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Specific binding sites for 3,3′-diiodo-L-thyronine (3,3′-T_2) in rat liver mitochondria

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Abstract Specific binding sites for 3,3′-T_2 can be detected in swollen and osmotically treated mitochondria (OTM) from normal and hypothyroid rat liver. In hypothyroid animals, maximal values of binding were obtained at 0°C and 20°C while values were lower at 37°C and no specific binding could be observed at 60°C. Binding was maximal at pH 6.4 and the mean values for the apparent association constant (K_a) and the binding capacity were on average 0.5×10^8 M^{-1} and 2.4 pmol/mg mitochondrial protein, respectively. No differences were observed between normal and hypothyroid rats with the exception of the capacity that was higher in normal animals (5.5 pmol/mg mitochondrial protein). The specificity of 3,3′-T_2 binding, examined in competition studies, followed this order: 3,3′-T_2 > 3,5-T_2 > rT_3. The other iodothyronines (3′,5′-T_2, T_3, T_4, 3-T_1, 3′-T_1, 3,5-Diac and 3,3′-Diac) showed only a small competition or none at all.

Key words: Diiodothyronine; Thyroid hormone; Mitochondria; Receptor; Cytochrome *c* oxidase

1. Introduction

Thyroid hormones are involved in the regulation of growth, development and cell differentiation in many organisms and, in addition, they have notable effects on respiration and energy metabolism. In this context the question of a direct action of the hormones on mitochondria is so far unresolved. In fact, in spite of several reports showing rapid effects of triiodothyronine (T_3) on mitochondria, the occurrence and the physiological significance of these effects is a long standing controversial issue of thyroid hormone action. In recent years, however, several reports have shown that two diiodothyronines (3,3′-T_2 and 3,5-T_2), under certain conditions, are the only iodothyronines which rapidly stimulate in vivo and in vitro mitochondrial respiration or cytochrome oxidase (COX) activity [1–6]. At the moment, the physiological significance of these effects is not clear and no insight into the mechanism by which diiodothyronines stimulate mitochondrial energy metabolism is available. As diiodothyronines have a rapid effect in vitro on rat liver mitochondrial COX activity these results should be the consequence of a direct interaction of diiodothyronines with specific mitochondrial binding sites. In this study, because we observed that the effect of 3,3′-T_2 could not be shown when added to intact mitochondria but was evident when added to isolated swollen and osmotically treated mitochondria (OTM), we have tried to show the presence of specific binding sites for 3,3′-T_2 in OTM from the liver of normal and hypothyroid rats.

Hypothyroidism was induced by propylthiouracil (PTU) administration for two reasons: (a) PTU is an inhibitor of some deiodinating enzymes [7] and this allows us to inhibit a possible pathway of T_2 metabolism; (b) after three weeks of PTU administration the presence of endogenous iodothyronines is strongly reduced [1] and the endogenous occupation of the binding sites should consequently be lowered.

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Abbreviations: T_4, thyroxine; T_3, 3,3′,5-triiodo-L-thyronine; rT_3 or revers T_3, 3,3′,5′-triiodo-L-thyronine; 3,3′-T_2, 3,3′-diiodo-L-thyronine; 3,5-T_2, 3,5-diiodo-L-thyronine; 3′,5′-T_2, 3′5′-diiodo-L-thyronine; 3-T_1, 3-monoiodo-L-thyronine; 3′-T_1, 3′-monoiodo-L-thyronine; 3,5-Diac, 3,5-diiodothyroacetic acid; 3,3′-Diac, 3,3′-diiodothyroacetic acid.

2. Materials and methods

2.1. Materials

3,[3′-^{125}I]T_2 (3000 μCi/μg); 3,5-T_2; 3,3′-T_2; 3′,5′-T_2; T_4; T_3; rT_3; 3,5-Diac; 3,3′-Diac; 3′-T_1; 3-T_1 were from MMDRI, Henning Berlin R&D (Berlin, Germany) and > 99.5% pure.

2.2. Animals

Male Wistar rats weighing approximately 300 g were used throughout the study. In one group of animals hypothyroidism was induced by the addition of 0.1% (w/v) PTU to the drinking water for 3 weeks.

At the end of treatment, both normal and hypothyroid rats were sacrificed by decapitation. Liver was quickly removed and placed in the appropriate medium for mitochondria separation.

2.3. Preparation of mitochondrial fraction

Liver mitochondria were isolated at 3000 × *g* according to the procedure described in [8]. The mitochondrial pellet was further fractionated using an osmotic shock according to a slightly adapted method previously described [9]. Briefly, 3 mg of mitochondrial protein were swollen for 30 min at 37°C in 1 ml of 0.1 M potassium phosphate buffer (pH 7.4) to disrupt and partially eliminate the outer membrane and also some cytoplasmic contaminants [10,11]. After collection, swollen mitochondria were treated by 10 min incubation in a hypotonic phosphate buffer (8 mM; pH 7.4). Osmotically treated mitochondria (OTM) were spun down by centrifugation and resuspended in isosmotic buffer to give a final protein concentration of 10 mg/ml, determined by the method of Hartree [12] using bovine serum albumine (BSA) as a standard.

Published procedures were used to measure the activities of uricase [13], glucose-6-phosphatase [14], acid-phosphatase [15] and succinic dehydrogenase [16].

2.4. Cytochrome oxidase activity

Aliquots of OTM (2.5 mg/ml) were incubated at 0°C with 3,3′-T_2 in a final concentration of 10^{-6} M. After the addition of 3,3′-T_2, the mixture was blended in a vortex and incubated for 30 min before the cytochrome oxidase activity assay.

Cytochrome oxidase activity in the OTM was determined polarographically at 25°C using a Clark oxygen electrode according to the method of Grav et al. as modified by Aulie and Grav [17,18]. Briefly: in 1,5 ml of reaction medium containing 30 μM cytochrome *c*, 4 μM

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rotenone, 0.5 mM dinitrophenol, 10 mM Na malonate, 75 mM HEPES buffer, pH 7.4. Cytochrome oxidase activity was measured as the difference between the rate of oxygen consumption observed after the addition of substrate (4 mM sodium ascorbate with 0.3 mM *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine (TMPD)) and mitochondria (50 μg of protein), and the rate of oxygen consumption observed after the addition of substrate alone, in order to take into account the auto-oxidation of ascorbate.

2.5. Incubation procedure for binding studies

Incubations with 3,[3'-^{125}I]T_2 (T_2^*) (3000 μCi/μg 3,3'-T_2, 0.05 μg/ml) were carried out for 30 min at 0°C in 1 ml of buffer using 10–100 nmol/l T_2^* and OTM (0.7–1.4 mg of protein). T_2^* bound to OTM was separated from free T_2^* by centrifugation. Residual unbound or loosely bound -T_2^* was removed from mitochondria by three washes in the presence of 0.5% bovine serum albumine, as previously described [9]. Non-specific binding was determined in parallel incubations with a 1000-fold excess of unlabelled 3,3'-T_2. K_a and capacity were calculated according to Scatchard [19]. The specificity of 3,3'-T_2 binding to mitochondria was examined in competition studies using one low concentration of 3,[3'-^{125}I]T_2 and increasing concentrations of cold analogues. Radioactivity was measured in a Beckman 4000 spectrometer with 70% of efficiency.

Values are expressed as mean ± S.E.M. Statistically significant differences were examined using Student's *t*-test.

3. Results

3.1. Enzymatic profile of the swollen mitochondrial preparation

Due to the low gravitational force used to obtain mitochondria, the mitochondrial fractions utilized in our experiments were almost pure. In fact, both in intact mitochondria and in OTM the values were below the detection limit for uricase and glucose-6-phosphatase, while acid phosphatase activity was 0.65 μg P_i/mg protein·min and 0.4 μg P_i/mg protein·min for intact mitochondria and OTM, respectively. (These activities represent 5% and 3% of the mean values in a lysosomal fraction [8]. Succinic dehydrogenase activity, on the other hand, was 0.088 μmol/min·mg protein in the homogenate, 0.28 μmol/min·mg protein in intact mitochondria and 0.32 μmol/min·mg protein in OTM.

3.2. Effect of 3,3'-T_2 on OTM cytochrome oxidase activity from normal and hypothyroid rats

The effect of 3,3'-T_2 on intact mitochondria and OTM cytochrome oxidase activity is shown in Table 1. 3,3'-T_2 (10^{-6} M) had no effect when added to intact mitochondria while on OTM caused a marked stimulation of cytochrome oxidase activity (+60% and 95% in normal and hypothyroid mitochondria, respectively).

Table 1
In vitro effect of 3,3'-T_2 on cytochrome oxidase activity of liver intact mitochondria and OTM from normal (N) and hypothyroid rats (PTU)

		Mitochondrial COX activity (natoms O/min·mg protein)	
		Controls	+ 3,3'-T_2 (10^{-6} M)
N	OTM	650 ± 63	1040 ± 96* (+60%)
	Intact	415 ± 22	435 ± 28
PTU	OTM	541 ± 52	1054 ± 100* (+95%)
	Intact	312 ± 7	300 ± 10

Data represent the mean ± S.E.M. of six experiments. *$P < 0.01$ vs. controls.

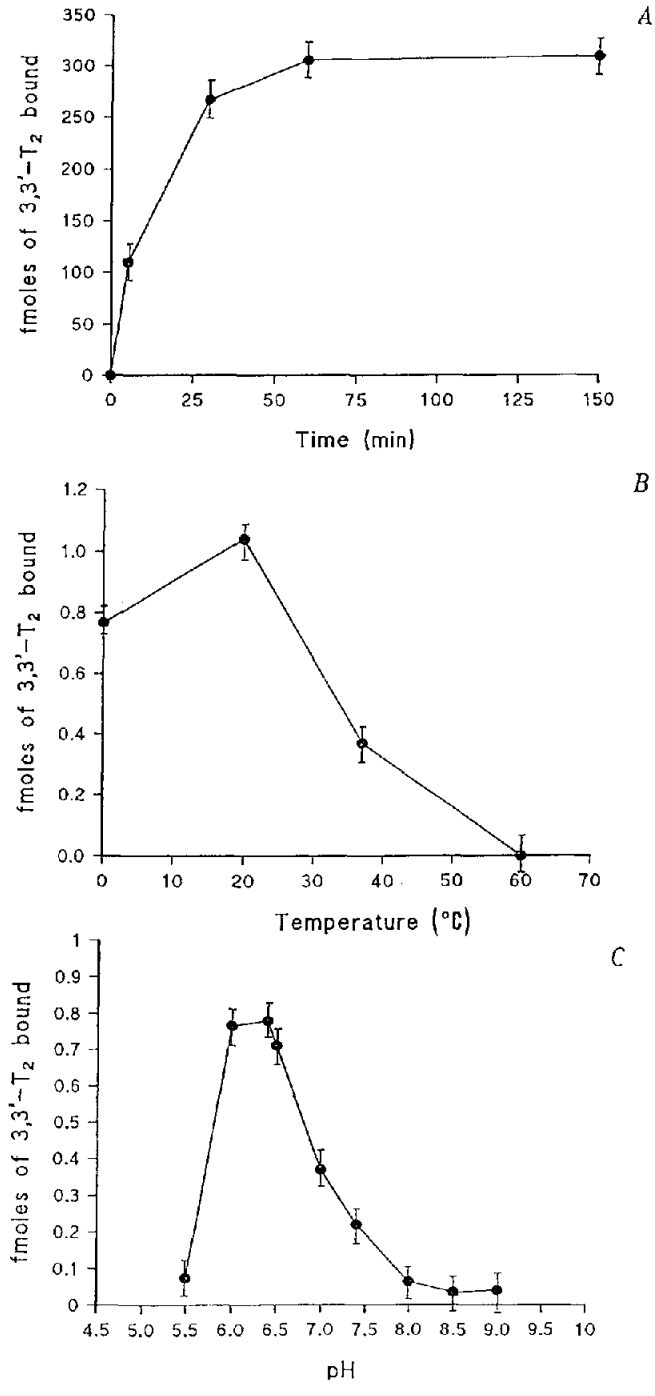

Fig. 1. Influence of time (A), temperature (B) and pH (C) on specific 3,3'-T_2 binding. Incubation with 0.85×10^{-8} M 3,[3'-^{125}I]T_2 was carried out for 30 min at 0°C in 1 ml of homogenate at various times, temperatures and pH values. Non-specific binding was determined in parallel with an excess of 3,3'-T_2 as described in section 2. Assays were performed in mitochondria after remotion of residual unbound or loosely bound 3,3'-T_2 by three washes in the presence of 0.5% BSA. Data are the mean ± S.E.M. of four experiments.

3.3. 3,3'-T_2 binding to mitochondria

After incubation, liver OTM bound a significant proportion of 3,[3'-^{125}I]T_2 added to the incubation medium (6% on average). The addition of 1000-fold unlabelled 3,3'-T_2 caused an average displacement of 70% of 3,[3'-^{125}I]T_2 bound.

3.4. Characteristics of 3,3'-T_2 binding to mitochondria

In hypothyroid rats specific 3,3'-T_2 binding to mitochondria occurred rapidly; after 5 min, 30% of binding was reached and a maximum was achieved after 60 min (Fig. 1A). The reversibility of 3,[3'-^{125}I]T_2 binding by isotopic dilution with unlabelled 3,3'-T_2 at equilibrium was of the order of 70%. Specific binding was destroyed at 60°C (Fig. 1B) while non-specific binding increased. Higher values of the specific binding were obtained after 60 min at 0°C and at 20°C, while at 37°C the values were significantly lower (Fig. 1B). Because of this and as the variability in binding studies is generally lower at 0°C [20] all further analyses were carried out at 0°C. Specific 3,3'-T_2 binding was maximal at pH 6.4 (Fig. 1C). No differences were observed in the characteristics of the binding between hypothyroid and normal mitochondria.

After having determined the optimal condition for specific 3,3'-T_2 binding all subsequent analyses were performed at 0°C and pH 6.4.

3.5. Saturation analyses

A series of saturation analyses were performed on OTM at pH 6.4 and at 0°C (Fig. 2A). In OTM from PTU treated rats

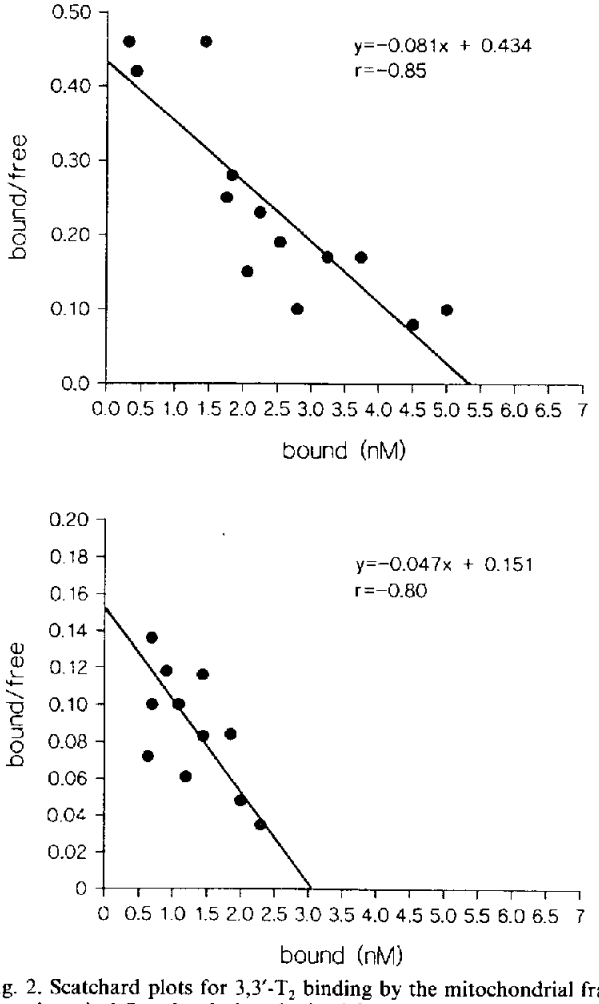

Fig. 2. Scatchard plots for 3,3'-T_2 binding by the mitochondrial fraction. A typical Scatchard plot obtained from normal (top panel) and hypothyroid (bottom panel) rat liver OTMs is shown. 3,[3'-^{125}I]T_2 concentrations ranged from 10^{-8} to 10^{-7} M in both cases. The mitochondrial protein concentration was 1.12 mg/ml and 0.8 mg/ml in hypothyroid and normal samples, respectively.

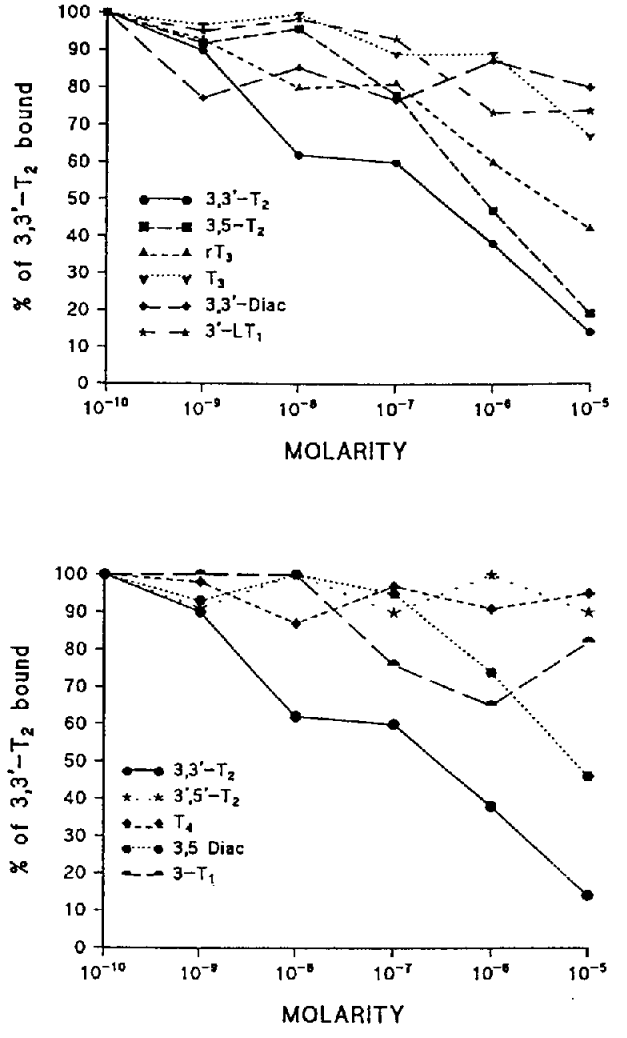

Fig. 3. Inhibition of specific 3,[3'-^{125}I]T_2 binding by increasing concentrations of various analogues added to the incubation medium. Incubations were performed in triplicate. Data are the mean ± S.E.M. of three different experiments. For a better representation of the results S.E.M.s have been omitted.

Scatchard plot of values gave a linear negative slope in a concentration range between 10^{-8} and 10^{-7} M. The mean values for the apparent association constant (K_a) and the binding capacity were 0.5×10^8 M^{-1} and 2.4 pmol/mg mitochondrial protein, respectively. The same experiment performed on OTM from normal animals gave slightly different results. In fact while K_a was 0.6×10^8 M^{-1} and did not differ from that measured in PTU animals, the binding capacity was higher (5.5 pmol/mg mitochondrial protein compared to 2.4 pmol/mg mitochondrial protein in hypothyroid animals).

3.6. Specificity of 3,3'-T_2 binding

The specificity of 3,3'-T_2 binding to OTM was examined in competition studies using one low concentration of 3,[3'-^{125}I]T_2 and increasing concentration of 3,3'-T_2 analogues (Fig. 3). The specificity for the tested iodothyronines followed this order: 3,3'-T_2 > 3,5-T_2 > rT_3. The other compounds (T_3, T_4, 3',5'-T_2, 3-T_1, 3'-T_1, 3,3'-Diac and 3,5-Diac) showed only a small competition or none at all.

4. Discussion

Several reports indicate that thyroid hormones have 'long-term' and 'short-term' effects on energy metabolism. These effects can occur at various levels. Triiodothyronine, the generally accepted active form of thyroid hormones, may alter the concentration of individual enzymes or may influence, directly or indirectly, their kinetic properties (for review see Brand and Murphy [21]). Recently, as mentioned before, several reports dealing with the action of iodothyronines at the cellular level, have shown that two diiodothyronines (3,3′-T_2 and 3,5-T_2) are the thyroid hormone analogues which are most effective at the cellular level [1–6]. In previous studies we hypothesized that T_3 could be responsible for the nuclear and long-term actions of thyroid hormones and diiodothyronines for the direct mitochondrial short-term actions [1,2]. This hypothesis presupposes the presence of specific binding sites for diiodothyronines in mitochondria. In accordance to the hypothesis, in the present study we are able to show the presence of specific binding sites for 3,3′-T_2 in rat liver mitochondria.

Both in normal and hypothyroid rats, specific binding to OTM occurred fairly rapidly and the reversibility of 3,[3′-^{125}I]T_2 binding by isotopic dilution with unlabelled 3,3′-T_2 at equilibrium was good and in the order of 70%. The binding of 3,3′-T_2 to OTM decreased at temperature higher than 20°C and was completely abolished at 60°C. In hypothyroid and normal animals, the apparent association constant (K_a) was 0.5×10^8 M^{-1} and 0.6×10^8 M^{-1}, respectively, while the capacity was 2.4 pmol/mg protein and 5.5 pmol/mg protein, respectively.

The mitochondrial origin of the described sites is highly probable regarding the high degree of mitochondrial purification (see enzymatic profile) and the characteristics which differentiate 3,3′-T_2 binding sites from contaminations by other sites able to bind other iodothyronines (nuclear and plasmic membrane binding sites): (1) from the nuclear sites which exhibit a different specificity toward analogues [22] and a different affinity [23]; (2) from plasma membrane binding sites which exhibit the highest affinity for T_4 [24].

Our data are not in agreement with current concepts with regard to biological activity of iodothyronines. In fact, previous reports said that diiodothyronines have no physiological potency in vivo [25,26]. However, in these reports parameters, such as growth promotion and antigoitrogenic activity, classically useful to evaluate T_4 or T_3 potency were considered. In effect, diiodothyronines either could influence other parameters or could not easily enter into the cells when injected in vivo. In addition, it has been recently demonstrated that diiodothyronines, when administered in vivo, stimulate liver cell metabolism [1,2,5].

The other noteworthy point of the present study is that, 3,3′-T_2 directly stimulates COX activity in isolated liver OTM. This rapid effect coupled with the presence of specific binding sites for 3,3′-T_2 induces us to suggest a direct interaction of this compound with COX complex. This hypothesis is supported by our recent data demonstrating that diiodothyronines are able to stimulate the activity and change the visible spectrum of the COX complex isolated from bovine heart mitochondria [27].

In particular, we suggest that 3,3′-T_2 alters the kinetic properties of the COX complex by interaction with non-catalytic subunits of COX. Interactions with non catalytic subunits of COX (VIa-H) have been recently described for adenine nucleotides [28,29].

In conclusion, the results reported here support our previous hypothesis of a direct interaction of 3,3′-T_2 with mitochondria and induce us to suggest that short-term effects of thyroid hormones on mitochondrial respiration could be at least partly due to the direct interaction of diiodothyronines with mitochondria.

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