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PARTIAL PURIFICATION AND CHARACTERIZATION OF NUCLEAR TRIIODOTHYRONINE BINDING PROTEINS

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SUMMARY. Nuclear high affinity triiodothyronine binding proteins from rat liver were purified about 500-fold over Triton-washed nuclei by a three-step procedure using gel filtration, DEAE-Sephadex and DNA-Sepharose chromatography. Although unstable, they retain most of the properties exhibited in crude nuclear extract : affinity and stereospecificity for T_3, thermolability, molecular size. Furthermore, their isolation from several chromatin constituents under reducing conditions allows their study with decreased aggregation risks.

L-triiodothyronine binds to protein "receptors" in the cell nuclei of several thyroid hormone responsive tissues from mammalian (1-5) and amphibian (6-7) origin. Nuclear thyroid hormone binding proteins (NTBP) are acidic proteins bound to chromatin from which they dissociate under moderately high salt conditions (8-10). Some properties of the solubilized NTBP have been described (11-14). Earlier work suggested that thyroid hormone may regulate gene expression (15). Recently, it was shown that T_3 could provoke a dose-dependent stimulation of growth hormone synthesis and a simultaneous depletion of NTBP (16). Progress in the understanding of thyroid hormone action at the nuclear level awaits the purification of the specific, high-affinity T_3 binder. The present report is the first description of a three-step purification procedure, using DNA-Sepharose chromatography, which results in a 500-fold enrichment of NTBP over purified nuclei.

MATERIALS AND METHODS

Rat liver nuclei were purified as previously described (10) and washed once with 0.25 % Triton-X100. Nuclear proteins were solubilized at 0°C with 0.35 ml/g liver equivalent of nuclei with 0.4 M KCl in TEM and immediately fractionated. In vivo formed T_3^*-NTBP complexes were obtained from rats injected with [^{125}I]T_3 2 hr before sacrifice (10). Total T_3 binding sites were

NTBP : nuclear thyroid hormone binding proteins; T_3 : 3,5,3'-triiodo-L-thyronine; T_3^* : [^{125}I]T_3; T_4 : 3,5,3',5'-tetraiodo-L-thyronine; triac : 3,5,3'-triiodothyroacetic acid; isopropyl-T_2 : 3'-isopropyl-3,5-diiodo-L-thyronine, rT_3 : 3,3',5'-triiodo-L-thyronine; et-SH : 2-mercaptoethanol; DTT : dithiothreitol; TEM : 20 mM Tris-Cl, 1 mM $MgCl_2$, 2 mM Na_2 EDTA pH 7.95 at 20°C; TEMS : TEM + 10 mM et-SH.

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roughly estimated by incubating the material at equilibrium (3 hr, 20°C) with nearly saturating concentrations of T_3^* (1-2 nM) (10, 12) and quantitated by saturation analyses plotted according to Scatchard (17). Unextracted NTBP were estimated in residual chromatin homogenized in TEMS, incubated as above and counted for bound T_3^* after centrifugation (5000 x g, 15 min) and two washes with 10 mM tris-Cl, 50 mM NaCl, 0.25 % Triton X-100 pH 7.4.

Gel filtration was performed in a Sephadex G-100 column (5x90 cm; Pharmacia) with 0.1 M KCl in TEMS. DEAE-Sephadex A_{50} (Pharmacia) was equilibrated in 1.6x8 cm columns with 0.1 M KCl-TEMS. Sample application, wash and elution with a KCl gradient in TEMS were done at a flow rate of 20 ml/hr. Salt molarity was checked with a Metrohm E518 conductivity meter. DNA-Sepharose was prepared as described by Arndt-Jovin et al. (18) with CNBr-activated Sepharose 4B (Pharmacia) and calf thymus DNA and used in 1.6 x 10 cm columns(about 10 mg covalently bound DNA) equilibrated with 0.1 M KCl-TEMS. Flow rates were of 7 ml/hr for sample application and 15 ml/hr for wash and KCl gradient elution. All steps were at 2°C.

Analytical studies, protein and DNA estimations and radioactivity measurements were performed as previously described (10, 12). [^{125}I] T_3, specific activity about 500 mCi/mg, was from Abbott Laboratories. Calf thymus DNA and histones, T_3, T_4, triac were from Sigma, rT3 was from Henning (Berlin); isopropyl-T_2 was a gift from Dr R. Michel to whom we are grateful.

RESULTS

1) Purification

NTBP were extracted from nuclei with 0.4 M KCl in TEM with a yield of 80 to 90 % and a purification of 1.7 to 2-fold. Solubilized NTBP exhibit a high tendency to aggregate which could be partly limited by reducing agents (12). Nuclear extracts were incubated with 5 mM DTT (30 min, 20°C) before Sephadex G-100 gel filtration. As expected from previous studies (12), two peaks of T_3-binding activity, with similar K_a for T_3, were obtained. A small excluded peak I contained 15-20 % of aggregated NTBP. The large included peak II contained 15.3 ± 1.3 % (mean ± SE, n = 9) of the extracted proteins and NTBP purified 3-to 4-fold over nuclear extract.

DEAE-Sephadex chromatographic behaviour of NTBP was first analyzed in nuclear extract containing *in vivo* formed T_3^*-NTBP complexes and after dilution with TEMS to 0.1 M KCl. Fig. 1 shows an elution profile obtained with a 0.1 to 0.3 M KCl gradient. Bound T_3^* and T_3-binding activity were retained at 0.1 M KCl. They eluted in two discrete peaks at 0.14-0.15 M and 0.17-0.18 M KCl, respectively, the T_3-binding activity peak generally showing a shoulder at 0.15 M KCl. When peak II material was analyzed under these conditions, only one symmetrical peak of T_3-binding activity was observed at 0.18 M KCl. NTBP purification was then 7.2 ± 1.5-fold (n = 5) over peak II. Since T_3^*-NTBP complexes eluted at a lower KCl concentration than free NTBP, a biospecific elution procedure was attempted; the washed column was eluted with, first, 0.15 M KCl-TEMS, then 0.15 M KCl-TEMS + 50 nM T_3. But the bulk of T_3-binding activity was eluted shortly after a protein fraction dissociated with 0.15 M KCl, in a large zone and independently of the presence of T_3. A stepwise elution of NTBP with 0.16 M KCl in TEMS was

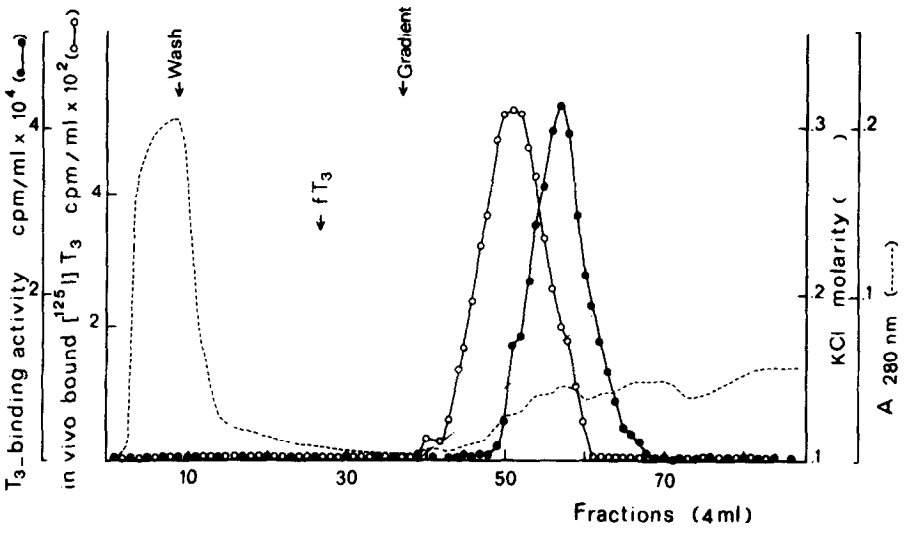

Fig. 1. DEAE-Sephadex chromatography of a nuclear extract containing in vivo formed T_3^*-NTBP complexes. Bound T_3^* was that which was not taken up by a Dowex anion-exchange resin in aliquots of each fraction. T_3-binding activity is expressed as T_3^* specifically bound after a 3 hr incubation at 20°C in 0,2 ml TEM containing 0.25 M KCl, 5 mM DTT, 1 nM T_3^* and 0.1 ml aliquots of each fraction. Non-specifically bound T_3 was determined in parallel incubations with 500 nM T_3.

adopted (purification over peak II : 16.6 ± 2 - fold, n = 4). NTBP could not be recovered in the absence of reducing agents.

As reported in a preliminary form (19), the material thus obtained is able to bind to DNA. NTBP could be retained by DNA-Sepharose columns, with a progressive saturation of DNA, and eluted as a single peak at about 0.2 M KCl (Fig. 2) with a further purification of 3.9 ± 0.5 - fold (n = 3). No discrimination could be obtained between free NTBP and preformed T_3^*-NTBP complexes suggesting that DNA binding of NTBP is poorly dependent upon the presence of bound T_3. Table 1 gives a representative purification of NTBP from purified nuclei.

2) Properties of the partially purified NTBP

When T_3-binding activity of purified NTBP was measured under standard incubation conditions with T_3^*, the complexes formed at short times were rapidly destroyed (Fig. 3). Among several protecting agents tested, proteins (ovalbumin, histones) and in particular a mixture of histones and DNA (see Fig. 3) were the most efficient. These agents do not bind T_3; DNA alone was without effect at the KCl concentration of the tests. When stored at 0°C, purified NTBP were highly unstable; the half-life of T_3-binding activity could be prolonged from 0.5 - 2 days to 10-15 days by addition of ovalbumin (0.2 mg/ml). DNA could have a partial protective role at low ionic strength (KCl < 0.1 M). Glycerol was ineffective.

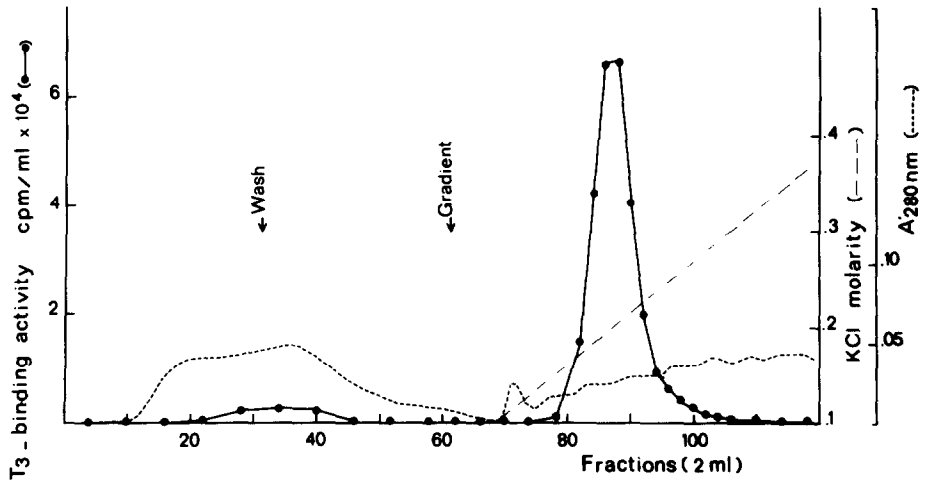

Fig. 2. DNA-Sepharose chromatography of NTBP recovered from DEAE-Sephadex step. Sample was applied after dilution to 0.1 M KCl with TEMS. Fractions, one on the other, were collected on ovalbumin (0.2 mg/ml, final concentration). T_3-binding activity was determined as in Fig. 1.

Table I. Purification of nuclear T_3 binding proteins.

Step	Total protein mg	Total receptor sites* pmoles	Specific activity pmole/mg	Purification fold	Ka* $M^{-1}x10^{10}$
Nuclei	488	99.9	0.20	1	$
Nuclear extract	222	79.9	0.36	1.8	0.91
Sephadex G-100	32.4	40.2	1.24	6.2	0.76
DEAE-Sephadex	1.11	27.7	24.95	125	0.90
DNA-Sepharose	0.18	18.8	104.4	520	1.01

* Receptor sites and affinity constant Ka determined by Scatchard plots

$ Ka for T_3 in residual chromatin : 0.37×10^{10} M^{-1}

Partially purified NTBP retain their thermolability at 36°C, even in the presence of the protecting mixture (Fig. 3), and their dependence upon the presence of reducing agents.

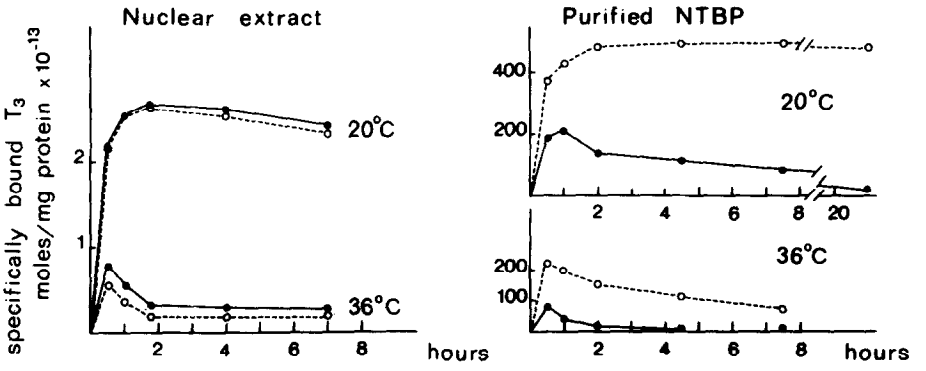

Fig. 3. Time-course of T_3 binding. Nuclear extract and NTBP purified after DNA-Sepharose were incubated at 20°C and 36°C under standard conditions (●—●) and with calf thymus DNA (0.075 mg/ml) and histones (0.15 mg/ml) added (o--o).

NTBP bind T_3 to one class of sites with the same apparent Ka whatever the purification may be (Table I). Saturation analyses of the purified NTBP were performed in the presence of DNA + histones which enhance total capacity without altering Ka values. The stereospecificity of NTBP for various thyroid hormone analogues is also fully maintained. In three experiments at pH 7.95, 50 % inhibition of T_3^* binding was obtained, as compared to T_3, with concentrations 1.8 ± 0.3 - fold lower for triac, equimolar for isopropyl-T_2, 13.3 ± 1.3 - fold higher for T_4 and 890 ± 106 - fold higher for rT_3

T_3^*-NTBP complexes in the purified material eluted from analytical Sephadex G-100 and Bio-Gel A-0.5 m columns in one included peak of Mr about 50 000, exactly as in nuclear extract (12). High Mr aggregates were almost completely absent. By contrast, in polyacrylamide gel electrophoresis at pH 8.6, T_3^*-NTBP complexes exhibited progressively higher Rf as the purification increased (Fig. 4). The most purified NTBP comigrated with rat serum prealbumin (Rf = 0.84 ± 0.006, n = 6). Moreover, Fig. 4 shows that when a mixture of DNA + histones was added during incubation with T_3^*, the T_3^*-NTBP complexes migrated less rapidly in heterogeneous zones.

DISCUSSION

The described procedure allows, in three steps, a 500-fold purification of nuclear T_3-binding proteins over purified nuclei. Assuming an approximate Mr of 50 000 and one T_3-binding site per molecule, one could estimate that a 100 000-fold purification will be necessary to obtain the pure NTBP. Attempts to reach higher specific activities, particularly using affinity chromatography with insolubilized T_3, were at present unsuccessful. A major difficulty is related to the instability of NTBP in solutions devoid of other chromatin components

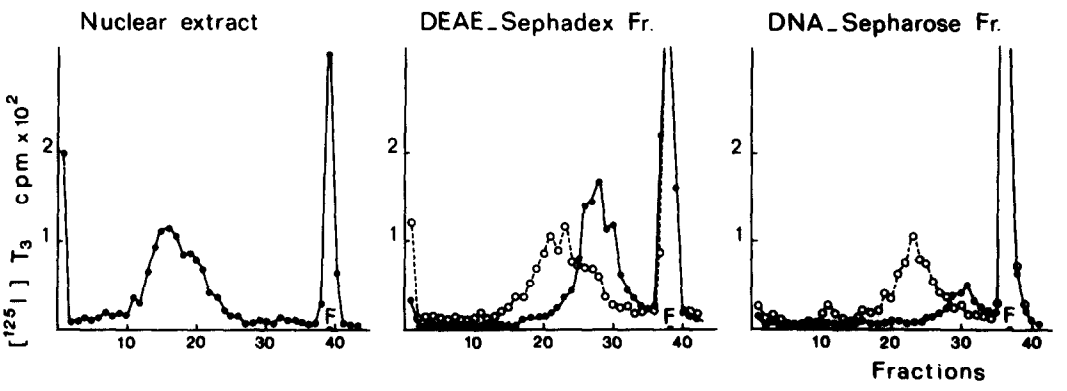

Fig. 4. Polyacrylamide gel electrophoresis of T_3^*-NTBP complexes formed after incubation of nuclear extract and purified NTBP with T_3^*. 5 % polyacrylamide gels were run at 2°C with 50 mM Tris-Glycine buffer pH 8,5, frozen and cut in 1.5 mm pieces for radioactivity measurement. Incubations were under standard conditions (●——●) or with added DNA + histones as in Fig. 3 legend (o----o). Rat serum and ovalbumin were run in parallel and stained with Coomassie blue. Mean Rf were : serum albumin : ≈ 0.70; prealbumin : 0.84 and ovalbumin 0.87. F is the bromophenol blue stained front were is located free T_3 and the whole radioactivity when incubation is performed with an excess of unlabelled T_3.

among which histones and DNA could be important. Nevertheless, after the three steps described, about 20 % of NTBP were recovered with intact T_3-binding activity and most of the molecular properties described in nuclear extract. At variance with some results from Latham et al. (11) are the maintenance of thermolability and the poor efficiency of rT_3 in inhibiting T_3^* binding to NTBP, whatever their purification state. It is also worth mentioning that NTBP electrophoretic mobility in polyacrylamide gels could be close that of rat serum prealbumin; the electrophoretic behaviour of NTBP reflects in most cases associations with other chromatin components, probably favoured by the low ionic strength conditions applied, and whose degree decreases with increasing purification.

From data obtained in crude nuclear extract (12) and purified fractions, only one class of nuclear T_3-binding proteins could be detected considering kinetics, gel filtration, sedimentation, salting-out and DNA-binding properties. The separate ion-exchange chromatographic behaviour of bound T_3^* and T_3-binding activity was also reported by Silva et al. (20); it might be related to some conformational changes of NTBP after binding of the hormone rather to the presence of T_3 which by itself would have given an opposite chromatographic shift or to discrete T_3-binding proteins otherwise undetectable. A T_3-induced conformational change could be an important event in initiating thyroid hormone action.

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