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Species differences in liver type I iodothyronine deiodinase

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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 ($\approx$ 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-1 activity was determined by incubation of the appropriate amount of microsomal protein for 20 min at 37°C with 10 μM rT_3 and 75 nCi [^{125}I]rT_3 in 200 μl 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 μl 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-1 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 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% 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-1 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-1 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

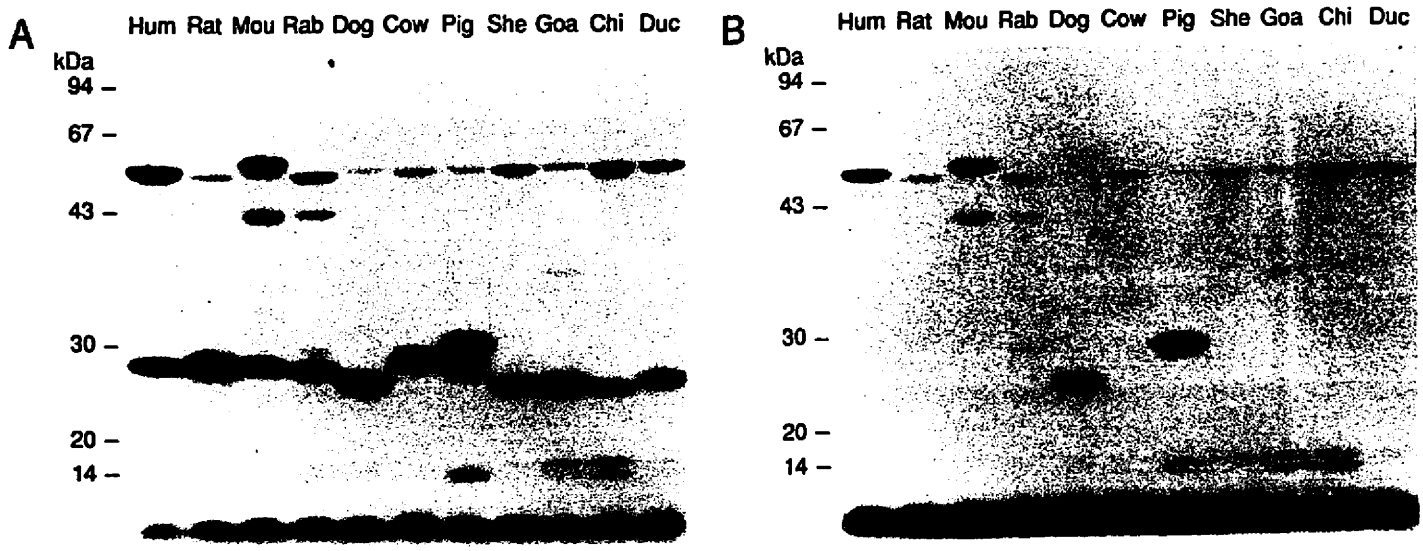

Fig. 1. Labeling of liver microsomal proteins of different species with BrAc[^{125}I]T_3. Microsomes (50 μg protein) were reacted for 10 min at 37 C with 0.1 μCi BrAc[^{125}I]T_3 in the absence (A) or presence (B) of 10 μM rT_3 and 100 μM PTU as described in Materials and Methods. After SDS-PAGE, film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

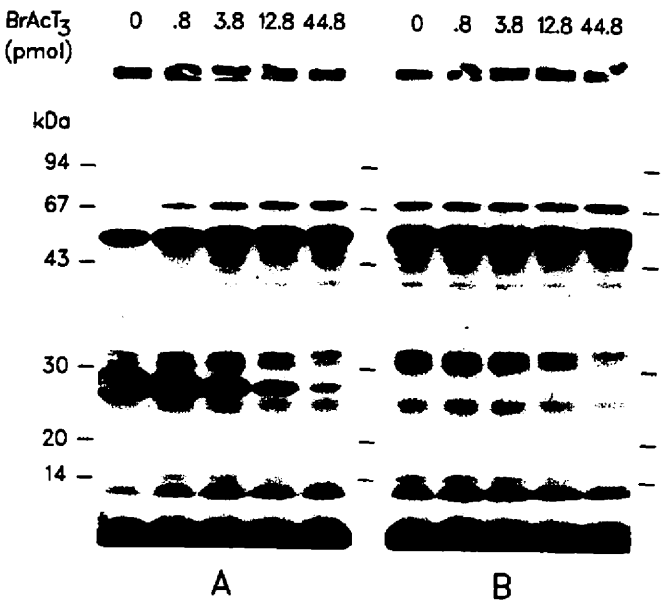

Fig. 2. Effect of increasing amounts of unlabeled $BrAcT_3$ on the labeling of rat liver microsomes by $BrAc[^{125}I]T_3$. Microsomes (100 μg protein) were reacted for 10 min at 37°C with 0.2 pmol (0.25 μCi) $BrAc[^{125}I]T_3$ in the presence of 0, 0.8, 3.8, 12.8 and 44.8 pmol of $BrAcT_3$ and in the 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

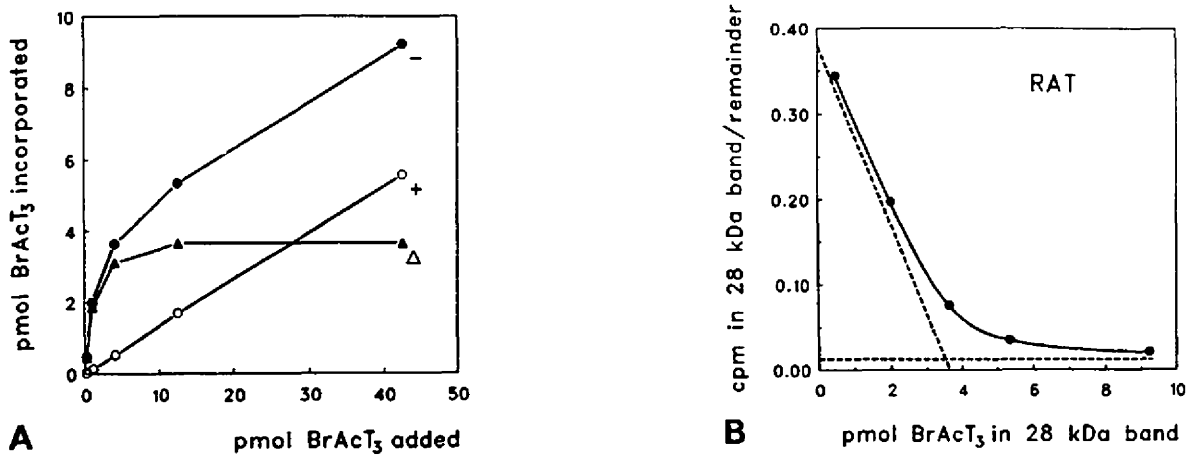

Fig. 3. Saturation analysis of the progressive incorporation of $BrAcT_3$ in rat liver ID-I with increasing amounts of added $BrAcT_3$. (A) Direct plot of $BrAcT_3$ incorporation in the ≈ 27 kDa band as a function of the amount of $BrAcT_3$ added in the absence (−) or presence (+) of 10 μM rT_3 and 100 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

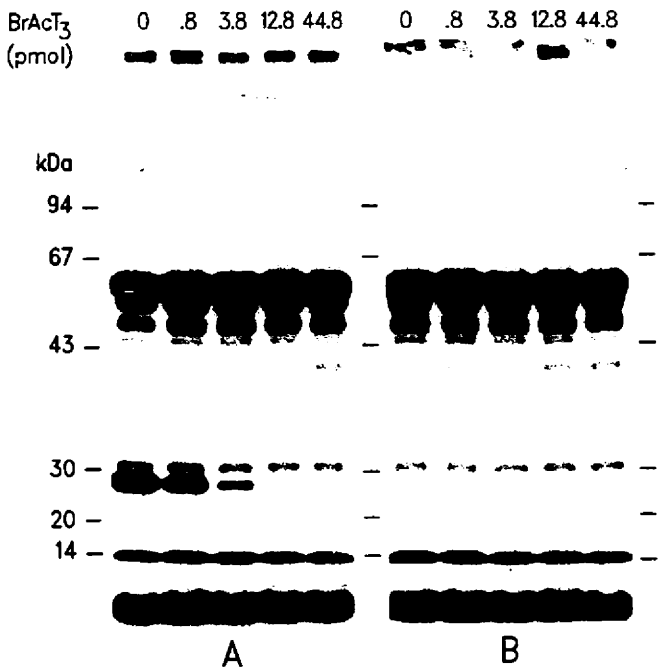

Fig. 4. Effect of increasing amounts of unlabeled $BrAcT_3$ on the labeling of human liver microsomes by $BrAc[^{125}I]T_3$ in the absence (A) or presence (B) of 10 μM rT_3 + 100 μM PTU. For further details, see Fig. 2.

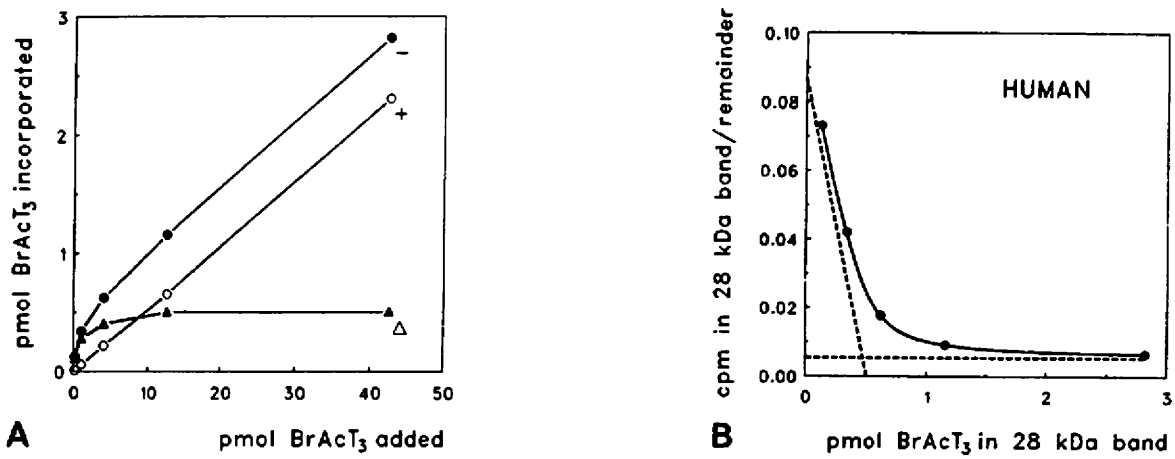

Fig. 5. Saturation analysis of the progressive labeling of human liver ID-I with increasing concentrations of $BrAcT_3$ by (A) direct plot or (B) Scatchard-like plot of the results depicted in Fig. 4. For further details, see Fig. 3.

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.

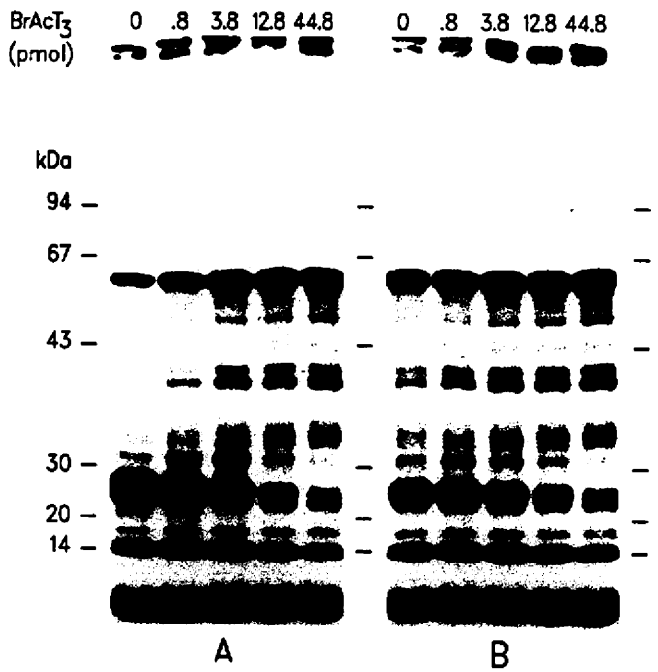

Fig. 6. Effect of increasing amounts of unlabeled $BrAcT_3$ on the labeling of dog liver microsomes by $BrAc[^{125}I]T_3$ in the absence (A) or presence (B) of 10 μM rT_3 + 100 μM PTU. For further details, see Fig. 2.

ID-I activity was determined at 10 μM rT_3, although in most species 1 μM 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 ≈ 5 μM (T.J. Visser, unpublished work) as opposed to ≈ 0.1 μM 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

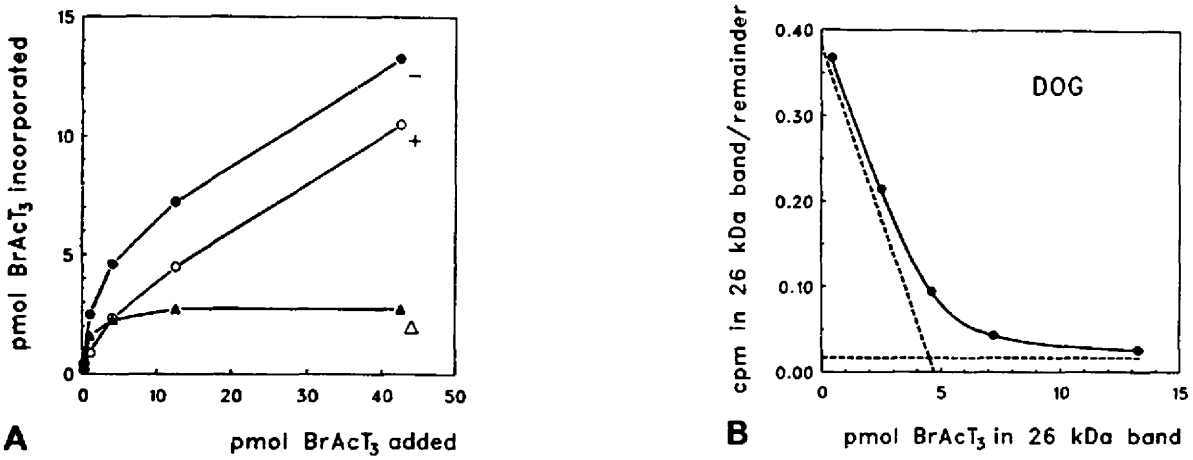

Fig. 7. Saturation analysis of the progressive labeling of dog liver ID-I with increasing concentrations of $BrAcT_3$ by (A) direct plot or (B) Scatchard-like plot of the results depicted in Fig. 6. For further details, see Fig. 3.

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 μM 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.

Acknowledgments

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