Volume 255, number 2, 385–390 FEB 07638 September 1989

Thyroid hormone effect on rat heart mitochondrial proteins and affinity labeling with *N*-bromoacetyl-3,3′,5-triiodo-L-thyronine

Lack of direct effect on the adenine nucleotide translocase

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Received 4 August 1989

N-bromoacetyl-3,3′,5-tri[3′-^{125}I]iodo-L-thyronine was used to label intact heart mitochondria from eu-, hypo- and hyperthyroid rats in order to identify proteins involved in T3-regulated mitochondrial processes. The results show strong labeling, competed for by T3 and other analogues, of two proteins with a molecular mass of 48 000 and 49 200 Da. No labeling is seen of the adenine nucleotide translocase, a likely target, neither at 0°C, at room temperature, nor after preincubation with the substrates or specific inhibitors. No difference in labeling intensity or distribution is seen in mitochondria from eu-, hypo- or hyperthyroid rats, and the abundance of the adenine nucleotide translocase is unchanged, but five other proteins show differential abundance.

Bromoacetyl-3,3′,5-tri[3′-^{125}I]iodo-L-thyronine, *N*-; Adenine nucleotide translocase; Thyroid hormone; (Rat heart mitochondria)

1. INTRODUCTION

Various effects of thyroid hormone on mitochondrial processes have been reported. In vivo 3,3′,5-triiodo-L-thyronine (T3) administration to rats induces a change in mitochondrial transcription and translation in heart, liver and kidney [1–4]. Also, change in the number and activity of respiratory components [5], increased oxygen consumption, a decrease in the ATP/ADP ratio inside the mitochondria and an increase in the cytosol are persisting effects [6]. Earlier reports suggested the adenine nucleotide translocase (ANT) as a target for direct T3 regulation of mitochondrial activity [6–8]. Since this protein has recently been well characterized from heart tissue by its activity [9], SDS-PAGE mobility [10], peptide maps and Western blotting [11], and by cDNA cloning [12], it would be of interest to further characterize a possible T3 binding site in the ANT. Experimentally induced hyper- and hypothyroidism in rats has been shown not to change the gene expression of the ANT [13]; a possible T3 effect is therefore expected to be directly on the catalytic activity of the ANT.

^{125}I-labeled *N*-bromoacetyl-T3 (BrAc[^{125}I]T3) was used for affinity labeling of intact mitochondria, since it has been used earlier to successfully label T3 receptors and T3 binding proteins in situ [14–16]. The integrity of BrAc[^{125}I]T3 was analyzed by high-performance liquid chromatography (HPLC) during the labeling of whole mitochondria. Labeling intensities and the abundance of the ANT and other proteins were compared in hypo-, eu-, and hyperthyroid rat heart mitochondria.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

2. MATERIALS AND METHODS

2.1. *Treatment of rats*

7-week-old male Lewis rats (approximately 200 g body wt) were used in all experiments. Hyper- and hypothyroidism was obtained by daily s.c. injections of 130 nmol L-thyroxine (T4)/kg body wt (Henning Berlin GmbH, Germany) and 1 μmol 6-*n*-propyl-2-thiouracil (PTU)/kg body wt (Sigma, St. Louis, MO, USA) for at least three weeks. Euthyroid rats received daily s.c. injections of 0.9% NaCl. After decapitation, the hearts were immediately used for preparation of mitochondria. The thyroid status of all animals was verified by radioimmunoassay (RIA) of serum T4 and T3 [17], liver enzyme activities and organ weights, table 1.

2.2. *Isolation of mitochondria*

The rat hearts were immediately placed in ice-cold freshly prepared isolation buffer (70 mM sucrose, 220 mM mannitol, 10 mM KMops, 0.2 mM EGTA, pH 7.2) and mitochondria were prepared as described [18], but without nagarse. Freshly prepared mitochondria in isolation buffer were diluted to 1.2 mg protein/ml and directly used for affinity labeling.

2.3. *Affinity labeling*

BrAc[^{125}I]T3 and BrAcT3 were synthesized as described [14] and purified by HPLC just before use. About 1.0 nCi of BrAc[^{125}I]T3 per μl of final assay volume (1.6 nM) was added to a polypropylene microtube. After the solvent (methanol) had been evaporated under a stream of nitrogen, the suspension of whole mitochondria was added to the tube, mixed thoroughly and incubated with occasional shaking on ice or at room temperature for the indicated time intervals. The reaction was stopped either by ethanol precipitation or by addition of sample buffer [19] to a final concentration of 20% and immediately loaded on an SDS-polyacrylamide gel. The ethanol precipitation was done by addition of two volumes of 100% ethanol, mixing, incubation on ice for at least 10 min and centrifugation at 16000 × *g* for 10 min. The pellet, which contained the covalently bound label, was counted (1260 Multigamma LKB, Bromma, Sweden) and the supernatant, which contained the unbound label, was used for HPLC analysis. In the competition experiments, either of the compuonds BrAcT3, *N*-acetyl-3,3',5-triiodo-L-thyronine (AcT3), 3,3',5'-triiodo-L-thyronine (rT3, Henning Berlin GmbH), iodoacetic acid, 3-methyl-4',6-dihydroxy-3',5'-dibromo-flavone (EMD 21388, Drs Pruecher and Irmscher, Merck, Darmstadt, FRG), ATP or ADP were added to the mitochondrial suspension to a final concentration of 5 μM and preincubated on ice for 2 h before affinity labeling as described above. Carboxyatractylate (Boehringer Mannheim, FRG) or bongkrekic acid (Dr Boos, Paderborn, FRG) were added to the mitochondrial suspension to a final concentration of 10 μM with or without 100 μM ATP, and incubation on ice was continued for 20 min before the affinity labeling.

2.4. *HPLC analysis*

Ethanol supernatants were diluted with 0.1% trifluoroacetic acid to a final concentration of 20% ethanol (v/v) and adsorbed on a μ-Bondapak C18 reversed-phase column (4.6 × 250 mm, Waters Associates, Milford, MA, USA) equipped with a 2 ml C18 guard column. Elution was done with a gradient of acetonitrile (with a constant concentration of 0.1% trifluoroacetic acid): 0–3 min 35% isocratic, 3–18 min 35–75% linear, 18–25 min 75–90% linear and 25–35 min 90% isocratic, with a flow rate of 1 ml/min. Radioactivity and UV absorption at 254 nm of the eluate were continuously monitored by the Berthold HPLC chromatography system LB 510.

2.5. *SDS-polyacrylamide gel electrophoresis and autoradiography*

SDS-PAGE and staining with Coomassie brilliant blue or silver was performed as described [19]. The dried gels were exposed to Kodak direct exposure films, DEF-2, for 24 h. By superimposing the film on the stained gel, the identity of the affinity-labeled bands was determined.

2.6. *Protein determination*

Determination of total protein was done with the Bio-Rad Protein Assay using bovine serum albumin as standard. Scann-

Table 1

Effects of T4 and PTU on serum hormone levels, heart weight, and hormone dependent enzymes in the liver

Treatment	Serum T3 (nM)	Serum T4 (nM)	Heart weight (g)	BW gain (g/day)	α-GPDH (mU/mg)	T4-5'-deiodinase (pmol T3·min^{-1}·mg^{-1}·protein)	Malic enzyme (mU/mg)
Saline	1.29 ± 0.24 (n = 7)	94 ± 48 (n = 7)	0.99 ± 0.07 (n = 3)	2.42 (n = 7)	2422 ± 8814 (n = 3)	28.3 ± 17 (n = 4)	12.2 ± 2.8 (n = 5)
L-Thyroxine	2.41 ± 0.16 (n = 5)	242 ± 100 (n = 5)	1.38 ± 0.16 (n = 3)	2.03 (n = 5)	8634 ± 4962 (n = 4)	105 ± 24 (n = 4)	74.3 ± 9.6 (n = 4)
Propylthiouracil	0.66 ± 0.21 (n = 3)	<20 (n = 3)	0.90 ± 0.0 (n = 3)	1.90 (n = 3)	357 ± 155 (n = 3)	<1 (n = 3)	6.7 ± 5.0 (n = 3)

15 male Lewis rats were treated as described in section 2 starting 7 weeks after birth. Initial body weight (BW) was 221 g (± 17, n = 15) and initial T3 and T4 serum levels were 1.36 nM (± 0.20, n = 15) and 131 nM (± 57, n = 15), respectively. The given values are from the day of decapitation. The values are calculated averages ± the range of individual measurements, and n = number of rats, α-GPDH = α-glycerol phosphate dehydrogenase

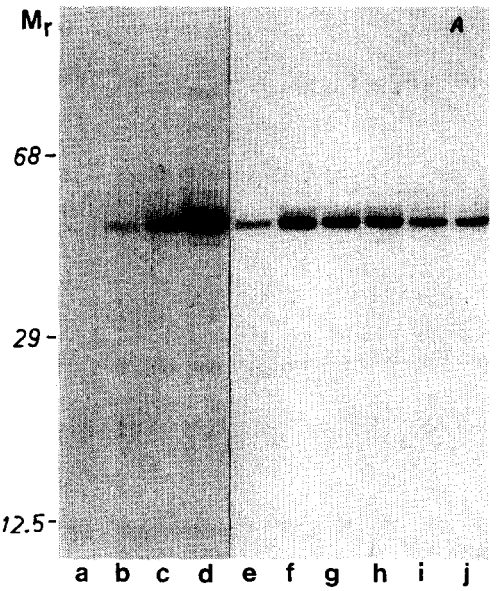
ing of the stained gels was performed by a laser densitometer (LKB, Ultroscan 2202) and relative protein quantitation by an automatic integrator (LKB, 2220 recording integrator).

3. RESULTS

3.1. *Affinity labeling of whole heart mitochondria*

No labeling of the ATN (7% of mitochondrial protein [19]) was seen when intact mitochondria were affinity labeled with BrAc[^{125}I]T3. Preincubation of the mitochondria with excess (5 μM) of either of the two specific ANT inhibitors, carboxyatractylate or bongkrekic acid [20], or with the substrates ADP or ATP, did not result in labeling of the ANT. Instead two closely migrating bands with an approximate molecular mass of 49200 and 48000 Da were strongly and equally affinity labeled, fig.1. The two labeled bands comigrated with stained bands 4 and 5 in fig.2, while the ANT was band 7. The abundance of bands 4 and 5 is about 2.8 and 3.0%, respectively of total mitochondrial protein, determined by averaging gel scanning values. Labeling intensity increases with time and temperature, fig.1A a–d, 1B a–c. Solubilization of the mitochondria with Triton X-100 abolished the binding of BrAc[^{125}I]T3 (not shown), indicating that the specific conformation of bands 4 and 5 in the intact mitochondrial membrane is essential for binding. No difference in the labeling pattern or intensity was seen in the hyper- or hypothyroid mitochondria, fig.1B, d–f. The stained bands 2 and 3 (fig.2) were weakly labeled in all the experiments.

Saturation of labeling is not reached before substantial degradation of the remaining unbound label is seen by HPLC analysis, 9% of the added BrAc[^{125}I]T3 is bound to mitochondria after 30 min incubation on ice and 85% of the remain-



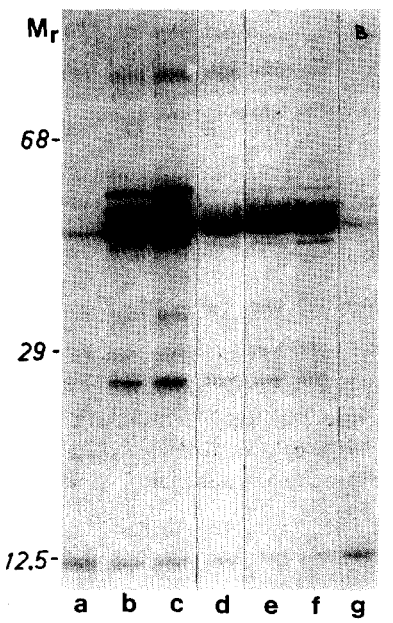


Fig.1. Autoradiography of affinity-labeled rat heart mitochondria separated by SDS-polyacrylamide gel electrophoresis. Preincubation, affinity labeling with BrAc[^{125}I]T3 (1.6 nM) of whole mitochondria (1.2 mg total protein/ml), SDS-PAGE and autoradiography were done as described in section 2. (A) Affinity labeling of normal rat mitochondria at 0°C for 5 s (a), 30 min (b), 2 h (c), and 5 h (d). Preincubation for 2 h with 5 μM of each BrAcT3 (e), AcT3 (f), T3 (g), rT3 (h), iodoacetic acid (i), and EMD 21388 (j) before affinity labeling for 5 h (as in d). (B) Affinity labeling of normal rat mitochondria at room temperature for 5 s (a), 30 min (b) and 5 h (c). Affinity labeling at 0°C for 5 h with normal rat mitochondria (d), mitochondria from T4-treated (e), and PTU-treated rats (f). Lane (g) was treated as in (d) but PAGE sample buffer was added before incubation with BrAc[^{125}I]T3. The strongly labeled band is actually two closely migrating equally labeled bands identified as bands 4 and 5 in fig.2 by superimposing the film on the stained gel. Film A and B were exposed for 24 h. $M_r \times 10^{-3}$ values are shown to the left.

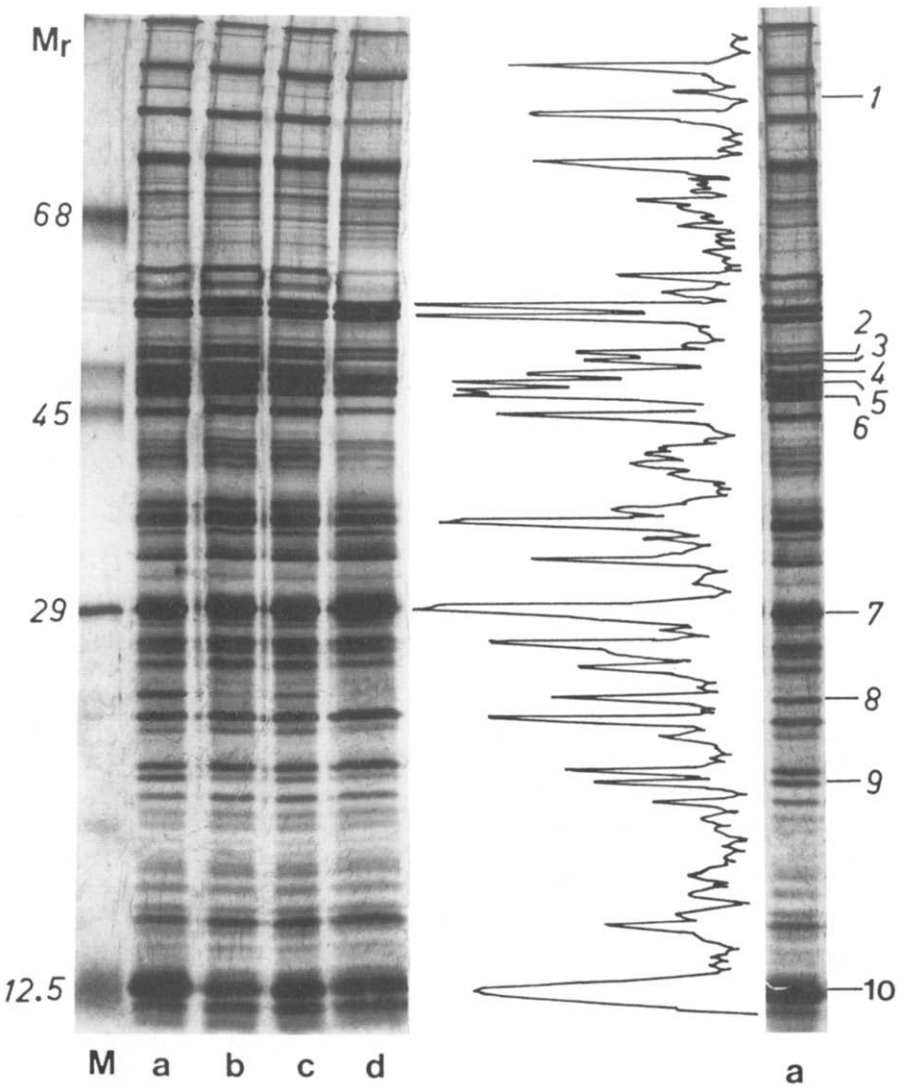


Fig.2. SDS-PAGE, silver staining and gel scanning of total rat heart mitochondria. 2.5 μg protein per lane from normal (a), T4-treated (b), PTU-treated (c) and solubilized normal (d) mitochondria. 55 ng of each (M) bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.5 kDa). A densitometric laser scan of lane (a) is shown to the right; the peak areas were automatically calculated by an integrator. The numbered bands are referred to in the text. Band 7 corresponds to the ANT. $M_r \times 10^{-3}$ values are shown to the left.

ing unbound BrAc[^{125}I]T3 is still intact. After 5 h, 30% is bound, but only 61% of the remaining unbound label is intact. After 5 h incubation at room temperature, 75% of the BrAc[^{125}I]T3 is bound and the remaining unbound label is completely degraded (fig.3).

3.2. *Competition with T3 and T3 analogues*

A 3000-fold excess of competitor was used in all experiments. Preincubation of mitochondria for 2 h with BrAcT3 showed 67% inhibition of BrAc[^{125}I]T3 binding after 5 h incubation. The same incubation conditions for the reversible binding compounds showed 35% inhibition by T3 or rT3, 30% by AcT3 and 52% by the flavonoid EMD 21388, a synthetic thyroxine antagonist and potent competitive inhibitor of the iodothyronine 5′-deiodinase enzyme [21]. Iodoacetic acid, which

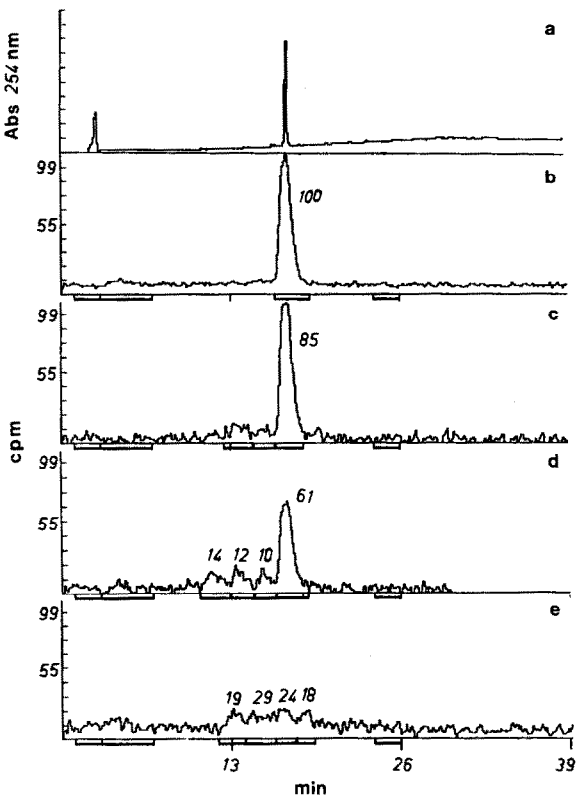


Fig.3. HPLC elution profile of purified affinity label and of supernatant after incubation with mitochondria. HPLC was performed as described in section 2. UV absorbance (a) and radioactivity profiles (b–e) are shown. Purified BrAc[^{125}I]T3 was mixed with 'cold' BrAcT3 and analyzed (a,b). Ethanol supernatants of the mitochondria after incubation with BrAc[^{125}I]T3 at 0°C for 30 min (c), 5 h (d), and after 5 h at room temperature (e) are shown. The numbers near the peaks show the percentage of total injected cpm.

irreversibly alkylates sulfhydryl groups, showed 45% inhibition of BrAc[^{125}I]T3 binding (fig.1A, d–j). Iodoacetic acid has been shown to be an active-site-directed reagent for iodothyronine 5'-deiodinase and T3-binding (or -metabolizing) proteins where a sulfhydryl group is in close proximity to the iodothyronine binding site [22].

3.3. *Effect of hyper- and hypothyroidism on heart mitochondrial proteins*

Table 1 shows the results of the treatment with T4 and PTU, an antithyroidal compound which blocks the synthesis and conversion of thyroid hormones. Experimental hyper- and hypothyroid conditions in the rats were verified by T4 and T3 serum levels, the activity of three hormone-dependent liver enzymes and the differences in heart weight. The overall pattern of the mitochondrial proteins is the same for the differently treated rats and the ANT abundance and mobility is clearly unchanged (fig.2). However, five bands show changes in relative abundance; these bands are labeled 1, 6, 8, 9 and 10. The functional identity of these proteins is unknown, thus they may be either nuclear or mitochondrial coded.

4. DISCUSSION

In vivo T3 administration to rats induces a decrease of the ATP/ADP ratio in the mitochondria and an increase in the cytosol [6], and the thyroid state has been reported to influence the kinetics of the ANT in liver [7]. We found no labeling of the rat heart ANT by BrAc[^{125}I]T3 using different experimental conditions. This indicates that the ANT is not a direct target for T3 binding, but its activity may instead be indirectly regulated, i.e. through contact with other T3-binding proteins or by T3-induced changes in the lipid matrix of the inner mitochondrial membrane [23]. Also, we found that the abundance and mobility of the ANT in the rat heart is unaffected by the thyroid state. A protein from rat liver mitochondria, which was reported to bind T3, has been purified by affinity chromatography and characterized; it has a molecular mass of 28000 Da and the amino acid composition is known [24]. The two most abundant proteins in this size range in rat heart mitochondria are the ANT and the closely migrating phosphate transport protein (PTP) [11]. Amino acid compositions are known for both the ANT and PTP protein from beef heart [19,25], but none of them are similar to the reported amino acid composition of the rat liver mitochondrial T3 binding protein.

Two closely migrating proteins with a molecular mass of 48000 and 49200 Da were strongly labeled, and labeling was competed for by T3 and other analogues. These two proteins may be directly involved in T3 regulation of mitochondrial activity. No difference in the labeling by BrAc[^{125}I]T3 was seen in hyper- or hypothyroid rats. The physiological function of the two affinity-labeled proteins described here, as well as the five proteins with changed abundancy in hypo- and hyperthyroid rat hearts still has to be resolved.

Acknowledgements: We thank Dr Hanno V.J. Kolbe for valuable suggestions and discussions during the study and Matthias Spanka for preparing some of the mitochondrial samples and help with animal treatments. This work was a cooperation research project between Bissendorf Peptide GmbH (U.B.R.) and the Medizinische Hochschule Hannover, supported by the Bundesministerium für Forschung und Technologie and Deutsche Forschungsgemeinschaft DFG He593/14-2.

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