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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 μM substrate analogs or inhibitors of ID-III, some of which completely inhibit ID-III activity at 1 μM. $BrAc[^{125}I]T_3$ 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-

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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

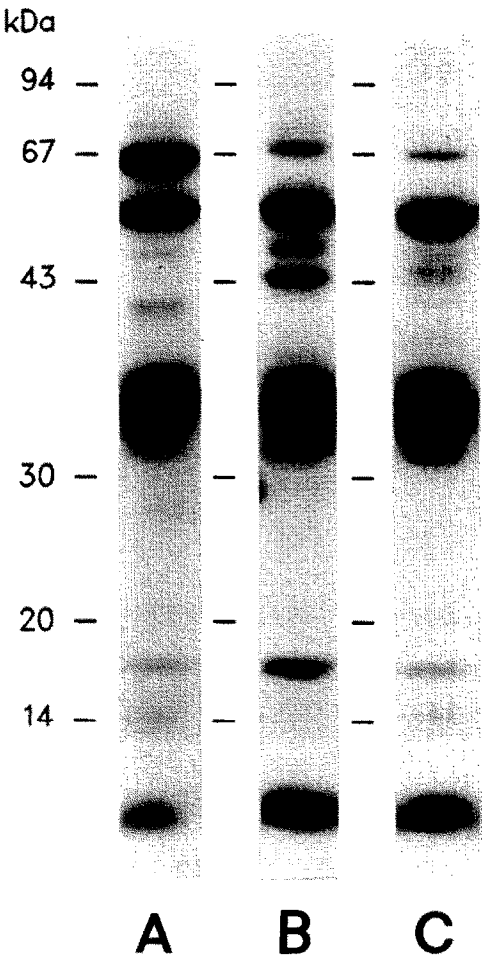

Fig. 1. Labeling patterns obtained after reaction of 50 μg of microsomal protein with 0.15 μCi BrAc[^{125}I]T_3. After SDS-PAGE film was exposed for 4 days. (A) Rat placenta. (B) Adult rat brain. (C) Fetal rat brain.

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

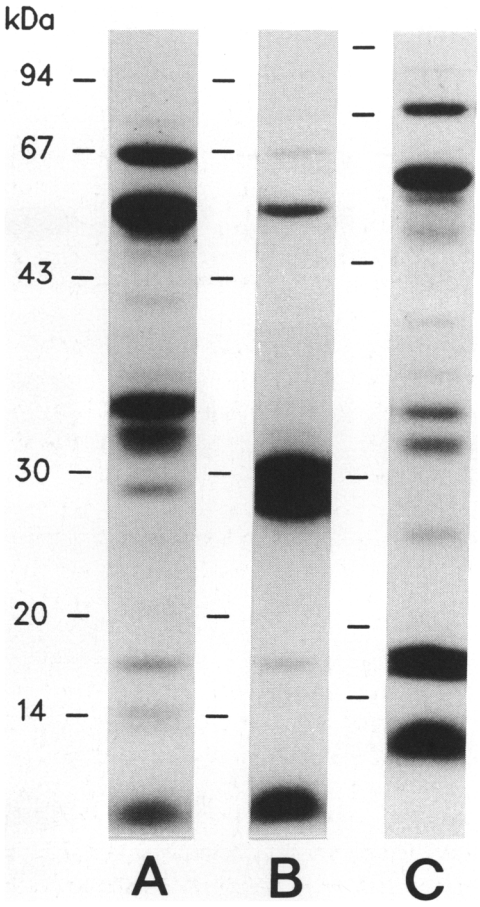

Fig. 2. Labeling patterns obtained after reaction of 50 μg of microsomal protein with 0.15 μCi BrAc[^{125}I]T_3. After SDS-PAGE film was exposed for 4 days. (A) Fetal rat liver. (B) Adult rat liver. (C) Adult rat liver in the presence of 10 μM rT_3 and 100 μM PTU.

of [^{125}I]T_3 by ID-III was largely inhibited by 1 μM 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 μM) or even rT_3 (0.06 μM) [3]. This is most likely caused by reaction of $BrAcT_3$ with the highly reactive selenocysteine residue in ID-I.

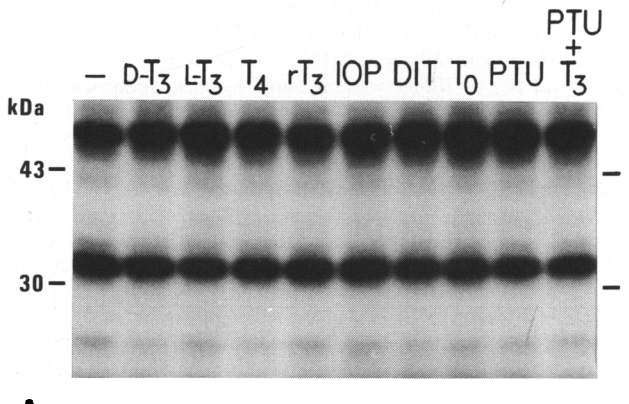

Fig. 3A.

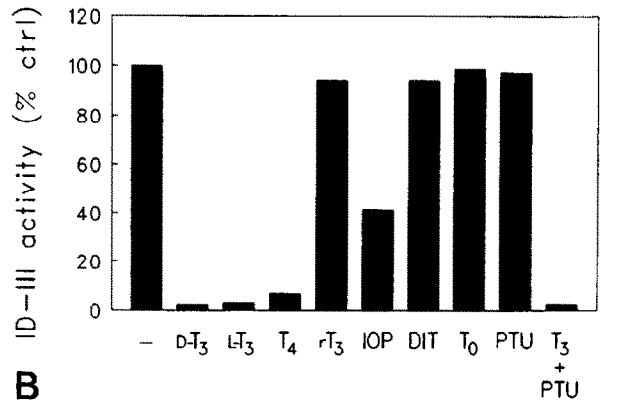

Fig. 3. (A) Labeling patterns obtained after reaction of 66 μg of rat brain microsomal protein with 0.1 μCi BrAc[^{125}I]T_3 in the absence or presence of 100 μM of various substrate analogs and deiodinase inhibitors. After SDS-PAGE film was exposed for 42 h. (B) Deiodination of 1 nM [^{125}I]T_3 by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

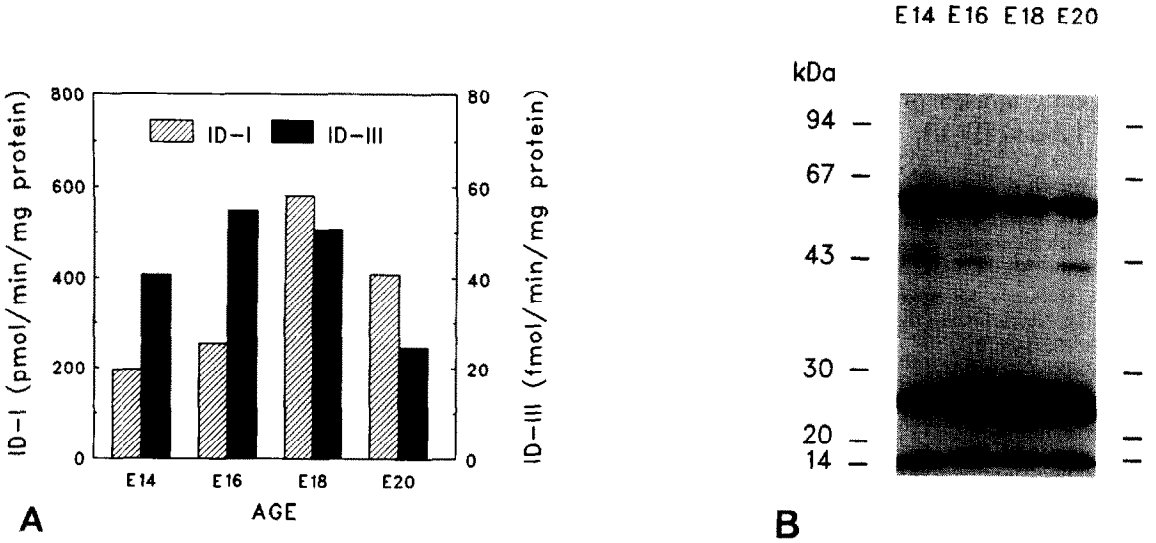

Fig. 4. (A) Ontogeny of deiodinase activities in chicken liver during the last week (E14–E20) of embryonic development. ID-I was assayed using 1 μM rT_3 and 10 μg/ml microsomal protein. ID-III was assayed using 1 nM T_3 and 100 μg/ml microsomal protein. (B) Autoradiogram of labeling patterns obtained after reaction of 100 μg embryonic chicken liver microsomal protein with 0.25 μCi 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 μM 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 μM 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

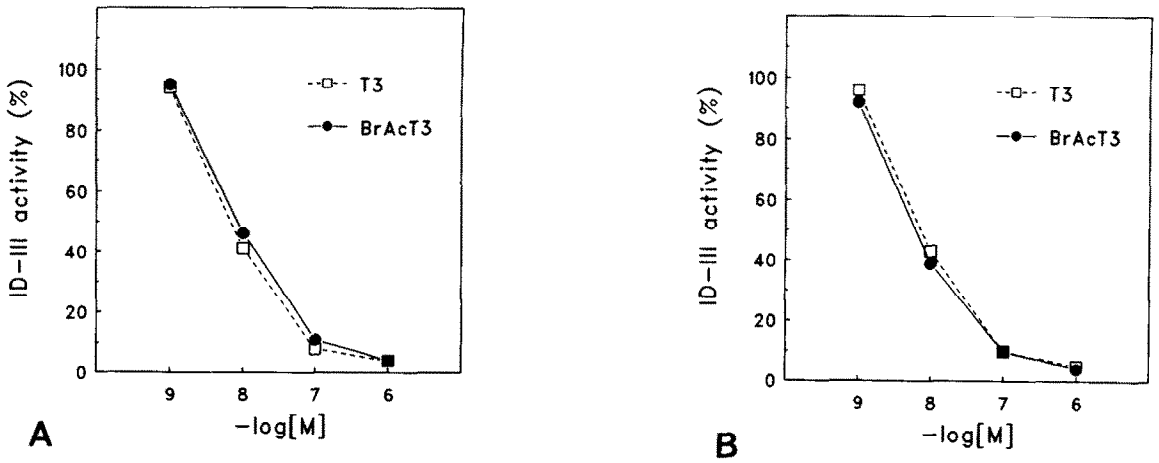

Fig. 5. Deiodination of 1 nM [^{125}I]T_3 by chicken E16 liver microsomes (A; 20 μg protein/ml) or rat placenta microsomes (B; 25 μg protein/ml) in the presence of increasing concentrations of $BrAcT_3$. Results are expressed as a percentage of that without additions.

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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