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HIGH AFFINITY THYROXINE BINDING TO PURIFIED RAT LIVER PLASMA MEMBRANES

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SUMMARY. Two sets of high-affinity thyroxine binding sites (K_D 0.39 ± 0.06 nM and 23 ± 5 nM) were detected on purified rat liver plasma membranes. Thyroxine is bound with high stereospecificity regarding iodine substituents and alanine side chain modifications of the molecule. Thyroxine binding is inhibited by -SH blocking agents and proteases. The highest affinity thyroxine binding site is also affected by phospholipase A and is distinct from triiodothyronine binding sites present in the membrane preparations; arguments are given for its plasmalemma origin.

Thyroid hormones, L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3) are transported to the tissues mainly bound to serum binding proteins. It is admitted that only their small free fraction is involved in cellular penetration, metabolism and action. The mechanism of action of thyroid hormones remains essentially unknown (1). Several cellular T_3 binding components have been described. T_3 binds to high-affinity nuclear sites which could represent a receptor mediating T_3 effects at a transcriptional level (2-4). T_3 also binds to a set of high-affinity sites in the inner mitochondrial membrane (5), to cytosol proteins with a lower affinity (6, 7) and to plasma membranes, as recently described (8, 9). Thyroxine, which generates a large part of peripheral T_3 through intracellular deiodination (reviewed in 10), is less strongly bound to nuclear sites and sometimes considered as a prohormone. Intracellular distribution of T_4 is less well-documented. This report demonstrates that purified rat liver plasma membranes contain two sets of specific high-affinity T_4 binding sites and argues in favor of the plasmalemma origin of at least the highest affinity site.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-250 g) were fasted overnight before being killed by decapitation. Plasma membranes were purified from the 1000 x g pellet of liver homogenate in hypotonic medium according to Ray (11) and stored in liquid nitrogen. The "plasma membrane" fraction collected at d 1.16/1.18 sucrose interface contained 1.3 ± 0.1 % (n = 5) of liver proteins estimated according to Lowry et al (12) using bovine albumin as reference. This fraction was enriched in glucagon-stimulated adenylate cyclase activity which was determined according to Orgiazzi et al. (13) using the cAMP radioimmunoassay of Cailla et

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al. (14) (in pmol cAMP/min/mg protein : 36.7 ± 5.3 as compared to 12.5 ± 1.7 for the homogenate in the basal state and 348.5 ± 67.7 versus the homogenate value of 28.0 ± 3.5 in the presence of 10 μM glucagon, in 5 preparations). Specific activities of cytochrome oxidase (15) and NADPH cytochrome c reductase (16), relative to values in homogenate, were 0.16 ± 0.06 (n = 3) and 0.36 ± 0.03 (n = 3) respectively. Electron micrographs revealed numerous empty vesicles and large sheets with junctional complexes and confirmed the scarcity of contamination with mitochondrial or rough endoplasmic elements. In some control experiments, plasma membranes were prepared from the 1000 x g pellet of liver homogenate in isotonic medium according to Aronson and Touster (17). Rough and smooth microsomal fractions were obtained from the post-mitochondrial supernatant as described by Dallner (18).

[^{125}I]T_4 (T_4^*; ∿ 4700 mCi/mg) and [^{125}I]T_3 (T_3^*; ∿ 2800 mCi/mg) were prepared by iodination of 3,5-diiodo-L-thyronine (0.5 nmol) with 1 mCi [^{125}I]NaI (Amersham, England) in the presence of 10 nmol chloramine T, and purified by filtration on Sephadex G-25 with 20 mM NaOH (19). T_3^* and T_4^* were neutralized and supplemented with propylene glycol (50 % v/v) immediately after collection. T_4^* and T_3^* were found to be pure when analyzed by thin-layer chromatography on cellulose plates in chloroform-tert.butanol - 2N ammonia (60 : 376 : 70) and in tert.amylol- 2N ammonia (v/v, upper phase), and stable for at least 3 weeks at 0°C.

Membranes (50 μg proteins) were incubated in 0.1 ml of 20 mM Tris-Cl-2 mM EDTA-1 mM $MgCl_2$-10 mM NaCl-0.2 M sucrose-1 mM dithiothreitol (DTT) pH 7.6, with T_4^* (0.1 nM) (or T_3^*) for 2 hr at 0°C. After incubation and addition of 1 ml incubation buffer, bound T_4^* was immediately separated by centrifugation (5 min, 10 000 x g, 2°C) and counted in a Packard autogamma spectrometer. Non-specific binding was determined in parallel incubations with 13 μM T_4 and subtracted from total binding to calculate specific binding. In control experiments using microsomal fractions, membrane pellets were spun down at 100 000 x g for 5 min in a Beckman Airfuge. Assays were performed in duplicate or triplicate. Values are expressed as mean ± SEM.

T_4, T_3 and analogs were from Sigma (St Louis, Mo) except for 3,3',5'-triiodo-L-thyronine (rT_3) and 3,3'-diiodo-L-thyronine (3,3'-T_2) which were from Henning (Berlin, W.G.) and 3,5,3',5'-tetraiodothyroformic acid which was from K and K (ICN Pharmaceuticals, Plainview, N.Y.)

RESULTS

Specific T_4 binding to purified rat liver plasma membranes occurs rapidly. Fig. 1 shows that at 0°C equilibrium was attained in 2 hr and remained stable for more than 6 hr. Similar specific T_4 binding values were obtained at 20°C with stability ranging from 1 to 3 hr of incubation. At 37°C, maximum binding was lower with non-specific binding rapidly increasing and specific binding decreasing after 30 min. Fig. 1 also shows that T_4 binding was rapidly reversed after addition of excess unlabelled T_4, the dissociation curve suggesting involvement of more than one binding component. After incubation with T_4^*, 95 % of bound radioactivity was extracted with ethanol and only T_4^* was detected by thin-layer chromatography.

Optimal conditions for specific T_4 binding require the presence of EDTA and reducing agents, as shown in Fig. 2. Uncomplexed Mg^{++} or Ca^{++} enhanced non-specific binding and decreased total binding. NaCl or KCl up to 100 mM were without effect. Specific T_4 binding was markedly inhibited by Na p-hydroxymercuribenzoate (PHMB) and N-ethylmaleimide (NEM) (Fig. 2), implying that such

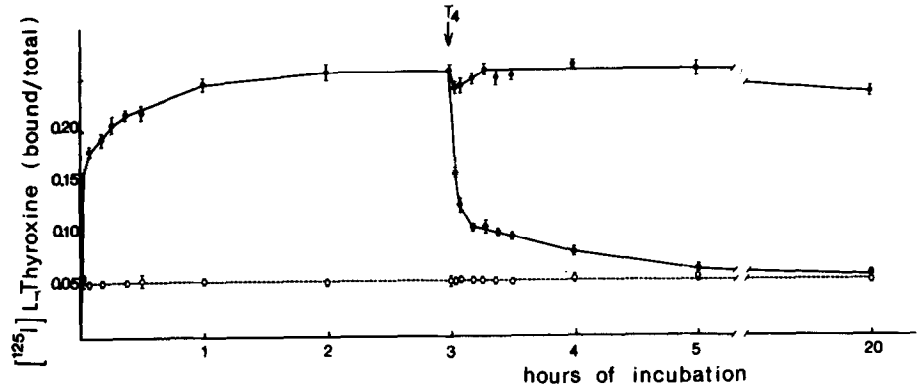

Figure 1. Time course of T_4 binding to purified rat liver plasma membranes at 0°C. Aliquots (0.1 ml) of the incubation medium were centrifuged at the indicated time points. Total binding (●——●) determined with 0.1 nM T_4^* and non-specific binding (o---o) with T_4^* + 13 µM T4. At 3 hr (arrow), incubation medium was divided into 2 parts to which was added 13 µM unlabelled T_4 (◎——◎) or the solvent alone (●——●). Each value is the mean of duplicate determinations.

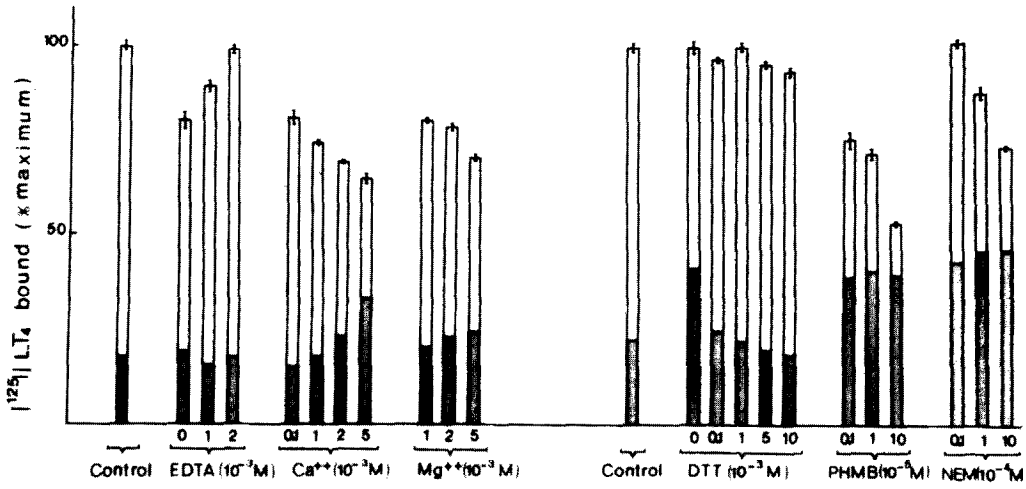

Figure 2. Influence of divalent cations and reducing agents on T_4 binding to purified plasma membranes. Total (empty bars) and non-specific (grey bars) T_4^* binding were determined under standard conditions for controls; left panel : Na_2EDTA, $CaCl_2$ or $MgCl_2$ were added to EDTA- and Mg^{++}-free incubation media and at the indicated final concentrations; right panel : DTT, PHMB or NEM were added to DTT-free incubation media and at the indicated final concentrations. Incubations, in duplicate, were for 2 hr at 0°C.

binding depends on the integrity of one or several - SH groups.

Specific T_4 binding rose linearly with increasing amounts of membrane proteins up to 500 µg/ml at 0.1 nM T_4 (Fig. 3). In saturation analyses, increasing concentrations of unlabelled T_4 progressively decreased T_4^* binding. When plotted according to Scatchard (20) (Fig. 4), data values were compatible with the existence of two sets of high affinity T_4 binding sites : K_1 with an apparent dissociation constant (K_D) of 0.39 ± 0.06 nM (n = 7) and a maximum

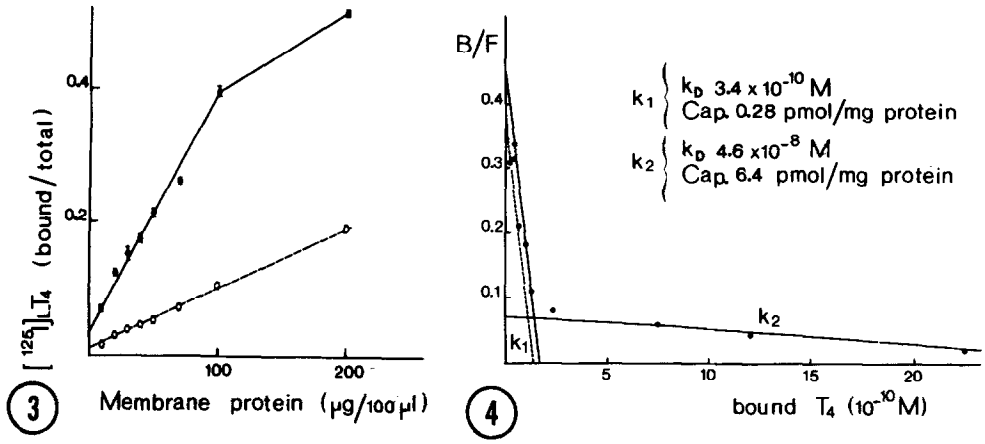

Figure 3. Effect of increasing membrane protein concentration on T_4^* binding. Total (●———●) and non-specific (o----o) binding were determined in triplicate incubations under standard conditions.

Figure 4. Scatchard plot of T_4 binding to purified rat liver plasma membranes incubated under standard conditions with T_4^* and increasing concentrations of T_4. Values of specifically bound T_4 are used. The high-affinity site, K_1 (----), was obtained by subtracting the contribution of the lower affinity site (K_2) from the curve.

binding capacity (MBC) of 0.32 ± 0.03 pmol T_4/mg membrane protein, and K_2 with a K_D of 23 ± 5 nM and MBC of 5.5 ± 0.5 pmol T_4/mg protein. Similar values were found in two plasma membrane preparations from liver homogenate in isotonic medium (K_D : 0.68 and 0.24 nM for K_1; 10 and 23 nM for K_2). The highest affinity site (K_1) could not be detected in rough and smooth microsomal fractions incubated under the same conditions. In 2 experiments with smooth microsomes, known to represent the major possible contaminant in plasma membrane preparations, K_D for T_4 binding were 26 ± 7 nM and 455 ± 5 nM with MBC of 6.2 and 54 pmol T_4/mg protein respectively.

Fig. 5 illustrates the high stereospecificity of T_4 binding to purified plasma membranes. When compared to L-T_4, about 200, 400 and 600 x fold higher concentrations of L-T_3, D-T_4 and r-T_3 respectively were needed to obtain a 50 % depression of T_4^* binding. Modifications of the alanine side chain (tetraiodothyroacetic acid, tetraiodothyroformic acid) lowered considerably the binding potency. Thyronine and diiodotyrosine were inactive. Binding studies with $[^{125}I]T_3$ were performed under the same standard conditions. Although specific T_3^* binding never exceeded 25 % of total T_3^* binding, high affinity T_3 binding sites were detected and displayed characteristics similar to those described by Pliam and Goldfine (9) : a first set with a K_D of 6.0 ± 1.0 nM (n = 3) and MBC of 0.65 ± 0.2 pmol T_3/mg protein and a second one with a K_D of 300 ± 10 nM and MBC of 35 ± 1 pmol T_3/mg protein. T_3^* binding was less inhibited by T_4 than by T_3 at 1.3 µM concentration.

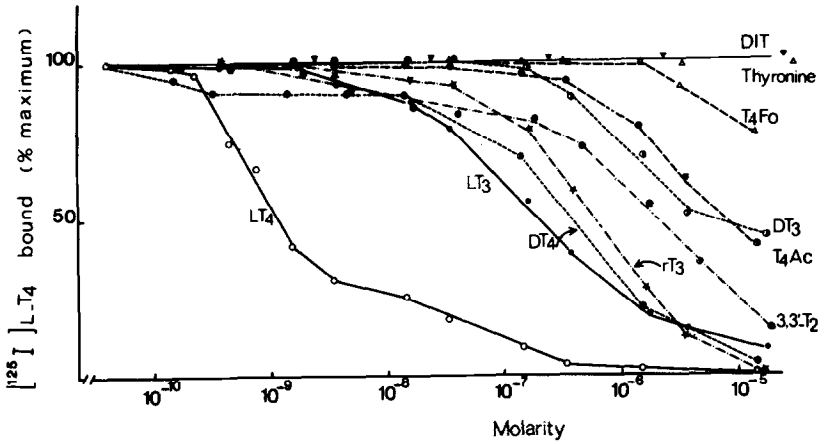

Figure 5. Inhibition of specific [^{125}I]T_4 binding by increasing concentrations of various analogs added to incubation medium : L-T4 (○——○); L-T3 (●——●); D-T4 (◐·····◐); D-T3 (◑·····◑); 3,3',5'-triiodo-L-thyronine (rT3) (✱—·—✱), 3,3'-diiodo-L-thyronine (3,3'-T_2) (⊙—·—⊙); tetraiodothyroacetic acid (T4AC) (⊕---⊕), tetraiodothyroformic acid (T4Fo) (△---△), diiodo-L-tyrosine (DIT) (▼——▼) and L-thyronine (△——△). Incubations, in duplicate, were performed under standard conditions.

Specific T_4 binding to purified plasma membranes was inhibited by 1,8-anilinonaphtalene sulfonate (18 % inhibition at 1 mM, 66 % at 5 mM) which is known to interact with T_4 binding to human thyroxine-binding globulin and prealbumin (21). Other inhibitors of T_4 binding to serum proteins (acetylsalicylic acid up to 5 mM, diphenylhydantoin up to 0.1 mM) were without effect. Propylthiouracile, an inhibitor of T_4 conversion to T_3 (10) did not interfere with T_4^* binding up to 0.1 mM and was slightly inhibitory at 1 mM (19 % inhibition). Enzymatic digestion studies indicate that specific T_4 binding probably involves protein components of the plasma membrane preparation and perhaps phospholipids (Table I). In 3 experiments with increasing concentrations of phospholipase A, inhibition of T_4 binding plateaued at 35-45 % of the initial level. Scatchard analysis of T_4 binding after phospholipase A digestion showed almost complete disappearance of the first set of T_4 binding sites (K_1) with maintenance of the second set, whereas both sites remained detectable in control membranes incubated without enzyme. Neuraminidase was also slightly inhibitory (although assayed at less than optimal pH).

DISCUSSION

Two sets of specific high-affinity thyroxine binding sites were detected in purified rat liver plasma membrane preparations, with apparent K_D of about 0.4 and 23 nM respectively. Furthermore, T_4 was bound with high stereospecificity regarding iodine substituents on the thyronine molecule and alanine side-chain modifications. These characteristics make unlikely a cross-contamination with T_4 binding components from rat serum which is devoid of the high affinity

Table I. Effect of enzymatic digestion on specific [^{125}I]T4 binding to purified rat liver plasma membranes.

Membrane treatment		[^{125}I]T_4 specifically bound % of control
None		100
Trypsin	50 µg/ml	18.3 ± 2.2
Pronase	50 µg/ml	15.0 ± 1.6
Phospholipase A	10 µg/ml	55.8 ± 3.3
	50 µg/ml	47.2 ± 2.7
	100 µg/ml	44.6 ± 1.9
Phospholipase C	50 µg/ml	85.0 ± 0.6
Neuraminidase	1mU/ml	76.5 ± 0.8
	2mU/ml	60.7 ± 1.2

Purified plasma membranes (500 µg/ml) were first incubated for 1 hr at 23-24°C in the presence of enzymes in the standard incubation medium pH 7.6 without EDTA and with added 1 mM $CaCl_2$, and then for 2 hr at 0°C after addition of 2 mM EDTA and 0.1 nM T_4^* or T_4^* + 13 µM T4 for determination of total and non-specific binding of T_4^*. Control membranes were similarly incubated but without enzyme. 1 mM DTT was present throughout the entire incubation except for the first incubation step with phospholipase C. Trypsin and pronase were from Sigma; purified Naja Nigricollis phospholipase A was the kind gift of Dr Kopeyan; Bacillus coeruleus phospholipase C was from Boehringer (Mannheim, W. Germany) and Vibrio cholerae neuraminidase from Behring-Hoechst. Assays were done in duplicate.

thyroxine-binding globulin (22), or from liver cytosol which only contains low-affinity T3 and T_4 binding components (7, and personal unpublished results obtained with T_4^*). The high-affinity T_4 binding sites displayed similar characteristics in plasma membrane fractions prepared under isotonic conditions where less nuclear disruption and less contamination with nuclear membranes are presumed. The highest affinity set (K_1 : $K_D \sim 0.4$ nM) was not found in fractions enriched in smooth microsomes, suggesting that at least K_1 represents a true plasmalemma constituent. Specific T_3 binding was also found as described by Pliam and Goldfine (9) in such plasma membrane preparations and was less influenced by T_4 than by T_3. The highest affinity T_4 binding site is likely to be different from T3 binding sites.

The physiological relevance of such specific high affinity T_4 and T_3 binding in plasma membranes is currently unknown. These sites could be involved in transport of the hormones themselves since T_3 transport has been shown to be a probably active phenomenon through plasma membrane proteins (23) and since in the intact rabbit adipocyte, T_4 and T_3 showed rapid uptake and binding to a small number of sites with similar high affinity for T_4 and T_3 (24). Such sites could play a role in the transport of small molecules. This has been suggested

for T_3 in amino acid transport by thymocytes (25) or sugar transport by embryonic cardiac cells (26), both of which are less influenced by T_4 than by T_3. Action on some membrane enzyme(s) or modulation of the membrane responsiveness to other hormonal influences are possible events but present studies on the adenylate cyclase system are contradictory. T_4-5'-deiodinase may be a plasmalemma enzyme (27) but its Km is about 3 μM thus rendering doubtful its relationship to the high-affinity T_4 binding sites. It has also been suggested that physiological concentrations of T_3 could alter both the cooperative behaviour of some membrane-bound enzymes and membrane fluidity (28); T_4 has been shown to present a stereospecific blocking action on such effects of T_3 (29).

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