Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 244-251

REVERSIBLE DISSOCIATION OF TRIIODOTHYRONINE-NUCLEAR RECEPTOR COMPLEXES BY MERCURIAL AND CHAOTROPIC REAGENTS

M.M. MENEZES FERREIRA, J. BISMUTH and J. TORRESANI

Laboratoire de Biochimie Médicale and Groupe U 38, INSERM, Faculté de Médecine 13385 Marseille Cédex 5, France.

Received January 6, 1982

SUMMARY. Triiodothyronine-nuclear receptor complexes from partially purified preparations of the receptor may be completely dissociated by 0.1 mM parahydroxymercuribenzoate (PHMB) or 0.5 M sodium thiocyanate (NaSCN). PHMB effect was totally reversed by addition of excess sulphydryl groups. NaSCN effect was also totally reversed by elimination of the reagent by dialysis. The same binding component was recovered as verified by chromatography on Sephadex G-100 columns, without changes of the binding parameters determined by Scatchard analysis. Furthermore, low concentrations of NaSCN (20 mM) were able to increase of about 1.5 fold the dissociation rate constant of T_3 binding to its partially purified receptor at 0°C, with minor alterations of maximum binding.

The nuclear receptor of 3,5,3'-triiodo-L-thyronine (T_3) is now commonly recognized as a major site of action of thyroid hormones at the cellular level (1-6). The T_3 receptor is an acidic non-histone protein loosely bound to chromatin. The receptor binds T_3 in vitro with an apparent affinity constant (Ka) of $0.5\text{-}3\times10^{10}$ M^{-1} and a capacity of about 1 pmol/mg DNA in the liver (4,7). The receptor was partially purified by conventional methods (8-10), being then highly unstable and dependent, for T_3 binding, on the presence of other nuclear components like histones and DNA (10,11). During purification procedures or physico-chemical studies, there was found the need to obtain the active receptor free from T_3 which occupied the binding sites or to alter temporarily the binding parameters. Chemical agents that could fulfill this requirements were investigated. T_3 binding was shown to be dependent on the presence of free-SH. We previously showed that -SH blocking reagents such as parahydroxymercuribenzoate (PHMB) could inhibit T_3 binding to the nuclear receptor (4) or dissociate the preexisting hormone-receptor complexes (7). Mercurial agents, PHMB included, are known to interact reversibly with -SH. Chaotropic ions such as thiocyanate (NaSCN), produce dissociation of proteins without drastic changes in protein conformation (12). NaSCN was shown to prevent time-dependent aggregation of the estrogen receptor in the cytosol (13) or increase the exchange rate of the estrogen binding to its receptor (14). In this paper we describe the reversible dissociation of the T_3-receptor complexes using partially purified preparations. The partially purified receptor exhibits a redu-

0006-291X/82/050244-08$01.00/0

ced tendency to aggregate compared to nuclear extracts (7) and is less stable (7, 15). Furthermore, alterations of T_3 binding kinetics with non dissociating concentrations of NaSCN are also presented. The enhanced dissociation rate of T_3 binding could be of interest when an exchange of previously bound T_3 is needed.

METHODS AND MATERIAL

^{125}I -L-T_3 (specific activity : 1,500-2,000 µCi/µg) was from the Radio-chemical Center (Amersham, GB) ; 3,5,3'-triiodo-L-thyronine, dithiothreitol (DTT), calf thymus DNA, calf thymus histones H2A, Dowex 1x8-400 (Cl^-), and sodium parahydroxy-mercuribenzoate were from Sigma chemicals (St Louis, Mo, USA), Sephadex G-100 was from Pharmacia (Uppsala, Sweden), sodium thiocyanate and the other chemicals of analytical grade were from Merck (Darmstadt, GFR).

Nuclear material. Sprague Dawley rats of 300-350 g were used to prepare purified nuclei from liver (16). Nuclear T_3 receptor was extracted from purified and Triton X-100 washed nuclei by solubilization with 0.4 M KCl in 20 mM Tris/Cl^- buffer, 2 mM EDTA, 1 mM $MgCl_2$ pH 7.9 (0.4 TKEM) as previously described (4). The solubilized T_3 receptor was further purified by filtration on Sephadex G-100 and ion exchange chromatography on DEAE-Sephadex A 50 (receptor partially purified, RPP) (7). With this procedure, an approximate 80 fold purification with about a 30 % yield of binding sites was obtained when refered to total nuclear content. For PHMB studies, RPP fraction obtained in 20 mM Tris/Cl^- buffer, 1 mM $MgCl_2$, 2 mM EDTA pH 7.9 containing 0.18 M KCl and 10 mM mercaptoethanol was previously dialyzed against the same buffer without mercaptoethanol. NaSCN studies used RPP directly as it was.

Binding assays. T_3 binding was generally analyzed by incubation of RPP fractions 3h at 20°C or 24h at 0°C (equilibrium conditions) with a single concentration of 0.2 or 0.3 nM ^{125}I-T_3 in 0.4 TKEM buffer containing 5 mM DTT, 75 µg/ml DNA and 50 µg/ml histones H2A (7). T_3 binding capacity and apparent affinity constants were determined by saturation analysis with 0.02-1 nM ^{125}I-T_3 in the same medium and conditions and data analyzed according to Scatchard (17). Non specific binding was always determined in parallel assays by isotopic dilution with unlabeled T_3 (750 nM) and subtracted from the correspondant total binding values. Free and protein-bound T_3 were separated by the Dowex resin method already described (4).

Analytical assays. RPP fractions were chromatographed on Sephadex G-100 columns (1x70 cm) in 20 mM Tris/Cl^- buffer, 1 mM $MgCl_2$, 2 mM EDTA, 0.25 M KCl pH 7.9, at a flow rate of 7 ml/h. With this system free T_3 is highly retarded and bound T_3 (as checked by the Dowex resin method), appears in a single peak at the ovalbumin molecular weight region (7,10). Columns were previously calibrated with 19S thyroglobulin and ovalbumin. Proteins were determined by the modified Lowry procedure according to (18), using bovine serum albumin as standard. Radioactivity was counted in a Biogamma Beckman Spectrometer.

RESULTS

Parahydroxymercuribenzoate (PHMB). PHMB is known to inhibit T_3 binding (4), and rapidly dissociate T_3 receptor complexes (7). The time-course of T_3-receptor dissociation by PHMB was studied with RPP previously dialyzed and incubated with ^{125}I-T_3. At 0°C and 0.05 mM or 0.1mM PHMB complete dissociation was rapidly obtained (within 15 min, not shown). The reversibility of the effect was assayed, after 30 min of PHMB contact, by the addition of 5 mM DTT and binding allowed to be restored. In Fig. 1A are represented the values of specifically bound T_3 from control, dissociated and reassociated samples and in B the elution profiles of the

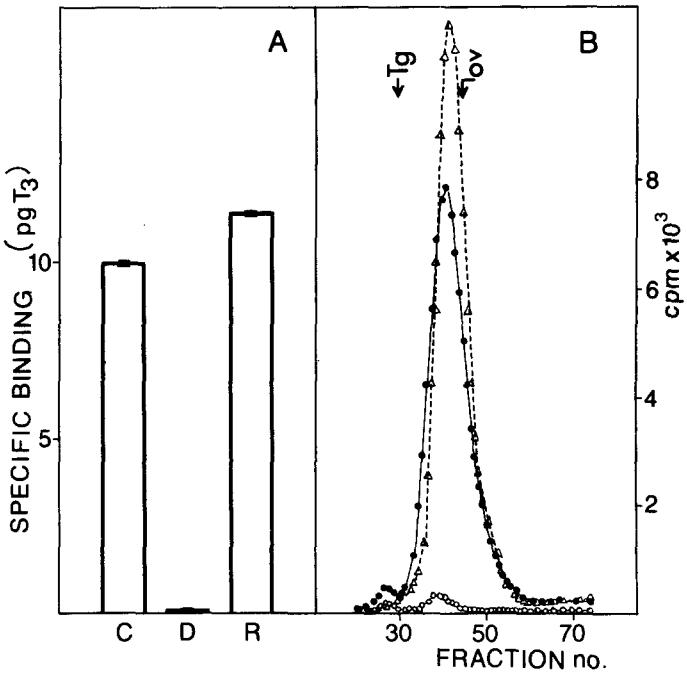

Fig. 1. Dissociating effect of PHMB on T_3-receptor complexes and reversibility. ^{125}I-T_3-receptor complexes in RPP (RPP 31.5 µg proteins/ml, dialysed against sample buffer without mercaptoethanol, incubated until equilibrium with 0.2 nM ^{125}I-T_3, DNA 75 µg/ml, histones H2A 50 µg/ml, with a specific binding of 5.3 pg T_3/30 µl) were dissociated with 0.1 mM PHMB at 0°C, 30 min. Reversion was studied by adding DTT (5 mM final concentration) to control and treated fractions. Reassociation of T_3 with the receptor was obtained after 24h at 0°C. A, specific binding of ^{125}I-T_3 to control (C) dissociated (D) and reassociated (R) aliquots (30 µl). Non-specific binding was substracted (3.8 % of total ^{125}I-T_3). B, fractionation of control (●), dissociated (○) and reassociated (△) fractions on Sephadex G-100 columns precalibrated with 19S thyroglobulin (Tg) and ovalbumin (ov). 1.1 ml fractions.

same samples from Sephadex G-100 columns. Total recovery of initial binding values was obtained. Furthermore, bound T_3 was eluted with the same elution volume before and after PHMB treatment in a single peak corresponding to the 50,000 molecular weight previously described for the T_3 receptor (7). The RPP fraction was dialyzed prior to the treatment in order to remove the mercaptoethanol needed for the purification. This provoked a slight aggregation of the receptor, as can be seen in the left part of the elution profile in Fig. 1B, corresponding to excluded material. This aggregation disappeared after reassociation, what might explain the slightly higher binding values obtained by the resin test (Fig. 1A). In order to check eventual changes in the receptor affinity after treatment with PHMB, Scatchard analysis of treated and non-treated dialyzed RPP were performed. No losses in capacity nor affinity for T_3 were observed (Table I). In exp. 2 where the RPP was analyzed after storage at 0°C, the lower Ka value before treatment could also be ascribed to the presence of age-dependent aggregates.

TABLE I. Effect of PHMB and NaSCN treatment on T_3 binding parameters of partially purified nuclear receptor.

	Experiment 1		Experiment 2	
	Ka x $10^{10}M^{-1}$	Capacity pmol T_3/ mg proteins	Ka x $10^{10}M^{-1}$	Capacity pmol T_3/ mg proteins
Control	1.6	11.6	1.4	5.6
PHMB	1.7	12.1	2.7	6.3
Control	2.6	9.0	1.9	5.7
NaSCN	2.3	11.0	2.2	5.9

Apparent affinity constants (Ka) and Capacity were determined by Scatchard analysis of different preparations of RPP (0.8-0.9 µg proteins per 0.2 ml incubate) before and after treatment. PHMB effect (0.1 mM) was reversed by 5 mM DTT (Fig. 1 legend) NaSCN was removed by dialysis (Fig. 3 legend). Incubations with ^{125}I-T_3 were in the presence of 5 mM DTT, DNA (75 µg/ml) and histones (50 µg/ml).

Thiocyanate. High concentrations of NaSCN inhibit T_3 binding to the nuclear receptor with a significant reduction from 50 mM and complete inhibition at 200 mM (not shown). Fig. 2 shows the time course of dissociation of T_3-receptor complexes by different NaSCN concentrations at 0°C. Dissociation was completed rapidly at high concentrations (200-500 mM) ; it was slower and stabilized at decreasing intermediate levels with concentrations from 20 to 100 mM. We found by Scatchard analysis that NaSCN produced a progressive decline in the apparent affinity constants with Ka values of $2.8x10^{10}$, $2.2x10^{10}$, $0.36x10^{10}M^{-1}$ without or with

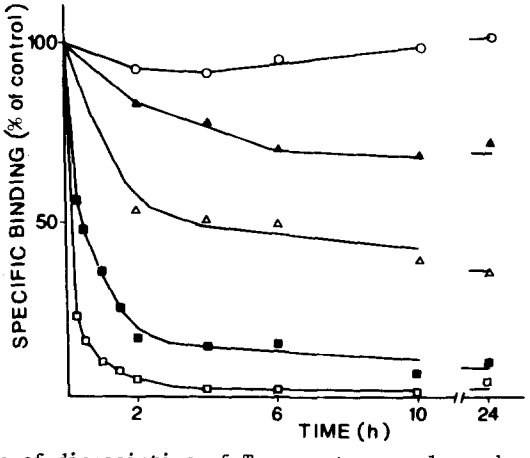

Fig. 2. Time course of dissociation of T_3-receptor complexes by different NaSCN concentrations. ^{125}I-T_3-receptor complexes in RPP (6.5 µg proteins/ml incubated with 0.2 nM ^{125}I-T_3 until equilibrium) were treated with 10 (○), 20 (▲), 100 (△), 200 (■) and 500 (□) mM NaSCN at 0°C for different time intervals. Non-specific binding was determined for each time interval and NaSCN concentration by isotopic dilution with 750 nM T_3 and subtracted from total binding. Values never exceeded 4 % of total ^{125}I-T_3.

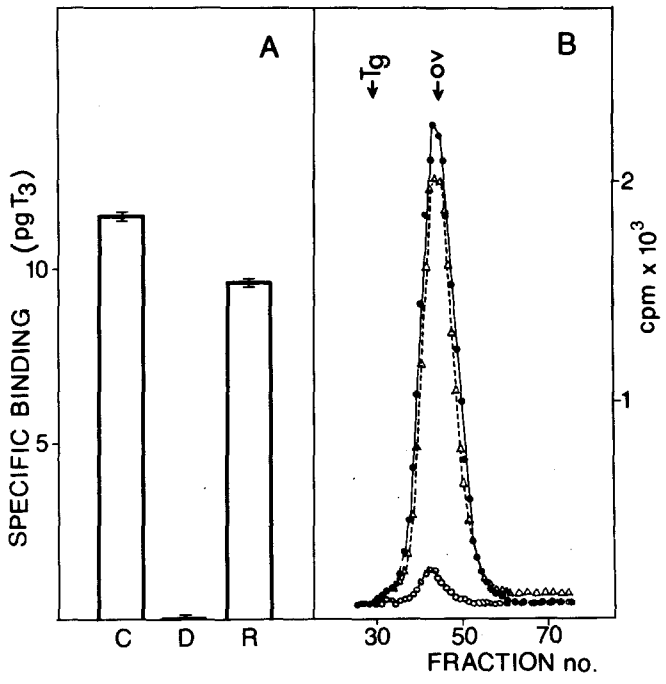

Fig. 3. Dissociation of T_3-receptor complexes by NaSCN and reversibility. ^{125}I-T_3-receptor complexes in RPP (RPP 31.6 µg proteins/ml incubated until equilibrium with 0:2 nM ^{125}I-T_3 at 0°C) were treated with 0.5 M NaSCN, 30 min, 0°C. Reversion was obtained by dialysis of treated fraction against sample buffer with 10 mM mercaptoethanol and reincubation 24h at 0°C with 0.2 nM ^{125}I-T_3. A, Specific binding values of control (C), dissociated (D) and reassociated (R) fractions obtained by the resin test as in Fig. 1. Non-specific binding (3.5 % of total ^{125}I-T_3) was subtracted. B, Sephadex G-100 elution profiles of control (●), dissociated (○) and reassociated (△) fractions as in Fig. 1.

20 and 100 mM NaSCN respectively. In order to study the reversibility of the dissociation obtained with 0.5 M NaSCN, dissociation and reversion experiments similar to the one described for PHMB were performed. RPP fractions complexed with ^{125}I-T_3 were treated with 0.5 M NaSCN and reversion analyzed after removing NaSCN by dialysis. Fig. 3, shows that complete dissociation was obtained. After elimination of NaSCN, binding activity was restored and the reassociated T_3-receptor complexes exhibit the same behaviour as control in Sephadex G-100 filtration. The lower binding levels measured by the resin test (Fig. 3A) with reversion samples (R) may be due to losses of receptor during the dialysis step obligatory for NaSCN removal instead of incomplete reversion. Total reversion without changes in capacity nor affinity was obtained by saturation analysis of RPP, treated or not with 0.5 M NaSCN and both samples dialysed. (Table I). The effect of NaSCN upon the receptor binding parameters at non-dissociating concentrations (20 mM NaSCN) was also analyzed. T_3 receptor from a RPP fraction was allowed to bind T_3 in the absence or presence of NaSCN at 0°C (Fig.4). Binding was estimated at different time intervals and, when equilibrium was reached dissociation was produced by adding 750 nM

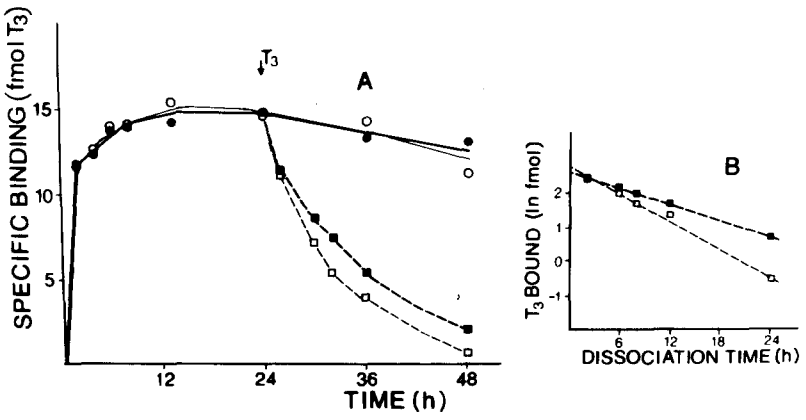

Fig. 4. Effect of NaSCN on the binding kinetics of T_3 to its nuclear receptor. RPP (64 µg proteins/ml) was incubated with 0.3 nM ^{125}I-T_3, 5 mM DTT, 75 µg/ml DNA, 50 µg/ml histones H2A at 0°C in the presence (○,□) or absence (●,■) of 20 mM NaSCN. After 24h, dissociation was produced by the addition of 750 nM T_3 (■,□). At each incubation time binding values were determined by the resin test. A, values expressed as specific binding. B, determination of dissociation rate constants (k_{-1}) by plotting ln of dissociation values versus time.

T_3. Differences in the rate of association were difficult to estimate although it seemed that it was slightly enhanced in the presence of NaSCN. Maximum binding was comparable. More important differences were obtained with the dissociation rates and the dissociation rate constants were 0.09 and 0.13 h^{-1} without and with NaSCN respectively. NaSCN effect was independent of the presence of DTT, known to increase the association and dissociation rates of T_3 binding to nuclear extracts (7). The dissociation rate constants in the absence of DTT were in the absence or presence of NaSCN respectively : 0.05 and 0.08 h^{-1}. NaSCN effect upon the dissociation rate was of the same order of magnitude at 20°C and 0°C or with T_3 receptor in nuclear extracts. It is to note that dissociation rates of bound T_3 were always lower in RPP than in nuclear extracts (about two fold, not shown).

DISCUSSION

Several methods for the reversible dissociation of T_3-receptor complexes were investigated. Some proved to be non dissociating like changes in KCl concentration ; others were unsufficiently reversible like alteration of pH (19). Parahydroxymercuribenzoate and thiocyanate, described here, completely dissociated the T_3 receptor complexes in a fully reversible fashion. PHMB acts by blocking the free sulphydryl groups needed for receptor activity, one at least being probably located at the active site (7). When excess sulphydryl groups were added, complete recovery of the initial T_3 binding level was obtained. This treatment did not lead to alterations of binding parameters, nor of the apparent molecular size. PHMB effect can therefore be considered as fully reversible. The same reversible effect was described for progesterone and vitamine D receptors (20), two steroid

receptors known to be also dependent on free sulphydryl groups for binding activity. Thiocyanate acts by weakening or disrupting hydrophobic and hydrogen bonds (21). The dissociation of T_3 receptor complexes was totally reversed by removal of the reagent by dialysis. Total recovery of initial binding was observed with no alterations of the binding parameters even when the receptor was not protected by the bound hormone. The 0.5 M concentration used was previously described as non dissociating for the estrogen receptor complexes (14) during sucrose gradient centrifugation and a permissible concentration of 0.1M for Sephadex chromatography. In that range of concentrations and under our conditions the T_3-receptor complexes were dissociated. The degree of dissociation obtained varied with NaSCN concentration. A limit concentration of 20 mM may be defined as uneffective concerning the stability of T_3 receptor complexes. At that concentration the kinetics of association and dissociation was nevertheless altered, being the dissociation rate increased of 1.4 fold, in the presence or absence of reducing agents. The effect is less spectacular than with steroid receptors where the dissociation rate is increased of 10 fold with 0.5 M NaSCN (14). It may still be useful for facilitation of the exchange of ^{125}I-T_3 into binding sites previously saturated with unlabeled T_3, particularly when combined with reducing agents (7). The NaSCN effect may be also helpful for T_3 binding site estimations of purified receptor considering its lower dissociation rate when compared to the receptor from nuclear extracts.

The reversible dissociating effect of 0.1 mM PHMB or 0.5 M NaSCN may be helpful to clear the receptor from bound T_3, applicable in the elution of the receptor from an affinity support with immobilized T_3, as described for the estrogen receptor (13). Of interest is also the probable reversion of age dependent aggregation of the T_3 receptor after treatment with these two agents.

ACKNOWLEDGMENTS

We are grateful to Prof. Lissitzky for helpful discussions and advices, S. Santelli for technical assistance and E. Giraud for typing the manuscript. This work was partially supported by CNRS (LA 178).

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