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Note

Multiple affinity classes of saturable triiodothyronine binding in mouse liver nuclear extract demonstrated using high-performance size exclusion chromatography

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Estimates of the concentration and affinity characteristics of nuclear thyroid hormone receptors in the liver and other thyroid hormone responsive organs are often obtained by saturation analysis of [^{125}I]triiodothyronine (T_3) binding in extracts of chromatin proteins solubilized in high concentrations of either potassium chloride or ammonium sulfate[1–3]. Separation of bound from free hormone on Sephadex G-25 or other resins can produce linear Scatchard plots, suggestive of a single affinity class of binding site[2,3]. However, in some cases, at least two classes of binding sites are suggested by a curvilinear T_3-binding plot[4,5].

Since [^{125}I]T_3-binding sites in solubilized nuclear extracts may then represent more than one affinity class, it would be advantageous to separate in some way these types and perform a saturation analysis of each, individually. In theory, this could be accomplished by separating various forms of T_3 binding by gel chromatography (*e.g.* Sephadex G-100) in a series of reactions containing [^{125}I]T_3 and nuclear extracts in competition with different concentrations of unlabeled hormone; saturation analysis of the binding data under each peak could then provide a maximum binding capacity (MBC) and dissociation constant (K_d) for each component resolved by the chromatography. In practice, the slow flow-rates involved in conventional gel chromatography would make impractical this type of saturation analysis.

High-performance liquid chromatography (HPLC) using molecular exclusion columns (TSK SW-3000)[6] would, however, be ideal for this sort of analysis since all the assays necessary for the binding analysis could be run in a single day. We have undertaken in the work reported here to use HPLC to resolve both the native thyroid hormone receptor (and other T_3-binding sites) and to perform a saturation analysis of each using data from the [^{125}I]T_3 elution profile.

EXPERIMENTAL

Materials

Female C3H/OUJ mice were kindly provided by Dr. Henry Outzen, Jackson Laboratories, Bar Harbor, ME, U.S.A. Samples of [^{125}I]T_3 (NEX-110H; specific activity, 1200 μCi/μg) were obtained from New England Nuclear Corp., Boston, MA,

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U.S.A., and unlabeled T_3 from Sigma. Gel filtration calibration kits (for high- and low-molecular-weight determinations) were purchased from Pharmacia.

Preparation of nuclear extract

Solubilized chromatin extract was prepared by a modification of a procedure described earlier[3]. Livers excised from four animals and immediately frozen in liquid nitrogen were pooled, thawed in 3 volumes of 2.1 *M* sucrose and 5 m*M* magnesium chloride at 37°C and homogenized with a Polytron homogenizer (PT-10 probe, Brinkman Instruments). The homogenate was filtered through a layer of Miracloth (Calbiochem), underlaid with cold 2.1 *M* sucrose and 5 m*M* magnesium chloride and centrifuged at 50,000 *g* for 60 min. The nuclear pellet was resuspended in 10 ml of wash buffer (20 m*M* Tris, 2 m*M* calcium chloride, 1 m*M* magnesium chloride, 0.5% Triton X-100; pH 7.6) and repelleted (2,000 *g* for 30 min). After a second nuclear wash, chromatin proteins were extracted from the nuclear pellet in 5 ml of a buffer containing a high salt concentration (20 m*M* Tris, pH 8.0, 0.2 *M* ammonium sulfate, 0.25 *M* sucrose, 1 m*M* ethylenediaminetetraacetate (EDTA), 5 m*M* dithiothreitol (DDT), 5% glycerol). This suspension was sonicated and centrifuged at 4000 *g* for 30 min and the supernatant nuclear extract was stored in liquid nitrogen.

Saturation analysis using TSK SW-3000 and Sephadex G-25 column chromatography

Aliquots (50 μl) of nuclear extract were incubated in Assay Buffer (50 m*M* sodium phosphate, pH 7.6, 0.2 *M* ammonium sulfate, 1 m*M* EDTA, 5 m*M* DDT, 10% glycerol) together with 0.1 n*M* [^{125}I]T_3 and unlabeled T_3 (12 concentrations, 0–1000 n*M*) in a total assay volume of 0.5 ml. Duplicate assay tubes allowed TSK SW-3000 and standard Sephadex saturation analyses to be performed in parallel. All assays were incubated at room temperature for 1 h, then at 4°C for 17 h.

In the standard saturation analysis[3] bound and free hormone were separated on Sephadex G-25 (medium) columns (bed volume, 2 ml) in the cold. The columns were developed with 0.4 ml of Assay Buffer and the protein peak (0.8 ml) was collected and counted for bound [^{125}I]-activity on a γ-spectrophotometer (Beckman).

To remove unbound [^{125}I]T_3 prior to TSK SW-3000 chromatography, thereby avoiding contaminating [^{125}I]T_3 in the column eluent, the second series of assays was also passed through Sephadex G-25 as described above. A 0.25-ml aliquot of the bound fraction from each tube was then loaded onto a Spherogel TSK SW-3000 molecular exclusion column (600 × 7.5 mm) (Altex Scientific, Berkely, CA, U.S.A.) and chromatographed (flow-rate, 0.5 ml/min) at 3°C using Assay Buffer modified to contain 0.1 m*M* DTT as the mobile phase. A total of 80 0.25-ml fractions was collected for each sample and counted on a γ-spectrophotometer.

In a second experiment, five [^{125}I]T_3 binding assays were set up as described above and loaded onto the TSK SW-3000 gel without prior elution through Sephadex. Since this analysis consisted of only five assays rather than twelve, the delayed elution of free [^{125}I]T_3 from the gel did not pose a problem.

Data analysis

A computer-assisted analysis was used to determine the K_d and MBC by linear transformation[8]. Since the elution positions of various proteins (as measured by 280 nm absorbance) were highly reproducible in the twelve HPLC chromatograms, sat-

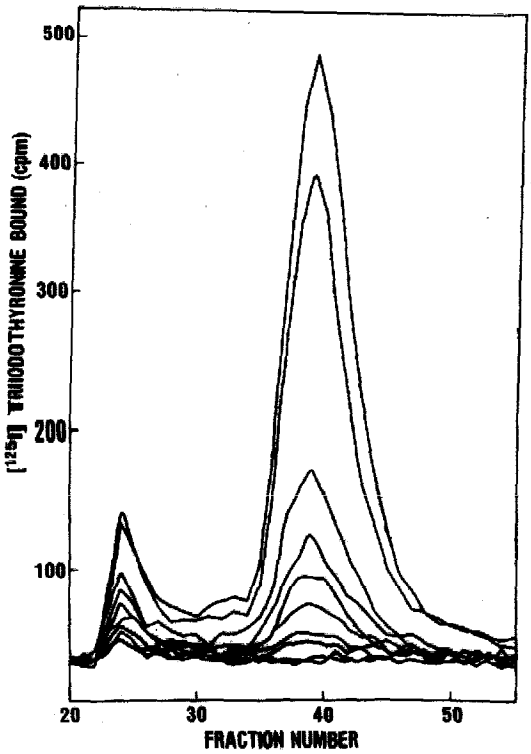

Fig. 1. TSK SW-3000 HPLC chromatography of mouse liver nuclear extract incubated with 0.1 n*M* [^{125}I]T_3 together with increasing concentrations of unlabeled T_3.

uration analysis could be performed for each fraction of eluted protein. In addition, binding data (cpm) integrated under the T_3 binding peaks was used to determine K_d and MBC values for each peak. Analysis of the distinctly non-linear Sephadex G-25 binding data was also performed using programs written by Munson and Rodbard[9] and Ulane *et al.*[10], on the assumption that multiple T_3 binding components were present in the assay.

RESULTS

HPLC of C3H/OUJ mouse liver nuclear extract on the TSK SW-3000 column resolved two major peaks of [^{125}I]T_3 binding: an excluded peak [molecular weight (M_r) > 450,000 daltons] and an included peak of *ca.* 50,000–55,000 daltons, the intensities of which both decreased with increasing concentrations of unlabelled T_3 (Fig. 1). The remarkable constancy of the chromatographic profiles, as indicated by the unvarying elution positions of the [^{125}I]T_3-binding peaks, permitted individual saturation analyses of the binding data for each fraction collected. The MBC and K_d for each fraction were estimated by best linear fit to Scatchard plots of the binding data. Fraction 43, for example, gave an MBC of 1.80 p*M* and a K_d of 0.07 n*M* (r = 0.93). Fig. 2 shows, as might be expected, that the peaks in a plot of MBC calculated for each fraction are coincident with the [^{125}I]T_3-binding peaks in Fig. 1. In addition, a small MBC peak at fraction 32 (M_r, 110,000 daltons), not readily dis-

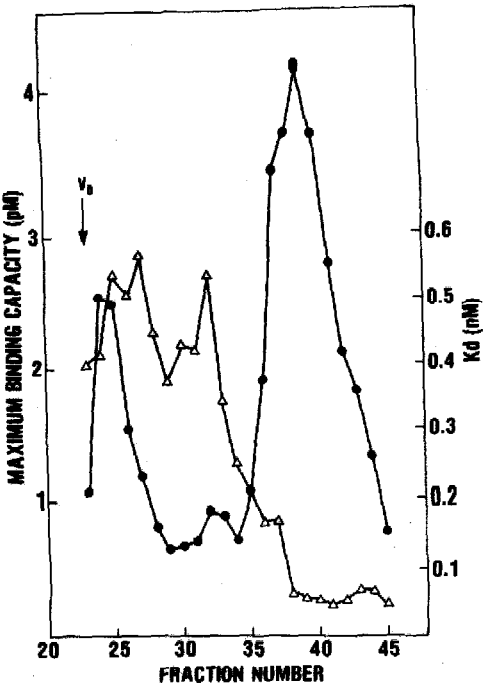

Fig. 2. MBC (●) and K_d (△) determined for each elution position of the TSK SW-3000 gel by saturation analysis of the binding data.

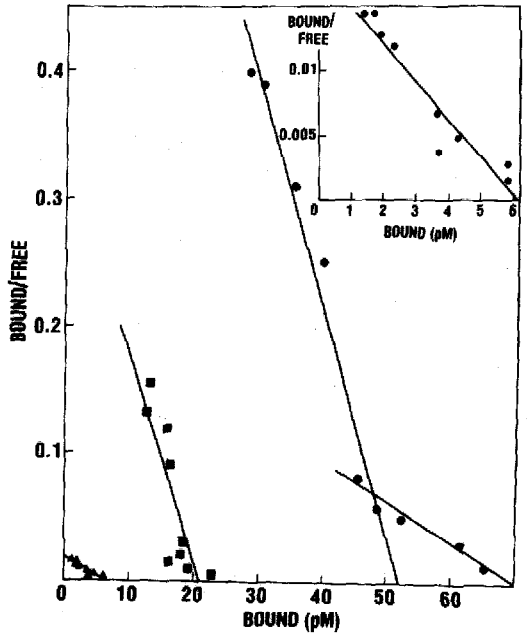

Fig. 3. Saturation analysis of mouse liver nuclear extract labeled in 0.1 nM [^{125}I]T_3 and separated on either Sephadex G-25 alone (●) or on a TSK SW-3000 gel following Sephadex G-25 elution (■, ▲ and inset). Binding plots for TSK SW-3000 are: peak 1 (fractions 23–26), ▲; peak 2 (fractions 38–45), ■. Inset; ●, peak 1 (scale expanded).

TABLE I

SATURATION ANALYSIS OF [^{125}I]T_3-BINDING IN MOUSE LIVER NUCLEAR EXTRACT USING TSK SW-3000 AND SEPHADEX G-25 ELUTION TO SEPARATE BOUND FROM FREE HORMONE

TSK SW-3000 peaks 1 (fractions 23–26) and 2 (fractions 38–45) were analyzed by linear fit to the data points shown in Fig. 3. Sephadex G-25 presented two apparent binding sites (sites 1 and 2) whose K_d and MBC were determined by linear fit to points 1–5 and 5–9 for sites 1 and 2, respectively.

Parameter	*TSK SW-3000*		*Sephadex G-25*	
	Peak 1	*Peak 2*	*Site 1*	*Site 2*
MBC (p*M*)	6.1	21.0	51.9	17.9
K_d (n*M*)	0.34	0.062	0.056	0.32
r	0.95	0.83	0.98	0.97

cernible as a binding peak in Fig. 1, is apparent. Data from a second experiment (not shown) confirm the presence and position of these three MBC peaks.

Fig. 2 also provides striking evidence that the hormone affinities of these T_3-binding components differ substantially. Fractions 23–32 exhibit K_d values in the 0.4–0.6 n*M* range whereas fractions 38–45 have values of *ca.* 0.05 n*M*. The increase in affinity (decreased K_d) between fractions 32 and 38 suggests a transition between two affinity classes in these fractions; dissociation constants of intermediate value result, which decrease as the high-affinity form becomes dominant. An increase in K_d at fraction 43, although slight (Fig. 2), was entirely reproducible in a second experiment (data not shown).

Saturation analysis of the integrated cpm in each major peak (peak 1, fractions 23–26; peak 2, fractions 38–45) was performed and the resulting Scatchard plots were compared with a Scatchard analysis of the data from a Sephadex G-25 binding assay performed in parallel (Fig. 3). Whereas peaks 1 and 2 produced linear Scatchard plots, the parallel G-25 assay resulted in a distinctly curvilinear plot indicative of at least two binding sites. Analysis of the Sephadex G-25 data with two-component models of [^{125}I]T_3 binding failed to differentiate the lower-affinity site. However, an approximation of the MBC and K_d of both higher- and lower-affinity sites was accomplished by a linear fit to the two "legs" of the binding data (Fig. 3). By this method, a high-affinity component with a K_d strikingly similar to that of HPLC peak 2 and a lower-affinity site with a K_d similar to that of peak 1 were found (Fig. 3, Table I). Owing to the greater stringency of the TSK SW-3000 in removing bound [^{125}I]T_3 from protein however, the MBC of peak 2 was only about 40% of that of the high affinity G-25 component.

DISCUSSION

The present study demonstrates that T_3 binding in salt-solubilized nuclear extracts of mouse liver is eluted from a TSK SW-3000 column in several macromolecular forms. An included form (peak 2) whose molecular weight of 50,000–55,000 daltons is consistent with previous descriptions of the nuclear thyroid hormone receptor[1,3,4,11,12] has been shown by HPLC saturation analysis (Fig. 3) to have a high

affinity for T_3 (K_d = 0.06 nM) similar to K_d values determined previously for the receptor in conventional analyses[4] and in a parallel Sephadex G-25 assay in the present report. Lower-affinity saturable T_3-binding sites were shown by HPLC analysis to be present predominantly in an excluded peak (peak 1) and in lesser concentrations in included fractions of a high molecular weight (Fig. 2).

Latham *et al.*[3] have proposed that the high-molecular-weight form of T_3 binding (excluded on Sephadex G-100), on the basis of its sensitivity to DNAase treatment, may, in part, represent the included receptor complexed with DNA and other chromatin proteins. The lower affinity of peak-1 binding sites for the hormone might, therefore, result from the allosteric hindrance of hormone–receptor interactions that are sequestered in chromatin.

Variations in the relative proportions of peak 1 to peak 2 T_3 binding in nuclear extracts obtained from a variety of tissues (and prepared using different methodologies) could explain the variation in form of the resultant Scatchard plots from rectilinear to curvilinear (multiple-component). We have earlier noted[7] that the relative contribution of peak 1 to peak 2 total T_3 binding is several-fold greater in the liver of the C3H/HeN mouse than in the mammary gland of the same species. Moreover, peak 1 can be substantially reduced by extended ultracentrifugation of the extract (unpublished results).

The primary advantage of performing saturation analyses with the TSK SW-3000 gel lies in the ability to determine rapidly and efficiently the K_d of any form of T_3 binding directly from its elution profile (Fig. 2). Moreover, our results suggest that this method of saturation analysis can distinguish separate affinity classes of T_3-binding sites which are too similar in affinity to be resolved by computer-assisted multiple-component modeling of conventional saturation analyses. This technique may thus be valuable for use in other ligand–receptor systems, where saturation analysis is complicated by multiple forms of ligand binding.

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REFERENCES

1 M. I. Surks, D. Koerner, W. Dillman and J. H. Oppenheimer, *J. Biol. Chem.*, 248 (1973) 7066.
2 H. H. Samuels, J. S. Tsai, J. Casanova and F. Stanley, *J. Clin. Invest.*, 54 (1974) 853.
3 K. R. Latham, J. C. Ring and J. D. Baxter, *J. Biol. Chem.*, 251 (1976) 7388.
4 J. W. Apriletti, N. L. Eberhardt, K. R. Latham and J. D. Baxter, *J. Biol. Chem.*, 256 (1981) 12094.
5 N. L. Eberhardt, J. C. Ring, L. K. Johnson, K. R. Latham, J. W. Apriletti, R. N. Kitsis and J. D. Baxter, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 5005.
6 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 190 (1980) 297.
7 D. F. Sellitti, Y. L. Tseng and K. R. Latham, *Cancer Res.*, 43 (1983) 1030.
8 G. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660.
9 P. J. Munson and D. Rodbard, *Anal. Biochem.*, 107 (1980) 220.
10 R. E. Ulane, J. E. Graeber, J. W. Hansen, L. Liccini and M. Cornblath, *Life Sci.*, 31 (1982) 3017.
11 A. J. Perlman, F. Stanley and H. H. Samuels, *J. Biol. Chem.*, 257 (1982) 930.
12 A. Pascual, J. Casanova and H. H. Samuels, *J. Biol. Chem.*, 257 (1982) 9640.