.

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## Materials and Methods

### *Materials*

$Na_2{}^{75}SeO_3$ (~450 Ci/mol), [3′,5′-$^{125}$I]$rT_3$, and [3′-$^{125}$I]$T_3$ (1700 Ci/mmol) were obtained from Amersham (Amersham, UK); unlabeled iodothyronines from Henning (Berlin, Germany); dithiothreitol (DTT) and PTU from Sigma (St. Louis, MO); Sodium dodecyl sulfate-polyacryl-

amide gel electrophoresis (SDS-PAGE) reagents from BioRad (Richmond, CA); BCA Protein Assay Reagent from Pierce Europe (Oud Beijerland, The Netherlands); molecular mass markers and Sephadex LH-20 from Pharmacia (Uppsala, Sweden); and Coomassie brilliant blue R-250 from Merck (Darmstadt, Germany).

*Mice and diets*

Two-month-old (25–30 g body wt) mice were obtained as follows: BALB/cAnNHSD, C3H/HeNHSD, and C57BL/6NHSD mice from Harlan Sprague Dawley (Indianapolis, IN; C3H/OuJ mice from Iffa Credo (L'Arbresle, France); and C3H/HeJ mice from The Jackson Laboratory (Bar Harbor, ME). All mice were kept in plastic cages with wood chip bedding at a constant temperature and humidity and a 12 h/12 h light/dark cycle. MilliQ (Millipore, Bedford, MA)-purified water and food (diet AMII 1410, Hope Farms, Woerden, The Netherlands) were available *ad libitum*. Se-deficient food supplemented with vitamin E (diet 170698) and control Se-sufficient food (same diet supplemented with 0.2 ppm sodium selenite, diet 170699) were purchased from Teklad Test Diets (Madison, WI). Se deficiency was induced in C3H and C57 mice by feeding dams during pregnancy and lactation with the Se-deficient diet. After weaning, the newborns were kept on the same diet for at least 8 weeks. Control groups received the Se-supplemented diet.

*Preparation of microsomes*

Mice were killed by cervical dislocation, and livers, kidneys, and brains were isolated. Microsomes were prepared in 10 mM Tris/HCl (pH 7.4), 3 mM EDTA, and 3 mM DTT (TED buffer) as previously described (12) and stored at −70 C. Protein content was measured with the BCA protein assay, using BSA as the standard.

*Affinity labeling*

An ethanol solution of BrAc[$^{125}$I]$T_3$, prepared as previously described (13), was transferred to an Eppendorf tube, and the solvent was evaporated under a stream of nitrogen at 42 C. A microsomal suspension in 100 μl TED buffer was added to the residue, and the mixture was vortexed for 30 sec. After further reaction for 10 min at 37 C, labeling was stopped by addition of 50 μl SDS sample buffer containing 30% β-mercaptoethanol and treatment for 5 min at 100 C. Proteins were separated by overnight SDS-PAGE in a 14-cm 10% T, 3% C gel, [T = total concentration (wt/vol) of acrylamide + bisacrylamide: C = concentration of bisacrylamide (wt/vol) as percentage of T], overlaid by a 2-cm 3% T, 3% C stacking gel (14). Gels were stained with Coomassie brilliant blue R-250 at 60 C and dried under vacuum. Autoradiography of labeled proteins was done by exposure to Kodak T-MAT G film (Eastman Kodak Co., Rochester, NY) at -70 C. Apparent molecular mass was determined by interpolation with protein markers.

*$^{75}$Se labeling*

Se-deficient or control mice received a single ip injection with 50 μCi $Na_2{}^{75}SeO_3$. After 4 days the animals were killed, and liver microsomes were prepared for examination of $^{75}$Se-labeled proteins by SDS-PAGE.

*Deiodinase assays*

ID-I activity was measured by incubation of 2 μg liver or kidney microsomal protein for 60 min at 37 C with 0.1 μM $rT_3$ and 75 nCi [$^{125}$I] $rT_3$ in 200 μl 0.2 M phosphate (pH 7.2), 4 mM EDTA, and 5 mM DTT as previously described (10). The reactions were stopped by addition of 750 μl 1 M HCl on ice. The $^{125}I^-$ released was isolated on Sephadex LH-20 and corrected for nonenzymatic deiodination determined in incubations without microsomes. ID-III activity was measured by incubation of 200 μg brain microsomal protein for 60 min at 37 C with 1 nM $T_3$ and 75 nCi [$^{125}$I]$T_3$ in 200 μl 0.1 M phosphate (pH 7.2), 2 mM EDTA, and 10 mM DTT. The reactions were stopped by addition of 300 μl methanol on ice. After centrifugation of precipitated proteins, the supernatants were analyzed for [$^{125}$I]3,3′-diiodothyronine formation by HPLC on a 100 × 3.0 mm Chromspher C18 column (Chrompack, Middelburg, The Netherlands), eluted with a 45:55 (vol/vol) mixture of methanol and 20 mM ammonium-acetate (pH 4.0) at a flow of 0.8 ml/min. Parallel incubations were done with 1 μM $T_3$, which saturates the low-$K_M$ ID-III but not the high-$K_M$ ID-I (3) ($K_M$ = Michaelis-Menten Constant), showing negligible type I and nonenzymatic deiodination of $T_3$ by brain microsomes.

*Northern blot analysis*

RNA was isolated from liver by acid guanidinium thiocyanate-phenol-chloroform extraction (15). Samples of 20 μg RNA were separated by agarose gel electrophoresis and transferred to a GeneScreen hybridization transfer membrane (DuPont, Boston, MA) (16). Rat ID-I (rID-I) complementary DNA (cDNA) probe (6) was kindly donated by Drs. Marla Berry and P. Reed Larsen (Harvard Medical School, Boston, MA) and radioactively labeled using random primers and Klenow polymerase (17). After hybridization at 42 C in the presence of 50% formamide and 6% dextran sulfate (18), autoradiographs were quantified using a BioRad model 620 video densitometer. In order to standardize the ID-I messenger RNA (mRNA) signal, the blots were stripped for 5 min at 100 C successively with 0.1× SCC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) and water, and hybridized with a β-actin cDNA probe.

## Results

In both C3H and C57 mice ID-I activity was much lower in kidney than in liver microsomes (Table 1), which is in contrast to rats and humans, where kidney and liver display similar ID-I activities (3, 4). Mean ID-I activities in both liver and kidney microsomes were significantly lower in C3H than in C57 mice (Table 1). In contrast, ID-III activity in brain microsomes was the same in C3H and C57 mice (Table 1).

Hepatic ID-I activity was not uniformly impaired in C3H mice but showed a marked heterogeneity independent of the supplier. In this study, ID-I activity was determined in a total of 47 C3H mice and 25 C57 mice. In each experiment, enzyme activity in individual mouse livers was expressed as a percentage of the mean control value in C57 mice, and the results are presented as histograms in Fig. 1. This figure clearly demonstrates the widely different distribution of hepatic ID-I activity in C3H and C57 mice. ID-I activity is severely impaired in most C3H mice, but occasionally fairly normal levels are encountered, *i.e.* similar to those in C57 mice.

Iodothyronines were measured in serum by specific RIAs. Table 2 shows that low ID-I activity in C3H mice is not associated with changes in serum $T_3$ or $T_4$ concentrations. However, serum $rT_3$ levels are significantly higher in C3H mice than in C57 mice.

Figure 2A shows typical examples of the labeling patterns

**TABLE 1.** Iodothyronine deiodinase activities in male C57BL/6N and C3H/He mice

| | C57BL/6N | C3H/He |
|---|---|---|
| ID-I in liver (n = 12) | 35.7 ± 9.6 | 5.6 ± 6.4[a] |
| ID-I in kidney (n = 6) | 7.6 ± 1.6 | 2.2 ± 0.6[a] |
| ID-III in brain (n = 4) | 41.2 ± 2.1 | 38.5 ± 14.0 |

ID-I and ID-III activity are given in picomoles of $rT_3$ per min/mg protein and femtomoles of $T_3$ per min/mg protein, respectively (mean ± SD).

[a] $P < 0.001$.

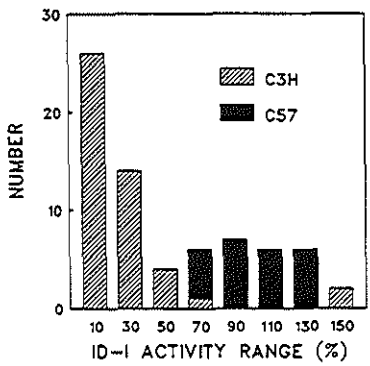


FIG. 1. Histograms of the distribution of hepatic ID-I activity in 47 C3H/He and 25 C57BL/6N mice. Liver microsomal ID-I activities in individual mice were expressed as a percentage of the mean enzyme activity of control C57 mice in each experiment. The results represent the number of mice with ID-I activities ranging from 0–20%, 20–40%, *etc.* of control.

**TABLE 2.** Serum iodothyronine concentrations in male C57BL/6N and C3H/He mice

| | C57BL/6N | C3H/He |
|---|---|---|
| $T_4$ | 29.0 ± 9.3 | 31.6 ± 7.5 |
| $T_3$ | 0.78 ± 0.23 | 0.88 ± 0.27 |
| $rT_3$ | 0.05 ± 0.02 | 0.17 ± 0.06[a] |

Concentrations are given in nanomoles per liter (mean ± SD, n = 12).
[a] $P < 0.001$.

obtained after reaction of BrAc[$^{125}$I]$T_3$ with liver microsomes of C57 and C3H mice, followed by SDS-PAGE and autoradiography. Using rat liver microsomes, the prominent 56-kilodalton (kDa) band has previously been identified as protein disulfide isomerase (5). The 28-kDa band represents ID-I (5, 10) and is labeled to highly variable extents in C3H samples. Figure 2B shows that BrAc[$^{125}$I]$T_3$ incorporation in the 28-kDa band closely parallels ID-I activity. The virtually undetectable labeling of this protein in C3H liver samples with low ID-I activity suggests a strong decrease in the amount of enzyme.

In both C3H and C57 mice Se deficiency resulted in a profound decrease in hepatic ID-I activity compared with the control groups fed the Se-supplemented diet (Table 3). The data presented in Table 3 for Se-sufficient C3H mice are another example of the wide variation in ID-I activity in this strain, ranging from grossly impaired in two mice to levels similar to those in control C57 mice in two other animals. Figure 3 compares the ID-I activities of liver microsomes of Se-deficient and control C57 mice with the labeling patterns obtained after reaction of the corresponding samples with BrAc[$^{125}$I]$T_3$. The results demonstrate that the decreased ID-I activity induced by Se deficiency is paralleled by a decrease in the BrAc[$^{125}$I]$T_3$ labeling of the 28-kDa band.

Incorporation of $^{75}$Se in the 28-kDa ID-I protein was detected by SDS-PAGE of liver microsomes of [$^{75}$Se]selenite-

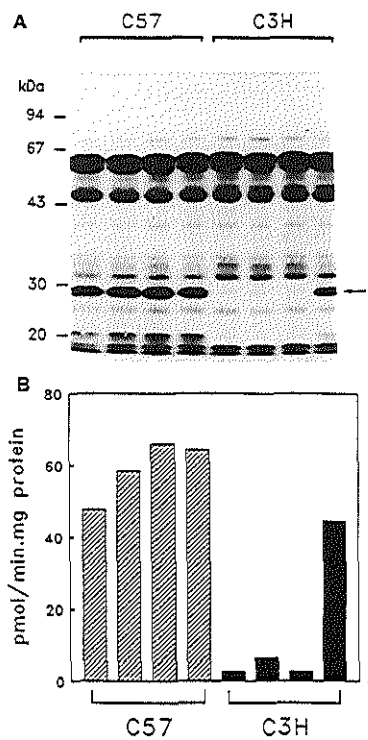


FIG. 2. A, Labeling patterns obtained after reaction of 50 μg liver microsomal protein from male C57BL/6N and C3H/He mice with 0.05 μCi BrAc[$^{125}$I]$T_3$. After SDS-PAGE, film was exposed for 26 h. B, ID-I activity of the same microsomal samples shown in A.

**TABLE 3.** Liver ID-I activity in control and Se-deficient male C57BL/6N and C3H/He mice

| | Control | Se-deficient |
|---|---|---|
| C57BL/6N | 52.7 ± 9.3 (n = 7) | 12.8 ± 1.5[a] (n = 4) |
| C3H/He | 40.5 ± 35.0 (n = 5) | 3.9 ± 0.7 (n = 4) |

ID-I activity is given in picomoles of $rT_3$ per min/mg protein (mean ± SD).
[a] $P < 0.001$.

injected mice (Fig. 4). The $^{75}$Se labeling of this band was markedly higher in Se-deficient than in Se-sufficient mice and was also markedly higher in C57 than in C3H mice.

To investigate if impaired ID-I activity in C3H mice is due to a structural defect in the ID-I gene or to decreased expression of the gene, Northern blot analysis of C3H and C57 liver RNA was performed using an rID-I cDNA probe. Figure 5 shows that differences in ID-I activity are closely paralleled by variation in liver ID-I mRNA ($r = 0.82$, $P < 0.005$). The mean ID-I mRNA level is significantly lower in C3H than in C57 mice (0.9 ± 0.2 *vs.* 1.8 ± 0.5; $P < 0.005$). When the ID-I mRNA signals were expressed relative to those for β-actin

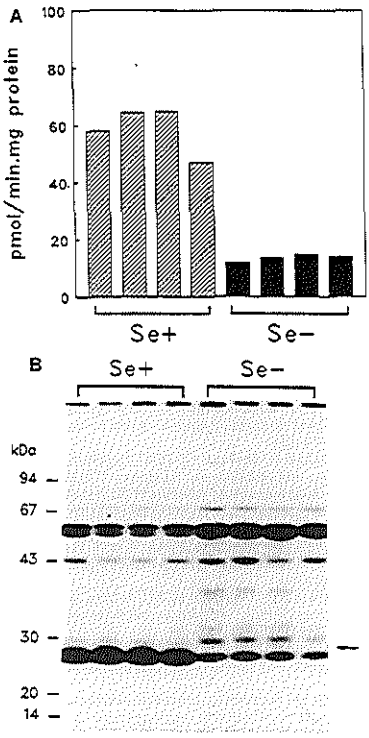


FIG. 3. A, ID-I activity in liver microsomes of Se-deficient and control male C57BL/6N mice. B, Labeling patterns obtained after reaction of the same microsomal samples (50 $\mu$g protein) shown in A with 0.13 $\mu$Ci BrAc[$^{125}$I]$T_3$. After SDS-PAGE, film was exposed for 4 h.

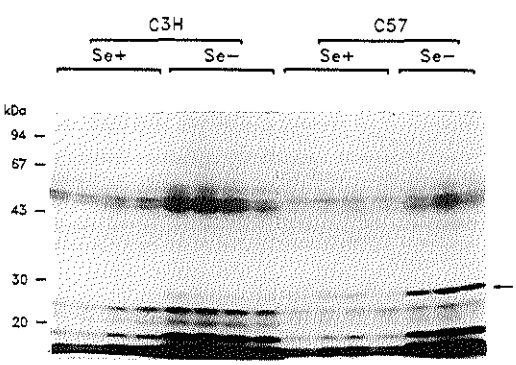


FIG. 4. $^{75}$Se labeling of liver microsomal proteins of Se-deficient and control male C3H/He and C57BL/6N mice. To each lane, 350 $\mu$g protein were applied. After SDS-PAGE, film was exposed for 34 days.

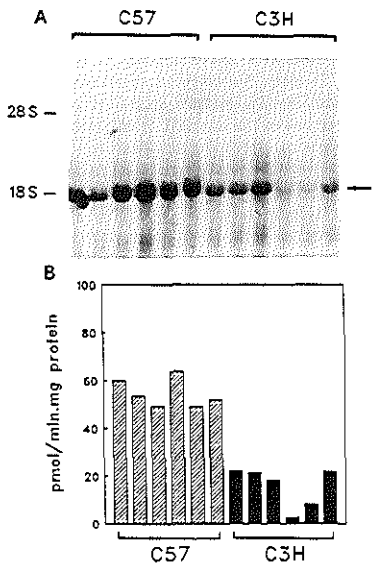


FIG. 5. A, Northern blot analysis of ID-I mRNA in livers from male C57BL/6N and C3H/He mice. B, ID-I activity in microsomes from the same livers shown in A.

mRNA, the resultant ratios were also significantly lower in C3H than in C57 mice (0.62 ± 0.19 *vs.* 1.24 ± 0.66; $P < 0.05$).

## Discussion

The present study represents the first full description of the defect in ID-I activity in an animal strain. The magnitude of this defect is not uniform in C3H mice but ranges from severely impaired to occasionally the same levels as observed in control C57 mice. In this initial work the emphasis was on the characterization of the defect rather than a detailed analysis of its biological consequences. The lack of a decrease in serum $T_3$ relative to serum $T_4$ in C3H mice with impaired ID-I activity is in contrast to findings in rats where the serum $T_3/T_4$ ratio is strongly reduced if ID-I activity is inhibited by PTU administration or Se deficiency (3, 4, 8, 9). This may be explained by: 1) a lesser importance of ID-I for plasma $T_3$ production in mice, which is supported by the much lower hepatic and renal enzyme activities compared with those in rats; and/or 2) a compensatory increase in alternative pathways of $T_3$ production, *i.e.* direct thyroidal $T_3$ secretion or $T_4$ to $T_3$ conversion by ID-II in tissues such as the brain. However, in the absence of data on $T_3$ secretion and peripheral production rates as well as of ID-II activity determinations, the importance of these routes remains speculative. The higher serum $rT_3$ levels in C3H mice compared with C57 mice are in keeping with the view that ID-I is important for the clearance of plasma $rT_3$ (3, 4).

Affinity labeling of the 28-kDa ID-I protein with BrAc[$^{125}$I]$T_3$ is decreased in C3H compared with C57 mice. Although this could be due to a lower affinity of the label for the enzyme in C3H mice, the correlation between the extent of labeling of the 28-kDa protein and ID-I activity strongly suggests a decrease in the amount of enzyme. This is further supported by the results of the analysis of ID-I labeling with $^{75}$Se as well as the quantification of ID-I mRNA (see below). The decreased ID-I activity induced by Se deficiency is paralleled by a decrease in the BrAc[$^{125}$I]$T_3$ labeling of the 28-kDa band representing ID-I. These findings confirm previous observations in rats (8, 9), indicating that Se deficiency results in a decrease of hepatic ID-I content.

$^{75}$Se labeling of the 28-kDa band was markedly higher in Se-deficient than in Se-sufficient mice, which is explained by the higher specific radioactivity of the selenium available for incorporation into selenoprotein. $^{75}$Se labeling of the 28-kDa protein was also markedly higher in C57 than in C3H mice. These findings suggest that: 1) mouse liver ID-I is a selenoprotein in accordance with the structure reported for rat and human ID-I (6, 7); and 2) synthesis of this protein is defective in C3H mice.

Northern blot analysis of C3H and C57 liver RNA using a rID-I cDNA-probe revealed a strong hybridization signal with RNA of 2.1 kilobases, *i.e.* the same size as rID-I mRNA (6). This suggests a high degree of homology between rat and mouse ID-I, which has also been found between the sequences of human and rat ID-I (6, 7). Differences in ID-I activity in both C3H and C57 mice are closely paralleled by variation in liver ID-I mRNA, and the mean ID-I mRNA level is significantly lower in C3H than in C57 mice. This is true irrespective of whether ID-I mRNA is expressed in absolute terms or relative to $\beta$-actin mRNA. However, since it is unknown if $\beta$-actin gene expression is the same in C3H and C57 mouse livers, it is questionable if the ID-I/$\beta$-actin mRNA ratio is an appropriate means to standardize the results. Our findings indicate that ID-I activity is impaired in C3H mice because of decreased transcription of the ID-I gene or decreased stability of the mRNA. C3H mice show a high prevalence of spontaneous liver tumor formation (19), and reduced expression of ID-I may thus reflect dedifferentiation of preneoplastic liver cells. However, heterozygosy for an ID-I gene defect in C3H/He mice cannot be excluded entirely. It is unlikely that reduced hepatic $T_3$ formation has a role in hepatocarcinogenesis, since this is not a general consequence of hypothyroidism.

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## Chapter 5

# REACTION OF THE TYPE III DEIODINASE WITH THE AFFINITY LABEL N-BROMOACETYL-TRIIODOTHYRONINE

Chapter 5

# Reaction of the type III iodothyronine deiodinase with the affinity label *N*-bromoacetyl-triiodothyronine

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The type III iodothyronine deiodinase (ID-III) catalyzes the inner ring deiodination and, thus, the inactivation of the thyroid hormones $T_4$ and $T_3$. ID-III activity in rat brain, rat placenta and embryonic chicken liver is inhibited by the affinity label *N*-bromoacetyl-$T_3$ ($BrAcT_3$) with an affinity similar to that of $T_3$. Reaction of rat brain and placenta microsomes with $BrAc[^{125}I]T_3$ resulted in the extensive labeling of a 32 kDa protein (p32). However, p32 was also prominently labeled in fetal rat liver microsomes which have no ID-III activity. Labeling of p32 was not influenced by 100 $\mu$M substrate analogs or inhibitors of ID-III, some of which completely inhibit ID-III activity at 1 $\mu$M. $BrAc[^{125}I]T_3$ labeling of embryonic chicken liver microsomes did not reveal p32 or another protein possibly related to ID-III. In contrast to previous suggestions, it is unlikely that p32 represents ID-III or a subunit thereof.

Thyroid hormone; Iodothyronines; Deiodination; Rat; Chicken; Liver; Placenta; Brain; Affinity-labelling; Bromoacetyl derivative

## 1. INTRODUCTION

The thyroid predominantly secretes the inactive prohormone thyroxine ($T_4$). Therefore, extrathyroidal outer ring deiodination to bioactive 3,3′,5-triiodothyronine ($T_3$) is an essential step in the exertion of thyroid hormone action. Alternatively, $T_4$ is converted by inner ring deiodination to the inactive metabolite 3,3′,5′-triiodothyronine (reverse $T_3$, $rT_3$). Inner ring deiodination also inactivates $T_3$ by converting it to 3,3′-diiodothyronine (3,3′-$T_2$) [1,2].

Three types of iodothyronine deiodinases have been identified in mammals. The type I deiodinase (ID-I) is capable of both inner and outer ring deiodination and is responsible for the greater part of peripheral $T_3$ production from $T_4$. However, the preferred substrate of the enzyme is $rT_3$. The enzyme is located in the microsomal fractions of liver, kidney and thyroid [1,2]. The iodothyronine derivative *N*-bromoacetyl-$T_3$ ($BrAcT_3$) has proved to be a very useful affinity-label for ID-I, resulting in the identification of the ≈ 27 kDa ID-I protein by SDS-PAGE [3–5]. Human, rat and mouse ID-I have been shown to contain the rare amino acid selenocysteine [6–8].

The type III iodothyronine deiodinase (ID-III) is the least well described deiodinase. It catalyzes the inactivation of thyroid hormone by inner ring deiodination of $T_4$ and $T_3$ [1,2]. In mammals, ID-III is mainly located in the microsomal fractions of brain [9], placenta [10], skin [11] and fetal intestine [12]. In chicken liver, the enzyme is mainly present during embryonic development, with the highest activity on day 16 and decreasing rapidly thereafter until very low levels are reached at hatching on day 21 [13]. In this study we tested if $BrAcT_3$ is a useful affinity-label for ID-III in rats and chickens.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

[3,5-$^{125}$I]$T_3$ (≈ 30 Ci/mmol) was generously provided by Drs. C. Horst and R. Thoma, Henning (Berlin, Germany); [3′,5′-$^{125}$I]$rT_3$ and [3′-$^{125}$I]$T_3$ (≈ 1700 Ci/mmol) were obtained from Amersham (Amersham, UK); thyronine ($T_0$) and iodothyronines from Henning; dithiothreitol (DTT), 6-*n*-propyl-2-thiouracil (PTU), 3,3′,5-triiodothyroacetic acid (Triac) and 3,5-diiodotyrosine (DIT) from Sigma (St. Louis, MO); iopanoic acid (IOP) from Sterling Winthrop (Newcastle, UK); electrophoresis grade SDS-PAGE reagents from Bio-Rad (Richmond, IL); BCA Protein Assay Reagent from Pierce Europe (Oud Beijerland, The Netherlands); $M_r$ markers and Sephadex LH-20 from Pharmacia (Uppsala, Sweden); and Coomassie brilliant blue R-250 from Merck (Darmstadt, FRG).

### 2.2. *Preparation of microsomes*

Wistar rats were purchased from Harlan Sprague–Dawley (Zeist, The Netherlands). Liver and brain were isolated from 10-week-old male rats after decapitation. Pregnant rats and fetuses were sacrificed on day 20 of gestation, and the placentas, fetal livers and brains were isolated. Fertilized chicken eggs were obtained from a local supplier and incubated for 14, 16, 18 or 20 days in a forced-draught incubator at 37°C and 80% humidity. Eggs were opened, the embryos were decapitated, and the livers were isolated. All tissues were immediately frozen in liquid nitrogen and stored at −70°C until further use. Micro-

somes were prepared in buffer A (10 mM Tris-HCl (pH 7.4), 3 mM EDTA and 3 mM DTT) as previously described [14] and stored at −70°C. Protein content was measured by Pierce BCA protein assay, using bovine serum albumin as the standard.

2.3. *Affinity-labeling*

BrAc[$^{125}$I]$T_3$ and non-radioactive $BrAcT_3$ were prepared essentially as previously published [3]. HPLC analysis demonstrated that the purity of BrAc[$^{125}$I]$T_3$ was ≥ 85%, with unreacted [$^{125}$I]$T_3$ as the main contaminant, while non-radioactive $BrAcT_3$ was > 95% pure. Solutions of BrAc[$^{125}$I]$T_3$ and $BrAcT_3$ in ethanol were pipetted into an Eppendorf tube, and the solvent was evaporated at 42°C under a stream of nitrogen. The desired amount of microsomal protein in 100 μl buffer A was added to the residue, and the mixture was vortexed for 30 s. After further incubation for 10 min at 37°C, labeling was stopped by addition of 50 μl of SDS-sample buffer containing 30% β-mercaptoethanol and treatment for 5 min at 100°C. Proteins were separated overnight by SDS-PAGE in a 14 cm 10% or 15% T, 3% C gel, overlaid by a 2 cm 3% T, 3% C stacking gel [15]. Gels were stained with Coomassie brilliant blue R-250, dried under vacuum and autoradiographed at −70°C with Kodak T-mat G film. $M_r$ was determined by interpolation with protein markers.

2.4. *Deiodinase assays*

The activity of ID-I was measured by incubation of 10 μg/ml microsomal protein for 20 min at 37°C with 1 μM $rT_3$ and 75 nCi [3′,5′-$^{125}$I]$rT_3$ in 200 μl 0.2 M phosphate (pH 7.2), 4 mM EDTA and 5 mM DTT, followed by isolation of the $^{125}I^-$ released on Sephadex LH-20 as previously described [16].

The activity of ID-III was measured by incubation of different amounts of microsomal protein for 60 min at 37°C with 1 nM [$^{125}$I]$T_3$ in 200 μl 0.2 M phosphate (pH 7.2), 4 mM EDTA and 20 mM DTT. Both inner and outer ring labeled [$^{125}$I]$T_3$ were used, yielding identical results. When [3′-$^{125}$I]$T_3$ was used, the reactions were stopped by addition of 300 μl methanol on ice. After centrifugation, the supernatants were analyzed for [3,3′-$^{125}$I]$T_2$ formation by HPLC on a 100 × 3.0 mm Chromspher $C_{18}$ column (Chrompack, Middelburg, The Netherlands), eluted with a 45:55 (v/v) mixture of methanol and 20 mM ammonium acetate (pH 4.0) at a flow of 0.8 ml/min. When [3,5-$^{125}$I]$T_3$ was used, reactions were stopped by addition of 100 μl pooled human serum on ice, followed by precipitation of protein-bound iodothyronines by addition of 500 μl 10% trichloroacetic acid. After centrifugation, the supernatants were analyzed for $^{125}I^-$ formation on Sephadex LH-20. Parallel incubations were done with 1 μM $T_3$, which saturates the low-$K_m$ ID-III but not the high-$K_m$ ID-I [1,2], showing negligible type I or non-enzymatic deiodination of $T_3$.

## 3. RESULTS

In the rat, high ID-III activities are found in the microsomal fraction of placenta and brain, while the enzyme is more abundant in brain of fetal than of adult rats. After reaction of rat placenta and brain microsomes with BrAc[$^{125}$I]$T_3$ and subsequent analysis by SDS-PAGE, a predominant radioactive band of 32 kDa (p32) was observed, with higher levels in fetal than in adult rat brain (Fig. 1). In none of these microsomes significant ID-I activity was found, which was associated with the absence of a 27 kDa band (p27), representing $BrAcT_3$-labeled ID-I, in the corresponding autoradiograms.

Both fetal and adult rat liver microsomes showed little ID-III activity. However, reaction of fetal liver microsomes with BrAc[$^{125}$I]$T_3$ resulted in extensive labeling of p32 (Fig. 2A). This is quite different from adult

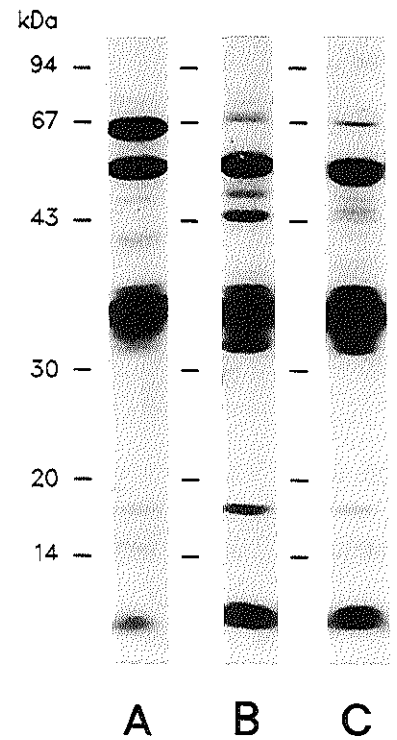


Fig. 1. Labeling patterns obtained after reaction of 50 μg of microsomal protein with 0.15 μCi BrAc[$^{125}$I]$T_3$. After SDS-PAGE film was exposed for 4 days. (A) Rat placenta. (B) Adult rat brain. (C) Fetal rat brain.

rat liver microsomes, where labeling of p32 is nearly absent (Fig. 2B). In contrast with fetal liver, microsomes of adult rat liver contain high levels of ID-I activity, leading to extensive labeling of p27, which could interfere with labeling of p32. However, inhibition of p27 labeling by addition of $rT_3$ and PTU resulted in only a minor increase in p32 labeling (Fig. 2C). Under these circumstances an increase was also observed in the labeling of a 56 kDa band, identified previously as protein disulfide isomerase [4], and of a 67 kDa band. These findings indicate that the level of p32 in adult rat liver microsomes is very low.

In spite of the discrepancies found between ID-III activity and p32 labeling, we further investigated their possible relationship by analyzing the effects of substrates and inhibitors of the enzyme on $BrAcT_3$ labeling of p32. Therefore, rat brain microsomes were reacted with BrAc[$^{125}$I]$T_3$ in the presence of 100 μM of various substrate analogs and deiodinase inhibitors. Labeling of the microsomal proteins was not influenced by any of the substances tested (Fig. 3A). However, deiodination

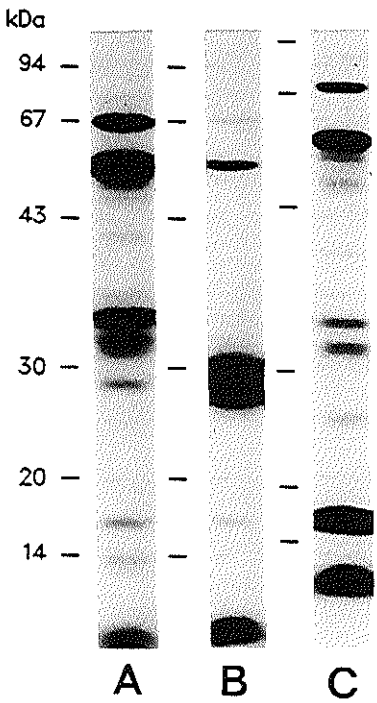


Fig. 2. Labeling patterns obtained after reaction of 50 $\mu$g of microsomal protein with 0.15 $\mu$Ci BrAc[$^{125}$I]$T_3$. After SDS-PAGE film was exposed for 4 days. (A) Fetal rat liver. (B) Adult rat liver. (C) Adult rat liver in the presence of 10 $\mu$M $rT_3$ and 100 $\mu$M PTU.

of [$^{125}$I]$T_3$ by ID-III was largely inhibited by 1 $\mu$M $T_3$, $T_4$, Triac or IOP (Fig. 3B).

In embryonic chicken liver, highest ID-III activity was observed at or around day 16 of embryonic development (E16); thereafter, ID-III activity decreases rapidly to become nearly undetectable in adult chicken liver microsomes (Fig. 4A); see also [13]). Reaction of embryonic and adult chicken liver microsomes with BrAc[$^{125}$I]$T_3$ did not reveal labeling of p32 or any other protein possibly correlated with ID-III activity (Fig. 4B). In adult chicken liver microsomes ID-I has been identified as a 26 kDa band [16]. Figure 4 shows the strong correlation between affinity labeling of this 26 kDa band and the varying ID-I activity in chicken liver during embryonic development.

ID-III activity of chicken E16 liver microsomes and rat placenta microsomes was measured in the presence of increasing concentrations of $BrAcT_3$ or $T_3$ (Fig. 5). In both tissues, deiodination of [$^{125}$I]$T_3$ was inhibited by similar concentrations of $T_3$ and $BrAcT_3$, with $IC_{50}$ values varying between 5.7 and 8.1 nM. After preincubation of rat placenta or E16 chicken liver microsomes with $T_3$ or $BrAcT_3$, time-dependent inactivation of ID-III was observed with $BrAcT_3$, but not with $T_3$, which persisted after removal of unreacted $BrAcT_3$ by Penefsky centrifugal column chromatography [17] (results not shown). These findings suggest the covalent modification of rat and chicken ID-III by $BrAcT_3$.

## 4. DISCUSSION

The low apparent $K_m$ of $T_3$ ($\approx$ 5 nM) for both rat and chicken ID-III estimated in the present study is in agreement with previously published data [19,20]. We also found that, although ID-III is irreversibly inactivated by $BrAcT_3$, the apparent $K_i$ of this compound is similar to the $K_m$ of the substrate $T_3$. This is quite different from inhibition of ID-I by $BrAcT_3$, which is half-maximum at $\approx$ 0.1 nM of the inhibitor, a concentration much lower than the $K_m$ for $T_3$ (6 $\mu$M) or even $rT_3$ (0.06 $\mu$M) [3]. This is most likely caused by reaction of $BrAcT_3$ with the highly reactive selenocysteine residue in ID-I.

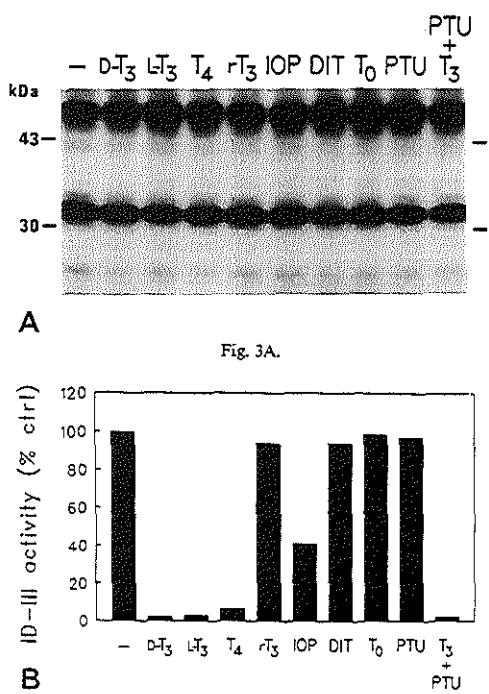


Fig. 3A.

Fig. 3. (A) Labeling patterns obtained after reaction of 66 $\mu$g of rat brain microsomal protein with 0.1 $\mu$Ci BrAc[$^{125}$I]$T_3$, in the absence or presence of 100 $\mu$M of various substrate analogs and deiodinase inhibitors. After SDS-PAGE film was exposed for 42 h. (B) Deiodination of 1 nM [$^{125}$I]$T_3$, by 500 $\mu$g/ml rat brain microsomal protein in the absence or presence of 1 $\mu$M of the compounds indicated, except that PTU was added at 100 $\mu$M. Activities are expressed as a percentage of that in the absence of the various additions.

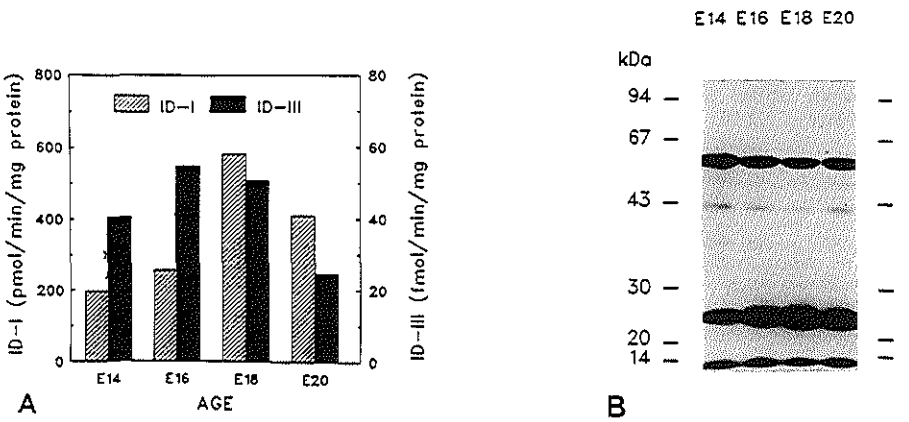


Fig. 4. (A) Ontogeny of deiodinase activities in chicken liver during the last week (E14–E20) of embryonic development. ID-I was assayed using 1 $\mu$M $rT_3$ and 10 $\mu$g/ml microsomal protein. ID-III was assayed using 1 nM $T_3$ and 100 $\mu$g/ml microsomal protein. (B) Autoradiogram of labeling patterns obtained after reaction of 100 $\mu$g embryonic chicken liver microsomal protein with 0.25 $\mu$Ci BrAc[$^{125}$I]$T_3$. After SDS-PAGE film was exposed for 25 h.

The difference in sensitivity to inactivation by $BrAcT_3$ between ID-I and ID-III may be explained by the finding that ID-III is apparently not a selenoenzyme [21].

Santini et al. have recently reported similar findings concerning the inhibition of ID-III activity as well as the labeling of a 31 kDa protein with $BrAcT_3$ in rat placental microsomes [18]. They suggested that this 31 kDa protein is the substrate-binding subunit of ID-III, although its labeling was only partially inhibited in the presence of as high as 150 $\mu$M $T_3$, while it was also observed in tissues with no ID-III activity. However, we have serious doubts about the relationship between p32 and ID-III. Firstly, $BrAcT_3$ labeling of p32 is observed not only in tissues with high ID-III activity (rat brain and placenta) but also in tissues with little or no enzyme activity (fetal rat liver and adult rat spleen) [22]. Secondly, $BrAcT_3$ labeling of p32 is not observed in embryonic chicken liver, which shows very similar levels and properties of ID-III activity as rat placenta, and which is as sensitive to $BrAcT_3$ inhibition as rat ID-III. Thirdly, $BrAcT_3$ labeling of p32 is not inhibited in the presence of 100 $\mu$M $T_3$, which is > $10^4$-fold higher than its apparent $K_m$ and, thus, should completely block modification of the substrate-binding site by $BrAcT_3$, which has a similar affinity for the enzyme as $T_3$. Therefore, our findings do not support the hypothesis that p32 is a subunit of ID-III.

Although ID-III is irreversibly inactivated by $BrAcT_3$, we were not able to identify the enzyme by affinity-labeling with this compound, which could be explained if: (1) BrAc[$^{125}$I]$T_3$ is degraded after coupling to ID-III, leading to loss of radioactivity; (2) the

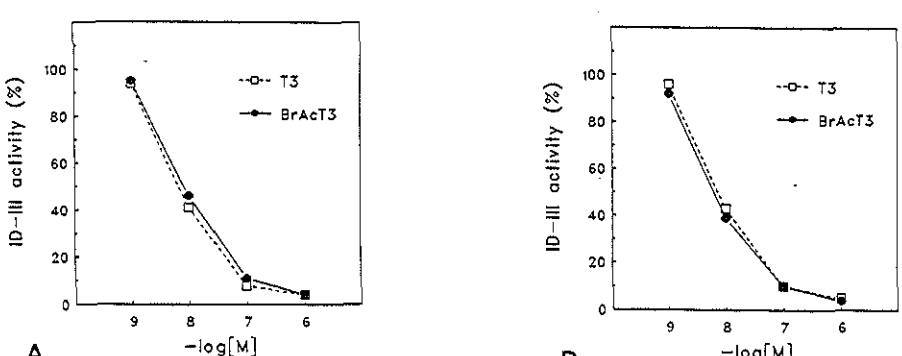


Fig. 5. Deiodination of 1 nM [$^{125}$I]$T_3$ by chicken E16 liver microsomes (A; 20 $\mu$g protein/ml) or rat placenta microsomes (B; 25 $\mu$g protein/ml) in the presence of increasing concentrations of $BrAcT_3$. Results are expressed as a percentage of that without additions.

amount of ID-III present in the various microsomes is too low to allow sufficient incorporation of BrAc[$^{125}$I]$T_3$; or (3) the $M_r$ of ID-III is close to that of another prominently labeled protein, interfering with the detection of labeled ID-III.

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## Chapter 6

# AFFINITY LABELING OF IODOTHYRONINE DEIODINASES IN RAT TISSUES WITH N-BROMOACETYL-IODOTHYRONINE DERIVATIVES

# AFFINITY-LABELING OF IODOTHYRONINE DEIODINASES IN RAT TISSUES WITH N-BROMOACETYL-IODOTHYRONINE DERIVATIVES

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SUMMARY: In the present study the hypothesis was tested that N-bromoacetyl-3,3',5-[$^{125}$I]triiodothyronine (BrAc[$^{125}$I]T3) is a useful affinity label for both type I and type III iodothyronine deiodinases (ID-I and ID-III). Therefore, the microsomal fractions of various rat tissues were tested for ID-I and ID-III activities, and microsomal proteins were labeled with BrAc[$^{125}$I]T3 and analyzed by SDS-PAGE. In agreement with previous observations, high ID-I activities were found in liver, kidney and thyroid, and high ID-III activities in brain, in particular fetal brain, and placenta. SDS-PAGE of BrAc[$^{125}$I]T3-labeled microsomes showed a prominent radioactive $\approx$27 kDa protein (p27) in liver, kidney and thyroid, which was previously identified as ID-I, and a $\approx$32 kDa protein (p32) in brain, in particular fetal brain, and placenta. A good correlation was found between the affinity-labeling of p32 and the inactivation of ID-III by BrAcT3, suggesting that p32 represents ID-III or a subunit thereof. After treatment of microsomes with 0.05% deoxycholate or carbonate buffer (pH 11.5) p32 was still labeled by BrAc[$^{125}$I]T3, indicating that p32 is a transmembrane protein. Although 3,3',5'-triiodothyronine (rT3) is not a substrate for ID-III, p32 was readily labeled with BrAc[$^{125}$I]rT3. Labeling of p32 in rat brain microsomes by BrAc[$^{125}$I]rT3 was not affected by addition of 100 $\mu$M unlabeled thyroxine (T4) or T3, whereas deiodination of [$^{125}$I]T3 by ID-III was inhibited by 91 and 96 % in the presence of 1 $\mu$M T4 and T3, respectively. The relationship between p32 and ID-III was further questioned by findings of prominent BrAc[$^{125}$I]T3 labeling of p32 in microsomes of spleen and fetal liver, which show very little ID-III activity. Peptide-mapping of p32 using trypsin or *Staphylococcus aureus* V8 protease yielded the same fragments in brain, placenta, spleen and fetal liver, suggesting that p32 is identical in all these tissues. In contrast to initial suggestions, therefore, we conclude that it is unlikely that the labeling of p32 by BrAc[$^{125}$I]T3 represents the identification of ID-III.

## INTRODUCTION

Differentiation, growth and basal metabolism are regulated by thyroid hormones, of which 3,3',5-triiodothyronine (T3) is the most bioactive form (1,2). The actions of T3 are initiated by its binding to nuclear receptors, which are encoded by the c-erbA $\alpha$ and ß genes, resulting in the stimulation or suppression of the transcription of thyroid hormone responsive genes (1,2). The thyroid predominantly secretes the biologically inactive prohormone thyroxine (T4), which is activated by outer ring deiodination (ORD) to T3. Alternatively, T4 undergoes inner ring deiodination (IRD) to the biologically inactive metabolite 3,3',5'-triiodothyronine (reverse T3, rT3). Both ORD and IRD are catalyzed by so-called iodothyronine deiodinases (3,4).

Until now three different types of iodothyronine deiodinases have been identified. Type I iodothyronine deiodinase

(ID-I) in mammals is found predominantly in the microsomal fractions of liver, kidney and thyroid, and is responsible for the largest part of peripheral production of T3 from T4. However, ID-I is capable of both ORD and IRD, and shows preference for rT3 as the substrate (3,4). ID-I is stimulated by small thiol compounds such as dithiothreitol (DTT) and is inhibited by 6-n-propyl-2-thiouracil (PTU) in an uncompetitive manner (3,4). N-Bromoacetyl-[$^{125}$I]T3 (BrAc[$^{125}$I]T3) has proven to be a very useful affinity-label of ID-I, allowing the specific identification of the enzyme in liver microsomes despite that it only constitutes $\approx$0.01% of the total protein (5,6).

We have previously shown that liver ID-I displays very similar structural and enzymatic properties in different species, except for the lesser substrate preference of dog ID-I for rT3 (7). The complete nucleotide sequences of rat, human and dog ID-I cDNA have been reported (8-10), showing a high degree of homology between these proteins, including the presence of the rare aminoacid selenocysteine (Sec). Mouse ID-I has also been shown to be a selenoprotein (11,12). In rats, the selenium (Se) status greatly influences the amount of ID-I in liver, kidney and thyroid, with ID-I activity being markedly decreased in Se-deficient animals (13,14).

Type II iodothyronine deiodinase (ID-II) in mammals is mainly found in the microsomal fractions of brain, brown adipose tissue and pituitary (3,4). It catalyzes only the ORD of iodothyronines, with T4 as the preferred substrate. ID-II is important for the local production of T3 from T4 to maintain the intracellular levels of this active hormone in critical tissues such as the brain (3,4). ID-II is also stimulated by small thiol compounds such as DTT, but it is insensitive to inhibition by PTU (3,4).

The type III iodothyronine deiodinase (ID-III) in mammals is mainly located in the microsomal fractions of brain (15), placenta (16), skin (17) and fetal intestine (18). This enzyme catalyzes the IRD and, thus, the inactivation of T4 and T3, the latter being the preferred substrate (3,4). It has recently been found that ID-III is readily inactivated by BrAcT3 (19), suggesting that BrAc[$^{125}$I]T3 may be an useful affinity-label for the identification of ID-III.

In this study, we compared the distributions of ID-I and ID-III in different rat tissues with the labeling patterns obtained after reaction of subcellular fractions of these tissues with N-bromoacetyl-[$^{125}$I]iodothyronine derivatives. This was done to investigate if ID-III can be identified using such affinity-labels.

## MATERIALS AND METHODS

*Materials.*

[3',5'-$^{125}$I]rT3 and [3'-$^{125}$I]T3 ($\approx$ 1700 Ci/mmol) were obtained from Amersham (Amersham, UK); unlabeled iodothyronines from Henning (Berlin, Germany); sequence grade trypsin and *Staphylococcus aureus* V8 protease from Boehringer Mannheim (Mannheim, Germany); DTT, sodium deoxycholate (DOC) and PTU from Sigma (St. Louis, MO); electrophoresis grade

SDS-PAGE reagents from Bio-Rad (Richmond, IL); BCA Protein Assay Reagent from Pierce Europe (Oud Beijerland, The Netherlands); protein markers and Sephadex LH-20 from Pharmacia (Uppsala, Sweden); and Coomassie Brilliant Blue R-250 from Merck (Darmstadt, Germany).

*Preparation of microsomes.*

Wistar rats were purchased from Harlan Sprague Dawley (Zeist, The Netherlands). Tissues were isolated from 10-week old male rats killed by decapitation. In addition, placenta and fetal liver were obtained from pregnant rats on day 20 of gestation. Immediately after isolation, all tissues were frozen in liquid nitrogen and stored at -70 C until further use. Microsomes were prepared as previously described (20) by homogenization of the tissue in 0.25 M sucrose, 10 mM HEPES (pH 7) and 10 mM DTT, and successive centrifugation for 10 min at 25,000x*g* and for 60 min at 100,000x*g*. The supernatants and pellets of these centrifugation steps were designated S25, P25, S100 and P100 (microsomes), respectively. The microsomes were suspended in 10 mM Tris/HCl (pH 7.4), 3 mM EDTA and 3 mM DTT (buffer A), and aliquots thereof as well as of the other fractions were stored at -70 C until further analysis. Protein content was measured by Pierce BCA protein assay using bovine serum albumin as the standard. Treatment of microsomes with deoxycholate (DOC) or high pH was done as follows. Microsomes were suspended in 10 ml 0.1 M sodium carbonate (pH 11.5) or in 25 mM HEPES (pH 7.4), 0.25 M sucrose and 50 mM KCl with or without 0.05% DOC. After occasional stirring on ice for 1 h, the mixtures were layered on 2 ml 50 mM HEPES (pH 7.4), 0.5 M sucrose, 50 mM KCl and 2 mM DTT, and centrifuged for 90 min at 105,000x*g* and 4 C. The resulting pellets were resuspended in 1 ml of buffer A, and stored in aliquots at -70 C until further examination.

*Affinity-labeling.*

Labeled and unlabeled BrAc-iodothyronine derivatives were prepared essentially as previously published (5,6). HPLC analysis demonstrated that the purity of BrAc-[$^{125}$I]iodothyronines was at least 85% with unreacted [$^{125}$I]iodothyronines as the main contaminant, while nonradioactive BrAc-iodothyronines were more than 95% pure. Ethanol solutions containing the desired amounts of BrAc-iodothyronine and BrAc-[$^{125}$I]iodothyronine were pipetted in an Eppendorf tube, and the solvent was evaporated under a stream of nitrogen at 42 C. The required amount of microsomal protein in 100 $\mu$l buffer A was added to the residue, and the mixture was vortexed for 30 s. After further incubation for 10 min at 37 C labeling was stopped by addition of 50 $\mu$l of SDS-sample buffer containing 30% $\beta$-mercaptoethanol and treatment for 5 min at 100 C. Proteins were separated overnight by SDS-PAGE in a 14 cm 15% T, 3% C gel, overlaid by a 2 cm 3% T, 3% C stacking gel (21), where T denotes the total concentration (wt/vol) of both monomers (acrylamide and bisacrylamide) and C denotes the concentration of bisacrylamide relative to the total concentration T. Gels

were stained with Coomassie Brilliant Blue R-250 at 60 C, dried at 60 C under vacuum and autoradiographed at -70 C with Kodak T-MAT G film. Apparent molecular mass (Mr) was determined by interpolation with protein markers. The correlation between the labeling of microsomal proteins and the inactivation of ID-III by BrAcT3 was examined as follows. 400 $\mu$g rat brain microsomal protein in 200 $\mu$l 0.1 M phosphate (pH 7.2), 4 mM EDTA and 20 mM DTT was reacted with 30 pmol BrAc[$^{125}$I]T3 for increasing time periods. Unreacted BrAc[$^{125}$I]T3 was subsequently removed by Penefsky centrifugal column chromatography (22). Control experiments revealed that in this way more than 99.9 percent of unreacted affinity-label was removed. The affinity-labeled microsomes were subsequently analyzed by SDS-PAGE and tested for ID-III activity.

*Peptide mapping.*

The desired amount of labeled microsomal protein was separated overnight by routine SDS-PAGE. Gels were stained for 5 min with Coomassie Brilliant Blue R-250 at room temperature. After destaining, lanes were excised from the gel and cut into 1 mm fractions, which were counted for radioactivity. Fractions containing the protein of interest were further analyzed as previously described (23). Using 1 $\mu$g of protease per 200 $\mu$g starting protein, digestion was done in a tricine-SDS-polyacrylamide gel running at 30 V constant (24). This gel was composed of a 12 cm 16.5% T, 3% C small-pore gel overlaid by a 2 cm 10% T, 3% C spacer gel, that again was overlaid by a 2 cm 4% T, 3% C stacking gel. Autoradiograms were prepared and Mr was determined as described above.

*ID-I assay.*

The activity of ID-I was measured by incubation of 2 $\mu$g microsomal protein for 30 min at 37 C with 0.1 $\mu$M rT3 and 75 nCi [$^{125}$I]rT3 in 200 $\mu$l 0.2 M phosphate (pH 7.2), 4 mM EDTA and 5 mM DTT as previously described (7). The reactions were stopped by addition of 750 $\mu$l 1 M HCl on ice. The $^{125}I^-$ released was isolated on Sephadex LH-20 and data were corrected for non-enzymatic deiodination determined in incubations without microsomes. Random labeling and deiodination of the 3' and 5' positions of [$^{125}$I]rT3 was taken into account for calculation of ID-I activity.

*ID-III assay.*

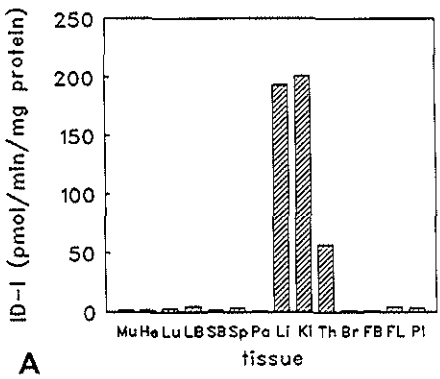
The activity of ID-III was measured by incubation of 20 or 200 $\mu$g microsomal protein for 60 min at 37 C with 1 nM T3 and 75 nCi [3'-$^{125}$I]T3 in 200 $\mu$l 0.1 M phosphate (pH 7.2), 4 mM EDTA and 10 mM DTT. The reactions were stopped by addition of 300 $\mu$l methanol on ice. After centrifugation of precipitated proteins, the supernatants were analyzed for 3,[3'-$^{125}$I]T2 formation by HPLC on a 100 x 3.0 mm Chromspher C18 column (Chrompack, Middelburg, The Netherlands), eluted with a 45:55 (vol/vol) mixture of methanol and 20 mM ammonium-acetate (pH 4.0) at a flow of 0.8 ml/min. Control incubations were done with 1 $\mu$M T3, which saturates the low-Km ID-III but not the high-Km ID-I (3,4), showing negligible type I or non-enzymatic deiodination of T3.

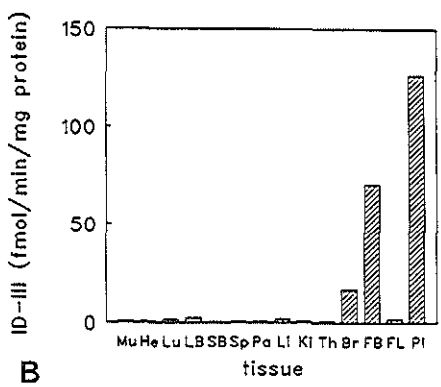
*Reproducibility.*

The data shown were obtained in representative experiments that were repeated 2 or 3 times with closely agreeing results.

## RESULTS

Figure 1 shows the ID-I activities determined in the microsomal fractions of the various rat tissues. It is clear that kidney, liver and thyroid microsomes contained high ID-I levels, whereas pancreas, spleen, small bowel, large bowel, lung, muscle, brain, placenta and fetal liver displayed very low activities. The ID-III activities of these same microsomal preparations are depicted in Fig. 1B. Brain and placenta microsomes exhibited high ID-III activities, in fetal brain higher than in adult brain, whereas the other tissues showed almost no activity. An autoradiogram of the SDS-PAGE separation of proteins labeled by reaction of the various microsomes with BrAc[$^{125}$I]T3 is presented in Fig. 1C. The film was overexposed in order to also reveal weakly labeled proteins. This is indicated by the very intense band at ≈27 kDa (p27) in



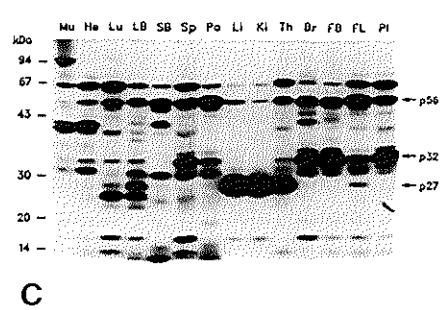




Fig. 1. (A) Tissue distribution of type I deiodinase activity in rat. ID-I was assayed using 0.1 $\mu$M rT3 and 10 $\mu$g/ml microsomal protein. (B) Tissue distribution of type III deiodinase activity in rat. ID-III was assayed using 1 nM T3 and 100 $\mu$g/ml microsomal protein.(C) Autoradiogram of labeling patterns obtained after reaction of 50 $\mu$g microsomal protein of rat tissue with 0.15 $\mu$Ci BrAc[$^{125}$I]T3. After SDS-PAGE film was exposed for 19 h.
Mu, muscle; He, heart; Lu, lung; LB, large bowel; SB, small bowel; Sp, spleen; Pa, pancreas; Li, liver; Ki, kidney; Th, thyroid; Br, brain; FB, fetal brain; FL, fetal liver; Pl, placenta.

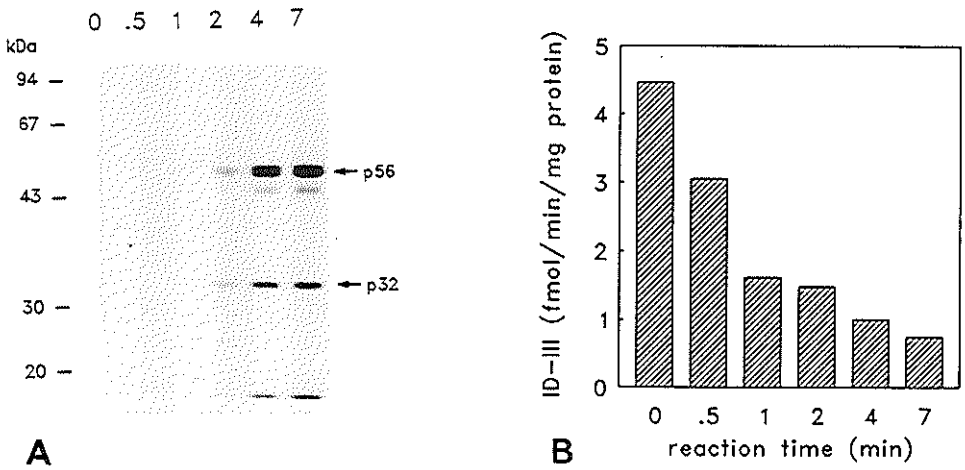



Fig. 2. (A) Autoradiogram of labeling patterns obtained after reaction of 30 pmol BrAc[$^{125}$I]T3 with 400 $\mu$g rat brain microsomal protein for increasing time periods. Reaction time is given in minutes above each lane. After SDS-PAGE film was exposed for 7 days. (B) Remaining ID-III activity of the same microsomal samples shown in A. ID-III was assayed using 1 nM T3 and 1 mg/ml microsomal protein.

liver, kidney and thyroid microsomes. These are also the tissues with high ID-I activity, and p27 has previously been identified as ID-I (6). In the tissues with high ID-III activities, i.e. brain and placenta, a prominent BrAc[$^{125}$I]T3-labeled protein of ≈32 kDa (p32) was observed, which was more pronounced in fetal brain than in adult brain. However, BrAc[$^{125}$I]T3-labeling of p32 was also observed in spleen and fetal liver microsomes, which contained little or no ID-III activity.

In order to investigate if p32 represents the labeling of ID-III, the correlation was examined between the extent of p32 labeling and that of ID-III inactivation after reaction of rat brain microsomes for increasing time periods with BrAc[$^{125}$I]T3 followed by removal of unreacted label. The results of these experiments are presented in Fig. 2, which demonstrates that there was indeed a close correlation between the time-dependent increase in p32 labeling and the decrease in ID-III activity. A similar relationship between p32 labeling and ID-III inactivation was found in experiments where the reaction time was kept constant but the amount of BrAc-[$^{125}$I]T3 was varied (results not shown).

Rat brain, placenta, spleen, and liver microsomes were treated with 0.05% DOC in order to remove lumenal proteins (25) or with carbonate buffer of pH 11.5 that detaches all but the very tightly bound transmembrane proteins (26), and the thus treated microsomes were labeled with BrAc[$^{125}$I]T3. Figure 3 shows that pretreatment of rat liver microsomes with 0.05% DOC resulted in the disappearance of the 56 kDa band, which has previously been identified as the soluble lumenal enzyme protein disulfide isomerase (PDI). Pretreatment with carbonate buffer of

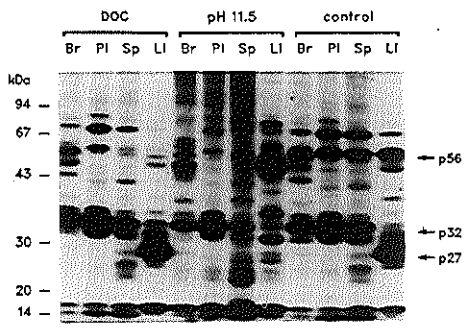



Fig. 3. Influence of pretreatment of rat brain, placenta, spleen and liver microsomes with 0.05% DOC or with carbonate buffer of pH 11.5 on labeling of proteins with BrAc[$^{125}$I]T3. Microsomes were processed as described under Materials and Methods. Of the resulting suspensions 15 $\mu$g protein was reacted with 0.25 $\mu$Ci BrAc[$^{125}$I]T3. After SDS-PAGE film was exposed for 22 h.

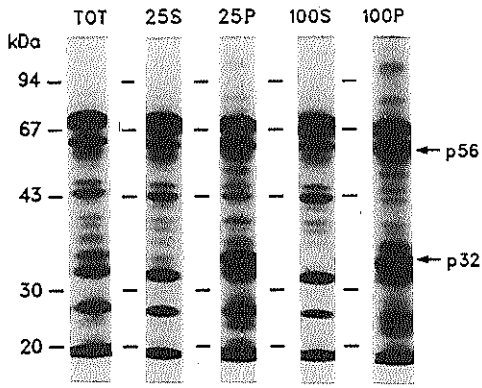



Fig. 4. Autoradiogram of labeling patterns obtained after reaction of 0.25 $\mu$Ci BrAc[$^{125}$I]T3 with 100 $\mu$g protein of different fractions of spleen tissue-homogenate obtained during preparation of microsomes using differential centrifugation. After SDS-PAGE film was exposed for 18h. TOT, total homogenate; 25S, 25,000x*g* supernatant; 25P, 25,000x*g* pellet; 100S, 100,000x*g* supernatant; 100P, 100,000x*g* pellet.

pH 11.5 resulted in the elimination of p27 labeling. Control experiments in which reaction with BrAc[$^{125}$I]T3 preceded treatment with carbonate buffer of pH 11.5 revealed that this is caused by the inactivation of ID-I rather than the removal of this enzyme from the microsomes, confirming that ID-I is a tightly bound transmembrane protein. Reaction of BrAc[$^{125}$I]T3 with brain, placenta and spleen microsomes after treatment with 0.05% DOC or pH 11.5 still resulted in the labeling of p32, indicating that p32 is a transmembrane protein. The strong association of p32 with the microsomal membranes was also demonstrated by the complete lack of p32 labeling in the final supernatant (S100) of the spleen homogenate obtained after centrifugation of the microsomes (P100; Fig. 4). Figure 4 also shows significant BrAc[$^{125}$I]T3 labeling of p32 in the low-speed pellet (P25) of spleen homogenate. A similar distribution of p32 over the different tissue fractions was seen in placenta, brain and fetal liver (not shown), but the exact subcellular location of this protein has not been further investigated.

If labeling of p32 by BrAc[$^{125}$I]T3 represents the covalent binding of the affinity-label to the substrate-binding site of ID-III, this labeling should be inhibited in the presence of substrates of the enzyme, as is the case with the BrAc[$^{125}$I]T3 labeling of ID-I (6). Furthermore, one would not expect to observe labeling of p32 with a

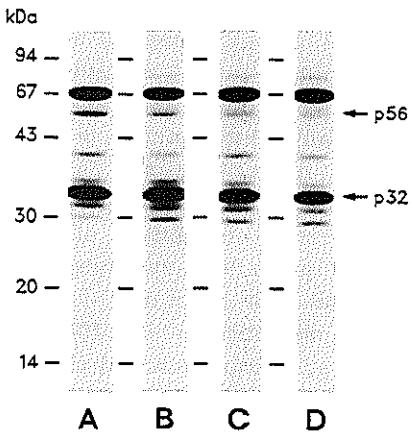



Fig. 5. Autoradiogram of labeling patterns obtained after reaction of 100 $\mu$g of rat placenta microsomal protein with 0.25 $\mu$Ci BrAc[$^{125}$I]rT3 in the absence (A) or presence of 100 $\mu$M T4 (B), 100 $\mu$M T3 (C) or 100 $\mu$M T3 + 100 $\mu$M PTU (D). After SDS-PAGE film was exposed for 4 h.

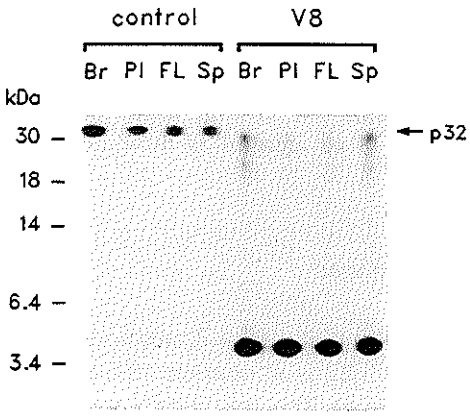



Fig. 6. Autoradiogram of labeling patterns obtained after peptide-mapping of p32 originating from brain, placenta, fetal liver and spleen. After reaction of 200 $\mu$g microsomal protein with 2.5 $\mu$Ci BrAc[$^{125}$I]T3 and subsequent SDS-PAGE, p32 was isolated from the gel and processed as described under Materials and Methods using 1 $\mu$g *Staphylococcus aureus* V8 protease. After tricine-SDS-PAGE film was exposed for 4 h.

BrAc-derivative of a iodothyronine such as rT3 which is not a substrate for ID-III. Therefore, rat brain microsomes were reacted with BrAc[$^{125}$I]rT3 in the absence or presence of excess unlabeled T4 or T3. Figure 5 shows that the labeling of p32 with BrAc[$^{125}$I]rT3 was as effective as the labeling of this protein with BrAc[$^{125}$I]T3. Moreover, labeling of p32 by BrAc[$^{125}$I]rT3 was not affected by addition of 100 $\mu$M T4 or T3 either in the absence or the presence of 100 $\mu$M PTU. However, deiodination of [$^{125}$I]T3 by ID-III was inhibited for 91 and 96 % by 1 $\mu$M T4 and T3, respectively. These findings are in agreement with previous observations that p32 labeling in rat brain microsomes by BrAc[$^{125}$I]T3 is not affected in the presence of 100 $\mu$M T3, T4, rT3, iopanoic acid, 3,5-diiodotyrosine, thyronine or PTU (19), suggesting that the binding of BrAc-[$^{125}$I]iodothyronines to p32 does not represent the affinity-labeling of the substrate-binding site of ID-III.

The prominent labeling of p32 in spleen and fetal liver microsomes despite their negligible ID-III activity (Fig. 1) further questions the possible relationship between p32 and ID-III. To exclude the possibility that the p32 labeled in brain and placenta is different from the p32 labeled in spleen and fetal liver, the labeled proteins were excised from SDS-PAGE gels and analyzed by peptide-mapping using *Staphylococcus aureus* V8 protease. This yielded identical radioactive peptide fragments in all four

tissues (Fig. 6). Peptide-mapping of p32 from these same tissues using trypsin also gave rise to peptide fragments of identical size (results not shown). Therefore, p32 appears to be identical in these different tissues.

## DISCUSSION

Previous studies have demonstrated that BrAc[$^{125}$I]T3 is an excellent affinity-label of ID-I (5,6). This was initially suggested by the findings that reaction of rat liver microsomes with BrAcT3 resulted in the irreversible inactivation of ID-I, and that the concentration of BrAcT3 required for this inactivation was very low ($\approx$0.1 nM) compared with the Km value for T3 ($\approx$10 $\mu$M) (5). Subsequent studies using SDS-PAGE have shown that mainly two proteins are radioactively labeled by reaction of rat liver microsomes with BrAc[$^{125}$I]T3, i.e. a $\approx$57 kDa protein (p57), which has been identified as PDI, and a $\approx$27 kDa protein (p27). Evidence that p27 represents ID-I or a subunit thereof can be summarized as follows. The labeling of p27 by BrAc[$^{125}$I]iodothyronines a) is only observed in tissues with ID-I activity (Fig. 1), b) is specifically inhibited by substrates and inhibitors of ID-I (6,27,28), c) is strongly correlated with ID-I activity under different pathophysiological conditions, such as starvation, hypothyroidism (29), and Se-deficiency (13,14,30), d) is strongly diminished in inbred mouse strains with ID-I deficiency (11,12), e) is identical by size and peptide mapping with ID-I protein labeled with $^{75}$Se (13,14), f) corresponds closely with the MW of 29.7 kDa predicted for ID-I by the rat ID-I cDNA nucleotide sequence (8), and g) is observed in cells transfected with ID-I cDNA but not in cells which do not express ID-I (31). The specific affinity-labeling of ID-I in tissue microsomal fractions despite the low deiodinase content (9) suggests an extremely high reactivity of the bromoacetyl group with an amino acid residue in the active center of ID-I. Since the high susceptibility of ID-I to inactivation by iodoacetate is explained by carboxymethylation of the Sec residue (32), it is tempting to speculate that the enzyme selenolate group is also the target for modification by BrAc-iodothyronines. However, the Sec126Cys mutant of ID-I is labeled as effectively by BrAc[$^{125}$I]T3 as the wild-type enzyme (31). Therefore, the molecular basis for this efficient affinity-labeling of ID-I remains to be established.

BrAc[$^{125}$I]T4 has also been used to affinity-label ID-II, resulting in the identification of a $\approx$29 kDa substrate-binding subunit of this multimeric protein (33). The specific affinity-labelling of ID-II by BrAc[$^{125}$I]T4 is not due to modification of a Sec residue, since there is strong evidence that ID-II is not a selenoprotein (30,34,35).

Initial findings suggested that the labeling of p32 by BrAc[$^{125}$I]T3 reported in the present and previous studies (19,36) represents the affinity-labeling of ID-III. The evidence for this hypothesis included a) the prominence of p32 in tissues such as brain, in particular fetal brain, and placenta with high ID-III activities (this paper,36), b) the close correlation between the progressive labeling of p32 and inactivation of ID-III by BrAcT3 with increasing

reaction time or BrAcT3 concentration (this paper), and c) the inhibition of p32 labeling and ID-III activity in rat placenta by $\mu$M concentrations of goldthioglucose (GTG) (36). These GTG concentrations are much higher than the nM concentrations of GTG required for inhibition of ID-I activity, which is explained by the high reactivity of GTG towards the Sec residue in the latter enzyme (8,35,36). The relatively low susceptibility of ID-III to inhibition by GTG is in agreement with other evidence that ID-III is not a selenoprotein (37,38).

However, there are major discrepancies which refute the possible relationship between p32 and ID-III, including a) the prominent labeling of p32 by BrAc[$^{125}$I]T3 in tissues such as spleen and fetal liver with very low ID-III activities (this paper,36), b) the lack of p32 labeling by BrAc[$^{125}$I]T3 in embryonic chicken liver despite its high ID-III activity (19), c) the prominent labeling of p32 by the BrAc-derivative of [$^{125}$I]rT3 which is not a substrate for ID-III (this paper), d) the lack of inhibition of p32 labeling by BrAc[$^{125}$I]T3 or BrAc[$^{125}$I]rT3 in the presence of unlabeled substrates and inhibitors of ID-III far in excess of the concentrations producing complete inhibition of the deiodination of [$^{125}$I]T3 (this paper,19,36).

We conclude, therefore, that it is unlikely that the labeling of p32 by BrAc[$^{125}$I]T3 represents the affinity-labeling of ID-III. Although ID-III is inactivated by BrAcT3 supposedly by covalent modification, we were unable to identify an alternative protein, the BrAc[$^{125}$I]T3 labeling of which conforms to the tissue distribution and substrate competition expected for ID-III. This may be due to the low abundance of ID-III even in microsomes of placenta and fetal brain, which would make it undetectable in the presence of other prominently labeled proteins. Therefore, additional investigations are required for the unequivocal identification of ID-III by affinity-labeling.

## ACKNOWLEDGMENTS

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# Chapter 7

## GENERAL DISCUSSION

At the start of the studies described in this thesis the identity of type I iodothyronine deiodinase (ID-I) was still to be determined. The enzyme had been identified with protein disulfide isomerase (PDI; 1-3), an abundant protein of the endoplasmic reticulum with a molecular mass of 56 kDa, but we considered this relation to be unlikely for various reasons. The reported protein concentration of PDI in liver microsomes (4,5) was much higher than the reported protein concentration of ID-I (6). Also, PDI had been shown to be a soluble acidic protein with a pI of $\approx$4.5 (7-9), whereas ID-I was found to be a transmembrane basic protein with a pI value of 9.3 in the delipidated state (10). Furthermore, a discrepancy was found in mRNA size as liver PDI is encoded by a 2.8 kb mRNA molecule (11), whereas ID-I activity was induced in *Xenopus laevis* oocytes by injection of a polyadenylated liver RNA fraction of 1.5-2.0 kb (12).

Closely followed by others (13,14), our group was the first to identify ID-I as the 27 kDa protein labeled in rat liver microsomes by reaction with BrAc[$^{125}$I]$T_3$. This identification, that is reported in Chapter 2, was done by showing selective inhibition of labeling of this protein in the presence of various substrates and inhibitors of ID-I. Furthermore, a very strong correlation was found between ID-I activity and labeling of the 27 kDa protein under widely varying conditions, such as trypsin treatment and procedures resulting in the stepwise removal of the various microsomal proteins. However, ID-I activity was not related to labeling of the 56 kDa PDI protein under any of the conditions tested. The finding that selenium deficiency leads to a decrease of ID-I activity and that the 27 kDa protein can be labeled with $^{75}$Se (15,16) further emphasized that ID-I and the 27 kDa protein are identical. Definitive prove came with the finding that ID-I is a selenocysteine-containing enzyme encoded by a mRNA of 2.1 kb from which a molecular mass of 29 kDa can be deduced (17).

Several of the possible strategies to elucidate the structure of the ID-I gene required purified ID-I protein. However, purification was found to be very difficult (18-20) because of the physico-chemical properties of the enzyme (14) and its low abundance (6,21). Chapter 3 describes the multi-species comparison of liver ID-I carried out to investigate possible other sources for the purification of the enzyme and to detect possible interspecies differences. Because the elucidation of the human ID-I gene was a major goal, it was not desirable to purify ID-I from a species

displaying characteristics widely different from human ID-I as this might indicate a low genetic homology between the two ID-I genes.

ID-I was characterized by evaluation of $rT_3$ ORD activity in liver microsomes and by affinity-labeling of microsomal proteins with BrAc[$^{125}$I]$T_3$. Using SDS-PAGE and autoradiography, ID-I was identified and subsequently quantified by saturation analysis of the progressive labeling with increasing BrAc$T_3$-concentrations. BrAc$T_3$ was found to be an effective affinity-label for ID-I from different species and a narrow range was found for subunit $M_r$ (25.7-29.1 kDa). Hepatic microsomal ID-I content varied significantly between the studied species, with dog and rat displaying the highest levels. The latter species also displayed highest ID-I activity. The turnover number of dog liver ID-I, using $rT_3$ as substrate, was found to be exceptionally low compared to the other species investigated. Labeling of dog liver ID-I by BrAc[$^{125}$I]$T_3$ was not completely prevented by the presence of 10 $\mu$M $rT_3$ plus 100 $\mu$M PTU, in contrast with the other species. These data suggest a high degree of homology for ID-I subunit structure between the various species. However, substrate specificity of dog ID-I is remarkably different. Others have also found dog ID-I to be considerably different from the rat liver enzyme with respect to substrate preferences (22).

Preceded by the elucidation of the genetic sequence of rat (17) and human (23) ID-I, dog ID-I was recently cloned in order to identify the amino acid residues critical for $rT_3$ binding by comparative analysis of function versus structure (24; Fig. 1). It was found that the $K_m$ for $rT_3$ deiodination of the dog enzyme was 25-fold higher than that of the human enzyme, whereas the $K_m$ for $T_4$ deiodination was only 3-fold higher in dog. This suggests that the differences between ID-I in these species affect $rT_3$ binding more than that of $T_4$. Using mutational studies it was shown that only three of the amino acid substitutions are mainly responsible for the observed differences in substrate preferences between human and dog ID-I. Replacement of these residues in dog ID-I with those of human ID-I yields an enzyme displaying human type characteristics and *vice versa*.

```
          1                                                  50
Human     MGLPQPGLWL KRLWVLLEVA VHVVVGKVLL ILFPDRVKRN ILAMGEKTGM
  Rat     ...S.LW... ...VIF.Q.. LE.AT....M T...E...Q. .....Q....
  Dog     ....R.V... R......Q.. .Q.A....F. K...A...QH .V..NG.---

          51                                                 100
Human     TRNPHFSHDN WIPTFFSTQY FWFVLKVRWQ RLEDTTELGG LAPNCPVVRL
  Rat     ....R.AP.. .V.....I.. .......... ....RA.Y.. .....T....
  Dog     --.....Y.. .A..LY.M.. .......Q.. ....R..P.. ..........

          101                                                150
Human     SGQRCNIWEF MQGNRPLVLN FGSCT*PSFM FKFDQFKRLI EDFSSIADFL
  Rat     ...K..V.D. I..S..... .........L L.........V D..A.T....
  Dog     ........D. .......... .........L .......... ...C.T....

          151                                                200
Human     VIYIEEAHAS DGWAFKNNMD IRNHQNLQDR LQAAHLLLAR SPQCPVVVDT
  Rat     I........T ........V. ..Q.RS.... .R........ ..........
  Dog     I......... ........VN ..T..T.... ....R...D. A.P.......

          201                                                250
Human     MQNQSSQLYA ALPERLYIIQ EGRILYKGKS GPWNYNPEEV RAVLEKLHS
  Rat     .......... .......V.. ....C....P .......... .......CIP
  Dog     .R.....F.. ......FVL. .........P .....H.... .........

          251
Human
  Rat     PGHMPQF
  Dog
```

Figure 1. Alignment of the predicted amino acid sequences of human, rat and dog type I deiodinases. For the sequences of the rat and dog enzymes, only those residues that are different from the human enzyme are shown; residues identical to the human enzyme are indicated by dots. The dashes indicate amino acids which are not present in the dog enzyme. Selenocysteine at position 126 is shown with an asterisk (taken from Ref. 24).

Various inbred mouse substrains differ markedly in hepatic deiodinase activity. Most mice of the C3H/He (C3H) substrain were found to be almost devoid of liver ID-I activity, whereas highly variable enzyme activities were found in the other animals. Chapter 4 reports the first full description of a defect in ID-I activity in an animal strain. A strong correlation was found between ID-I activity, labeling of the 28 kDa ID-I protein, and ID-I mRNA levels in animals of both C3H and C57BL/6N (C57) substrains. This suggests that the low ID-I activity in C3H mice is due to a decrease in transcription of the ID-I gene or reduced stability of the mRNA. Selenium deficiency resulted in a decrease of ID-I activity in both mouse substrains. After [$^{75}$Se]-selenite injection, it was found that incorporation of $^{75}$Se in ID-I was higher in Se-deficient than in control mice and was also higher in C57 than in C3H

mice, suggesting that mouse ID-I is a selenoprotein like rat, human and dog ID-I (24).

As ID-I plays a key role in thyroid hormone metabolism and specifically is responsible for the largest part of peripheral $T_3$ production, it was surprising that $T_4$ and $T_3$ levels in the circulation of affected C3H animals were found to be normal although the serum $rT_3$ concentration was elevated. These findings are in contrast with a recent publication of Berry et al. (25) in which the decrease of ID-I activity in C3H mice was accompanied by an increase in serum $T_4$. Furthermore, their population of over 40 C3H mice displayed a homogeneously decreased level of ID-I activity opposed to the extremely variable enzyme activity found in our animals. Animals from both studies originated from the same supplier, The Jackson Laboratory (Bar Harbor, ME). However, the animals used in our investigations were at least 8 weeks old whereas the mice used in the study of Berry et al. were sacrificed at 7 weeks. Perhaps this difference in age can account for the observed discrepancies.

The segregation characteristics of ID-I in inbred strains, derived from crosses between high and low ID-I activity strains, suggested a single allele difference (25). Furthermore, a restriction fragment length variant that segregated with ID-I activity was observed on Southern blots using a rat ID-I probe. Using recombination frequencies for previously mapped loci, the ID-I gene was assigned to mouse chromosome 4 and its approximate chromosomal position was determined. Because this region of mouse chromosome 4 was found to be homologous to a region of human chromosome 1 and these regions display uninterrupted conserved linkage, the ID-I gene was predicted to be located on human chromosome 1 (25).

Although $T_3$ is not the preferred substrate of ID-I, BrAc[$^{125}$I]$T_3$ has proven to be an excellent affinity-label of this enzyme (6,13,14; Chapters 2-4). This was also evident from the very low apparent $K_i$ as revealed by Lineweaver Burk analysis ($\approx$0.1 nM) (6), compared with the Km value for T3 ($\approx$10 $\mu$M) or even the preferred substrate $rT_3$ ($\approx$0.1 $\mu$M) (26). Using N-Ac$T_3$, it was found that introduction of the acetyl group already caused a decrease in the $K_i$ of $T_3$ to $\approx$0.1 $\mu$M, which is in agreement with the observed decrease in $K_i$ of other iodothyronines upon N-acylation (6). Although ID-I content in the microsomal fraction is very low (23), the enzyme is labeled and inactivated by BrAc$T_3$ in a highly specific manner. This suggests an extremely high reactivity of the

bromoacetyl group with an active center amino acid residue. A possible candidate would be the selenocysteine residue, thought to be responsible for the high susceptibility of ID-I to inactivation by iodoacetate by carboxymethylation (27). However, replacement of this residue by a normal cysteine residue, using site-directed mutagenesis, did not result in a decrease of labeling efficiency (28). Therefore, the molecular mechanism of the efficient affinity-labeling of ID-I remains to be elucidated.

In addition to the ≈27 kDa and ≈56 kDa proteins, another prominent band with $M_r$ of 32 kDa was observed after labeling of pig liver microsomes with BrAc[$^{125}$I]$T_3$ in the multi-species comparison of ID-I. The hypothesis that this 32 kDa band might represent the type III iodothyronine deiodinase (ID-III) seemed to be supported by the following observations: 1) significant ID-III activity is found in pig liver microsomes in contrast to rat, 2) a 32 kDa band is also seen in labeling patterns of rat brain and placenta microsomes that contain very high ID-III activities, 3) reaction with Br$AcT_3$ results in rapid and irreversible inactivation of ID-III, and 4) a good correlation can be found between the extent of labeling of the 32 kDa band and the extent of inactivation of ID-III, both in a dose and time dependent manner.

In Chapters 5 and 6, it is shown to be very unlikely that the 32 kDa protein and ID-III are identical, because 1) labeling of the 32 kDa protein is observed not only in tissues with high ID-III activity such as rat brain and placenta, but also in tissues lacking enzyme activity such as rat spleen and fetal liver, 2) it was shown by peptide mapping that this labeled protein is identical in all these organs, 3) labeling with BrAc[$^{125}$I]$T_3$ yields no 32 kDa band in embryonic chicken liver microsomes, in which ID-III activity shows similar levels and characteristics as rat placenta, and is as sensitive to inhibition by Br$AcT_3$ as rat ID-III, 4) although $rT_3$ is not a substrate for ID-III, the 32 kDa protein is readily labeled by BrAc[$^{125}$I]$rT_3$, 5) labeling of the 32 kDa band by both BrAc[$^{125}$I]$T_3$ or BrAc[$^{125}$I]$rT_3$ is not influenced by 100 $\mu$M concentrations of substrate analogs or inhibitors of ID-III, although some of these substances inhibit ID-III activity almost completely at 100-fold lower concentrations.

Part of these results were presented at the 19th annual meeting of the European Thyroid Association, August 25-30 1991, Hannover, Germany (29). After this presentation Santini et al. submitted a study in which they reported similar findings concerning the inactivation of ID-III as well as the labeling of a 31 kDa protein with Br$AcT_3$ in rat placental microsomes (30). They proposed that this 31 kDa protein

is the substrate-binding subunit of ID-III, although labeling of this protein was inhibited only partially by the presence of 150 $\mu$M $T_3$ and was also found in tissues devoid of ID-III activity. However, we strongly doubt that the 31-32 kDa protein represents ID-III or a subunit thereof.

In order to get a better understanding of the structure and catalytic mechanism of ID-I, future investigations could concentrate on the identification of the amino acid residues which are involved with the binding of the substrate or which participate in the deiodination process in a direct manner. It will also be interesting to find out which residues are responsible for the inactivation of ID-I by $BrAcT_3$ or PTU. Furthermore, until now it has not been proved that ID-I is indeed a homodimer of two 27 kDa subunits, as has been assumed. Such evidence could be provided by cross-linking experiments combined with label-incorporation studies using $BrAc[^{125}I]T_3$ and $^{75}Se$.

Future research might also focus on the elucidation of the genetic structure of ID-III. Possibly the *Xenopus laevis* oocyte expression system can provide the means to clone and investigate this enzyme. Recently the cDNA of a tadpole protein displaying characteristics of ID-III has been cloned and sequenced (31). Maybe, this cDNA can be used to characterize human ID-III. However, it has not been established if the cDNA cloned from *Xenopus laevis* represents the tadpole equivalent of mammalian ID-III or an evolutionary divergent form of ID-I with prevalent inner ring deiodinase activity.

The type I and III iodothyronine deiodinases are unique enzymes, playing major roles in thyroid hormone metabolism. Their activity is influenced by hypo- or hyperthyroidism and may be altered in pathophysiological conditions, such as starvation or non-thyroidal illness. Furthermore, the action of ID-I may be affected by drugs such as dexamethasone, propranolol, amiodarone and PTU. Knowledge of the catalytic mechanism and molecular structure of these enzymes is crucial for better understanding of their role in the regulation of thyroid hormone bioactivity. It may lead to the development of more potent and specific inhibitors of ID-I, which are potential drugs in the treatment of hyperthyroidism.

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## Summary

This thesis describes research on the enzymes responsible for deiodination of thyroid hormones or iodothyronines. In humans the thyroid secretes predominantly the prohormone thyroxine ($T_4$). Active thyroid hormone is generated when $T_4$ is converted to 3,3',5-triiodothyronine ($T_3$) by removal of an iodine atom. This deiodination is catalyzed by so called iodothyronine deiodinases - enzymes also capable of catalyzing other deiodination steps, resulting in the generation of the various other iodothyronines.

Next to a general introduction, Chapter 1 gives an overview of the current state of knowledge concerning the characterization of the three different types of deiodinases discovered until now. Furthermore, alternative metabolic routes for iodothyronines are discussed, together with their transport from the plasma to their target tissues and the molecular mechanism of action of thyroid hormone involving different thyroid hormone receptors and response elements.

The study described in Chapter 2 resulted in the unambiguous identification of type I iodothyronine deiodinase (ID-I). The study was undertaken to test the hypothesis put forward by others in a previous publication that ID-I was identical to protein disulfide isomerase (PDI). When rat liver microsomes were labeled by N-bromoacetyl-[$^{125}$I]$T_3$ (BrAc[$^{125}$I]$T_3$) and subsequently separated by SDS-PAGE, two prominently labeled bands with relative molecular mass ($M_r$) of 56 and 27 kDa were seen on the autoradiogram of the gel. Labeling of the 27 kDa band, but not that of the 56 kDa band, was inhibited by substrates and inhibitors of ID-I. Trypsin treatment of microsomes resulted in elimination of both labeling of the 27 kDa protein and ID-I activity. However, labeling of the 56 kDa protein was not diminished by trypsinization. After treatment of microsomes with carbonate buffer of pH 8.0-9.5 or with 0.05 % deoxycholate both PDI content and labeling of the 56 kDa protein were strongly diminished, whereas ID-I activity and labeling of the 27 kDa protein were uninfluenced. Above pH 10 the latter two decreased in parallel. Although rat pancreas microsomes contained high concentrations of PDI, they showed no ID-I activity. Upon BrAc$T_3$-labeling of these microsomes a strong 56 kDa band was seen, whereas no 27 kDa protein was labeled. Purified PDI with $M_r$ of 56 kDa displayed no ID-I activity but was readily labeled with BrAc$T_3$. These results prove that the 27 kDa band represents ID-I and, thus, that PDI is not identical

to ID-I.

Purification of ID-I from the membranous fraction of liver or kidney has proven to be very difficult because it is an extremely hydrophobic transmembrane protein that is present in very low concentrations. In the study described in Chapter 3, ID-I was investigated in liver microsomal fractions of different species to explore alternative sources for purification of this enzyme and to elucidate possible interspecies differences. ID-I was characterized by measurement of its activity using the preferred substrate 3,3',5'-triiodothyronine ($rT_3$) and by affinity-labeling with BrAc[$^{125}$I]$T_3$. ID-I was identified and quantified using SDS-PAGE and autoradiography. In the species studied the $M_r$ of ID-I varied between 25.7 and 29.1 kDa. Liver microsomes from rat and dog displayed a markedly higher ID-I content than liver microsomes from human, mouse, rabbit, cow, pig, sheep, goat, chicken or duck. Rat liver microsomes showed highest ID-I activity of all species examined. Turnover numbers for ID-I, for all species except dog, varied between 264 and 1059 $min^{-1}$ with rabbit and goat displaying highest values. Dog liver microsomal ID-I displayed an exceptionally low turnover number of 79 $min^{-1}$ and, unlike the other species, labeling of the enzyme could not be prevented by 10 $\mu$M $rT_3$ plus 100 $\mu$M 6-N-propyl-2-thiouracil (PTU). From these results it was concluded that ID-I has similar properties in all species investigated with exception of dog.

The interspecies comparison of ID-I had included the BALB/c mouse strain. However, large differences in ID-I activity were found between different mouse strains, with substrain C3H/He (C3H) displaying an exceptionally diminished ID-I activity compared with BALB/c and C57BL/6N (C57) mice. In the experiments reported in Chapter 4 it was investigated if this diminution correlates with the results of labeling experiments with BrAc[$^{125}$I]$T_3$ or $^{75}$Se. By this time it was found by others that ID-I in rat and human is a selenoenzyme that can be labeled by radioactive selenium. ID-I activity in liver microsomes of C3H mice was found to be highly variable with a median of only 18 % of that found in C57 mice. Surprisingly, $T_4$ and $T_3$ levels in the circulation were normal, although the serum $rT_3$ concentration was elevated. Mouse ID-I with a $M_r$ of 28 kDa was identified on autoradiograms of BrAc[$^{125}$I]$T_3$ labeled microsomes analyzed by SDS-PAGE. Labeling of the 28 kDa band was nearly absent in C3H samples with low ID-I

activity. In Se-deficient mice both ID-I activity and labeling of the 28 kDa band was strongly decreased compared to control mice. Labeling of ID-I with $^{75}Se$ was demonstrated by SDS-PAGE of liver microsomes isolated from [$^{75}Se$]-selenite injected mice. Labeling of ID-I with $^{75}Se$ was clearly higher in Se-deficient than in Se-sufficient mice and was also higher in C57 than in C3H mice. Using a rat ID-I cDNA probe, liver ID-I mRNA was quantified on Northern blots. It was found that mRNA concentrations correlated strongly with ID-I activity and that both were strongly decreased in most C3H mice. From these results it is concluded that ID-I in mouse is also a selenoenzyme. ID-I activity in C3H mice is decreased because of diminished transcription of the ID-I gene or reduced stability of the mRNA.

In the multi-species comparison in Chapter 3 it was noticed that $BrAcT_3$-labeled pig liver microsomes showed, next to the ID-I band of 27.6 kDa, a very strongly labeled band of $\approx$32 kDa. The possibility that this band might represent the type III iodothyronine deiodinase (ID-III) was supported by the findings that pig liver microsomes show high ID-III activity compared to rat, and that a 32 kDa protein is labeled in microsomes of rat brain and placenta, both displaying high ID-III activity. This possibility was further investigated as described in Chapter 5. ID-III activity in microsomes of rat brain, rat placenta and embryonic chicken liver is inhibited in an irreversible manner by $BrAcT_3$ with an affinity similar to that of $T_3$. Reaction with BrAc[$^{125}$I]$T_3$ resulted in the labeling of a 32 kDa band in microsomes of rat brain and placenta, but also in fetal rat liver microsomes that had no ID-III activity. Labeling of the 32 kDa band was not influenced by the presence of 100 $\mu$M concentrations of substrate analogs or inhibitors of ID-III, whereas some of these substances inhibited ID-III activity almost completely at 1 $\mu$M. Embryonic chicken liver microsomes showed high ID-III activity, but upon labeling with BrAc[$^{125}$I]$T_3$ no 32 kDa band could be demonstrated nor another protein possibly representing ID-III. From these results it is concluded that it is unlikely that the labeled 32 kDa protein is identical to ID-III or a subunit thereof.

Chapter 6 describes investigations in which the prevalence of the different types of deiodinases in various rat tissues was compared to the labeling patterns obtained when subfractions of these organs were reacted with N-bromoacetyl-[$^{125}$I]iodothyronine derivatives. This was done to find out if ID-III could be

identified by affinity-labeling with these substances. It was found that microsomes from rat kidney, liver and thyroid contained high ID-I activity and after reaction with BrAc[$^{125}$I]$T_3$ showed a prominent band of 27 kDa in their labeling pattern. Rat brain and placenta microsomes displayed high ID-III activity and a band of 32 kDa upon affinity-labeling with BrAc[$^{125}$I]$T_3$. The finding that ID-III was readily inactivated by reaction with $BrAcT_3$ indicated that this substance might be a useful affinity-label for the identification of this enzyme. A good correlation was found between the extent of labeling of the 32 kDa band and the extent of inactivation of ID-III. Treatment of rat brain or placenta microsomes with 0.05 % deoxycholate or carbonate buffer of pH 11.5 did not eliminate labeling of the 32 kDa band, indicating a transmembrane localization of this protein. The 32 kDa protein was readily labeled by BrAc[$^{125}$I]r$T_3$, although r$T_3$ is not a substrate for ID-III. Labeling of rat brain microsomes with this affinity-label was uninfluenced by the presence of 100 $\mu$M concentrations of different iodothyronines. However, deiodination of [$^{125}$I]$T_3$ by the same microsomes was reduced by 91 and 96 % by 1 $\mu$M $T_4$ and $T_3$, respectively. A 32 kDa band was also found in the labeling patterns of spleen and fetal liver microsomes, although neither preparation shows ID-III activity. Peptide mapping of the 32 kDa band from brain, placenta, spleen and fetal liver microsomes resulted in identical fragments in all four tissues, indicating that the labeled protein is identical in these cases. These results once more strongly suggest that the identity of ID-III has not been elucidated until now.

# Samenvatting

In dit proefschrift wordt onderzoek beschreven dat verricht is aan de enzymen die verantwoordelijk zijn voor de dejodering van schildklierhormonen oftewel jodothyronines. De humane schildklier scheidt voornamelijk het prohormoon thyroxine ($T_4$) uit. Actief schildklierhormoon ontstaat pas wanneer $T_4$ door het verwijderen van een jodium atoom, oftewel dejodering, wordt omgezet in 3,3',5-trijodothyronine ($T_3$). Deze dejodering wordt gekatalyseerd door zogenaamde jodothyronine dejodasen - enzymen die naast voornoemde omzetting ook nog andere dejoderingsstappen kunnen bewerkstelligen en daarmee verantwoordelijk zijn voor de vorming van de verschillende jodothyronines.

Naast een algemene inleiding, wordt in Hoofdstuk 1 een overzicht gegeven van de huidige stand van zaken betreffende de karakterisering van de drie verschillende typen dejodasen die tot op heden zijn aangetoond. Verder komen alternatieve afbraakroutes van jodothyronines aan bod, samen met hun transport vanuit het plasma naar de doelweefsels, het moleculaire werkingsmechanisme van schildklierhormoon en de verschillende schildklierhormoon receptoren en respons elementen die hierbij betrokken zijn.

De studie die wordt beschreven in Hoofdstuk 2 resulteerde in de onomstotelijke identificatie van het type I jodothyronine dejodase (ID-I). Kort daarvoor was namelijk door anderen in de literatuur beweerd dat het enzym protein disulfide isomerase (PDI) identiek aan ID-I was. Wanneer microsomale ratte lever eiwitten gelabeld werden met N-bromoacetyl-[$^{125}$I]$T_3$ (BrAc[$^{125}$I]$T_3$) en vervolgens gescheiden werden met SDS-PAGE, werden op het autoradiogram van de gel twee prominent gelabelde banden waargenomen met relatieve molecuulmassa's ($M_r$) van 56 en 27 kDa. Substraten en inhibitoren van ID-I waren in staat om de labeling te remmen van de 27 kDa band maar niet die van de 56 kDa band. Behandeling van microsomen met trypsine resulteerde in het uitblijven van labeling van het 27 kDa eiwit en het verdwijnen van ID-I activiteit, terwijl de labeling van het 56 kDa eiwit niet geremd werd. Na behandeling van microsomen met carbonaat buffers van pH 8.0-9.5 of met 0.05 % deoxycholaat waren zowel het PDI gehalte als de labeling van het 56 kDa eiwit sterk afgenomen terwijl de ID-I activiteit en labeling van het 27 kDa eiwit niet verminderden. Boven pH 10 namen de laatste twee grootheden parallel af. Ratte pancreas microsomen bevatten hoge concentraties PDI maar

vertoonden geen ID-I activiteit. Wanneer deze microsomen gelabeld werden met BrAc[$^{125}$I]$T_3$ resulteerde dit in een zeer sterke labeling van een 56 kDa eiwit terwijl geen 27 kDa eiwit kon worden waargenomen. Gezuiverd PDI met een molecuulmassa van 56 kDa werd uitstekend gelabeld door Br$AcT_3$ maar vertoonde geen ID-I activiteit. Deze resultaten bewijzen dat de 27 kDa band ID-I representeert en dat derhalve PDI en ID-I verschillende eiwitten zijn.

De zuivering van ID-I uit de membraanfractie van lever of nier is zeer moeilijk gebleken doordat het hier een uitermate slecht oplosbaar transmembraan eiwit betreft dat in zeer lage concentraties voorkomt. In de experimenten beschreven in Hoofdstuk 3 wordt ID-I in microsomale lever fracties van verschillende species onderzocht om mogelijke alternatieve bronnen voor de zuivering van dit enzym aan het licht te brengen en om eventuele interspecies verschillen te onthullen. ID-I werd gekarakteriseerd met activiteitsmetingen met het geprefereerde substraat 3,3',5'-trijodothyronine ($rT_3$) en door affiniteits-labeling met BrAc[$^{125}$I]$T_3$. Gelabelde ID-I werd geïdentificeerd en gekwantificeerd met SDS-PAGE en autoradiografie. In de bestudeerde species varieerde het $M_r$ van ID-I tussen 25.7 en 29.1 kDa. Ratte en honde lever microsomen vertoonden een opvallend hoger enzym gehalte dan de lever microsomen van mens, muis, konijn, koe, varken, schaap, geit, kip of eend. Ratte lever microsomen vertoonden de hoogste ID-I activiteit van alle onderzochte species. De omzettingssnelheid van ID-I lag voor alle species, behalve de hond, tussen 264 en 1059 $min^{-1}$ waarbij konijn en geit de hoogste waarden vertoonden. Honde lever microsomaal ID-I vertoonde de uitzonderlijk lage omzettingssnelheid van 78 $min^{-1}$ en labeling van het eiwit bleek, in tegenstelling tot de andere species, slecht rembaar met 10 $\mu$M $rT_3$ plus 100 $\mu$M 6-N-propyl-2-thiouracil (PTU). Uit deze gegevens is af te leiden dat ID-I gelijksoortige eigenschappen bezit in alle bestudeerde species met uitzondering van de hond.

Bij de interspecies vergelijking van ID-I was de BALB/c muize stam betrokken. Tussen de verschillende substammen werden echter grote verschillen gevonden in ID-I activiteit. Hierbij bleek de substam C3H/He (C3H) een uitzonderlijk lage ID-I activiteit te vertonen ten opzichte van BALB/c en C57BL/6N (C57) muizen. In Hoofdstuk 4 wordt onderzocht of deze verlaging correleert met de resultaten van labelingsexperimenten met BrAc[$^{125}$I]$T_3$ of $^{75}$Se. Ondertussen was namelijk door

anderen bekend geworden dat ID-I in rat en mens een seleno-enzym is dat met radioactief selenium gelabeld kon worden. ID-I activiteit in lever microsomen van C3H muizen bleek in hoge mate variabel met een mediaan van slechts 18 % van die gevonden in C57 muizen. De serum $T_4$ en $T_3$ concentratie van C3H muizen was echter normaal, alhoewel het serum gehalte aan $rT_3$ verhoogd was. Muize ID-I met een $M_r$ van 28 kDa werd geïdentificeerd met behulp van SDS-PAGE van BrAc[$^{125}$I]$T_3$ gelabelde lever microsomen. In C3H monsters met lage ID-I activiteit was deze labeling zo goed als afwezig. De ID-I activiteit was sterk verlaagd in Se-deficiënte muizen, wat vergezeld ging met een drastische afname van de labeling van de 28 kDa band vergeleken met controle muizen. Labeling van ID-I met $^{75}$Se kon worden aangetoond met SDS-PAGE van lever microsomen geïsoleerd uit [$^{75}$Se]-seleniet geïnjecteerde muizen. $^{75}$Se labeling was duidelijk hoger in Se-deficiënte dan in Se-sufficiënte muizen en was ook duidelijk hoger in C57 dan in C3H muizen. Lever ID-I mRNA werd gemeten op Northern blots met gebruikmaking van een ratte ID-I cDNA probe. Hieruit bleek dat de concentratie aan mRNA in sterke mate correleerde met de ID-I activiteit en dat beiden relatief laag waren in de meeste C3H muizen. Uit deze resultaten volgt dat ID-I ook in de muis een seleno-enzym is. Verder is de ID-I activiteit in C3H muizen relatief laag door een lage transcriptie van het ID-I gen of door een geringe stabiliteit van het mRNA.

In de multi species vergelijking in Hoofdstuk 3 viel het op dat in $BrAcT_3$-gelabelde varkens lever microsomen, naast de ID-I band van 27.6 kDa, ook een band van $\approx$32 kDa zeer sterk gelabeld werd. De mogelijkheid dat deze band het type III jodothyronine dejodase (ID-III) representeerde leek aanwezig, omdat varkens lever microsomen een verhoogde ID-III activiteit vertoonden vergeleken met de rat en omdat ook een 32 kDa eiwit gelabeld werd in microsomen van ratte hersenen en placenta die beiden een hoge ID-III activiteit bezaten. Deze mogelijkheid werd verder onderzocht in het onderzoek dat beschreven is in Hoofdstuk 5. De ID-III activiteit in ratte hersenen, ratte placenta en embryonale kippe lever werd irreversibel geremd door het affiniteits-label $BrAcT_3$ met een affiniteit vergelijkbaar met die van $T_3$. Reactie van ratte hersen en placenta microsomen met BrAc[$^{125}$I]$T_3$ resulteerde in sterke labeling van een 32 kDa eiwit. Dit eiwit werd echter ook gelabeld in foetale ratte lever microsomen die geen ID-III activiteit hadden. De labeling van de 32 kDa

band werd niet beïnvloed door de aanwezigheid van 100 $\mu$M concentraties van substraat analoga of remmers van ID-III tijdens de labelingsreactie, terwijl sommige van deze stoffen de ID-III activiteit nagenoeg volledig remden bij een concentratie van 1 $\mu$M. Wanneer embryonale kippe lever microsomen, met een hoge ID-III activiteit, gelabeld werden met BrAc[$^{125}$I]$T_3$ werd geen 32 kDa eiwit waargenomen noch enig ander eiwit dat mogelijk het ID-III representeert. Gezien deze bevindingen is het onwaarschijnlijk dat het 32 kDa eiwit identiek is aan het ID-III of een subunit hiervan.

In Hoofdstuk 6 wordt onderzoek beschreven waarin de prevalentie van de verschillende typen dejodasen in diverse ratte weefsels vergeleken werd met de labelingspatronen die verkregen werden na reactie van subfracties van deze weefsels met N-bromoacetyl-[$^{125}$I]jodothyronine derivaten. Het doel hiervan was om te achterhalen of ID-III geïdentificeerd kon worden door affiniteits-labeling met deze substanties. Het bleek dat nier, lever en schildklier microsomen een hoge ID-I activiteit bezaten, terwijl hersen en placenta microsomen een hoge ID-III activiteit vertoonden. Na labeling met BrAc[$^{125}$I]$T_3$ kon een prominente band van 27 kDa gezien worden in het labelingspatroon van de weefsels met hoge ID-I activiteit. In de organen die een hoge ID-III activiteit vertoonden werd een band van 32 kDa gelabeld. De bevinding dat ID-III snel geïnactiveerd werd door reactie met Br$AcT_3$ gaf aan dat deze substantie mogelijk een bruikbaar affiniteits-label was voor de identificatie van dit enzym. Een goede correlatie werd gevonden tussen de mate van affiniteits-labeling van de 32 kDa band met BrAc[$^{125}$I]$T_3$ en de mate van inactivering van ID-III. Na behandeling van ratte hersen of placenta microsomen met 0.05 % deoxycholaat of carbonaat buffer van pH 11,5 werd nog steeds een 32 kDa eiwit gelabeld door BrAc[$^{125}$I]$T_3$, wat aangaf dat het eiwit een transmembraan lokalisatie had. Alhoewel r$T_3$ geen substraat is voor ID-III werd het 32 kDa eiwit uitstekend gelabeld door BrAc[$^{125}$I]r$T_3$. De labeling van het 32 kDa eiwit in ratte hersen microsomen met dit affiniteits-label werd op geen enkele manier beïnvloed door de aanwezigheid van 100 $\mu$M concentraties van verschillende jodothyronines met PTU. De dejodering van [$^{125}$I]$T_3$ door ID-III in dezelfde microsomen werd echter voor 91 en 96 % geremd door respectievelijk 1 $\mu$M $T_4$ en $T_3$. Labeling van een 32 kDa band werd ook gevonden in microsomen van milt en foetale lever die

echter geen van beiden ID-III activiteit vertoonden. Peptide mapping van de 32 kDa band uit hersen, placenta, milt en foetale lever microsomen resulteerde in identieke fragmenten in alle vier weefsels, wat aangeeft dat het gelabelde eiwit in alle gevallen identiek is. Uit deze resultaten volgt wederom dat de identiteit van ID-III nog definitief vastgesteld moet worden.

## CURRICULUM VITAE

Christianus Hermann Hendrikus Schoenmakers werd op 27 maart 1964 geboren te Oss. Het Atheneum-B diploma werd in 1983 behaald aan het Maasland College te Oss. Aansluitend hierop begon hij aan de studie Scheikunde aan de Katholieke Universiteit te Nijmegen waar in 1988 het doctoraal-examen werd behaald. De doctoraalfase omvatte de hoofdrichting Biochemie (Prof.dr. W.J.M. van de Ven; begeleidster Dr.ir. A.M.W. van den Ouweland) en de nevenrichting microbiologie (Prof.dr. A.J.B. Zehnder; begeleider Dr. F.P. Houwen).

Per juli 1988 werd hij aangesteld als onderzoeker in opleiding (OIO) bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO; Gebied Medische Wetenschappen). In dit dienstverband is tot juni 1992 onderzoek gedaan op de Afdeling Inwendige Geneeskunde III en Klinische Endocrinologie van het Academisch Ziekenhuis Dijkzigt te Rotterdam. De resultaten van dit onderzoek zijn verwerkt in dit proefschrift.

Vanaf juni 1992 is hij aangesteld als Klinisch Chemicus In Opleiding bij de Afdeling Klinische Chemie (Dr. J. Lindemans) van het Academisch Ziekenhuis Dijkzigt/Sophia te Rotterdam.

## NAWOORD

Het moge duidelijk zijn dat een proefschrift niet een weerspiegeling is van het werk van één maar van velen. Daarom wil ik hierbij een ieder die direct dan wel indirect heeft bijgedragen aan de totstandkoming van dit proefschrift oprecht danken.

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Marjolijn Schalk: Beste Marjolijn, Het moet voor jou een flinke stap geweest zijn: vanuit een studie aan de Landbouw Universiteit te Wageningen verhuizen naar Rotterdam voor het volgen van een stage aan de afdeling Interne III. Ik dank je voor je bijdrage aan ons onderzoek, die je met zoveel inzet en enthousiasme hebt gedaan.

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