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INTRACELLULAR THYROXINE-BINDING PROTEINS IN RABBIT MUSCLE AND LIVER

KRISTIAN LIEWENDAHL*, **

Department of Medicine, School of Medicine, University of California and Veterans Administration Hospital, San Francisco, Calif. (U.S.A.)

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SUMMARY

Cellular thyroxine-binding proteins in rabbit skeletal muscle and liver were studied by gel filtration, ion-exchange chromatography and polyvinyl chloride electrophoresis. Soluble protein fractions with affinity for thyroxine were detected in muscle and liver extracts by gel filtration. The fraction with the highest relative affinity for thyroxine was further purified. In both tissues this fraction was found to consist mainly of two cellular thyroxine-binding proteins when analysed by ion-exchange chromatography. Electrophoresis showed that these fractions were not completely homogeneous. The cellular thyroxine-binding proteins thus isolated had different electrophoretic mobilities than rabbit serum thyroxine-binding protein. The thyroxine-binding capacities of the isolated cellular thyroxine-binding proteins were studied by electrophoresis. The maximal binding capacities varied from 3.5 to 10.5 μg thyroxine per mg protein. The total binding capacity of both muscle and liver extract was so large that it was not possible to completely saturate it with the electrophoretic technique. It was over 600 μg thyroxine per 100 ml supernatant. The easy release of thyroxine from cellular thyroxine-binding proteins during the purification procedure indicates a rather low affinity of cellular thyroxine-binding proteins for thyroxine. The physiological function of cellular thyroxine-binding proteins is not known.

INTRODUCTION

The importance of the interaction between thyroxine (T_4) and serum proteins for the transport of this hormone has long been recognized. However, little is known about the transfer of T_4 from the extra- to the intracellular compartment. As cellular T_4-binding protein(s) (C-TBP) may participate in this process, studies of the interaction between T_4 and tissue proteins are required.

Tata[1] presented evidence for the existence of a specific tissue protein in rat

* International Research Fellow, U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, U.S.A.

** Present address: K. Liewendahl, M. D., Senior Research Fellow, Endocrine Research Unit, University of Helsinki, P.O. Box 819, Helsinki 10, Finland.

muscle capable of binding T_4. This C-TBP had less affinity for T_4 than the T_4-binding protein in serum. A higher degree of purification of C-TBP from rat liver was reported by Hamada *et al.*[2]. Electrophoretic studies on C-TBP from rabbit muscle and brain performed by Manté-Bouscayrol *et al.*[3] revealed the protein fractions that bound T_4. These C-TBP also had less affinity for T_4 than serum T_4-binding proteins. Recently Hocman[4] performed a systematic gel filtration study on rat liver C-TBP. A number of T_4-binding protein fractions were observed.

The present paper describes gel filtration, ion-exchange column chromatography and polyvinyl chloride electrophoresis for the demonstration and further purification of soluble C-TBP from rabbit muscle and liver. The T_4-binding capacities for the crude extracts and the isolated C-TBP were studied.

MATERIAL AND METHODS

Preparation of muscle and liver

Rabbits weighing about 1.5 kg were used. Anaesthesia was obtained by intraperitoneal injection of Nembutal®, and the lower-limb muscles were perfused with 0.9% NaCl to obtain blood-free material. The rabbit was then killed by bleeding and the liver was dissected. The liver was further perfused by careful injection of saline through the portal vein with a syringe. Muscle tissue was freed from fasciae and fat before it was sliced. Homogenization was done in 0.25 *M* sucrose, using a muscle to sucrose ratio of 1:4 (w/v). The homogenate was centrifuged at high speed ($105.000 \times g$) for 30 min at 2° to obtain a supernatant free of any particulate material The supernatant was ultrafiltered at 4° to about one-fifth of its initial volume and stored at −20° before use. The perfused liver was sliced and homogenized in a similar way to muscle. Centrifugation caused some extracted fat from liver to rise to the surface; from beneath this layer a clear supernatant was collected.

Gel filtration on Sephadex G-200

The clear supernatant was incubated with carrier-free ^{125}I-labelled radiothyroxine (RT_4) (Abbott Labs., North Chicago, Ill., U.S.A.), using 0.2–0.4 μCi/ml supernatant, for 1 h in a refrigerated room (4°), where all subsequent fractionation of protein was performed. Sephadex G-200 gel particles were packed into a glass column (70×3 cm I.D.) in diethyl barbiturate (pH. 8.6; 0.5 *M*). A 5-ml sample of supernatant was applied to the column. Elution with a flow-rate of 30 ml/h was performed with the buffer and 5-ml fractions were collected automatically. After fractionation, radioactivity was counted in a well-type scintillation detector and the absorbance at 280 nm was measured. The protein fraction with the highest relative specific radioactivity was ultrafiltered for further processing. Relative specific radioactivity was defined as the ratio of counts per min to the relative protein concentration measured by absorbance at 280 nm.

DEAE-cellulose column chromatography

DEAE-cellulose, Whatman DE-52 (W. & R. Balston, Maidstone, Great Britain), was equilibrated with Tris–HCl buffer (pH 8.5; 0.25 *M*) and packed into a glass column (17×2.5 cm I.D.). A 3-ml dialysed sample from the gel filtration step was pipetted on to the column. Elution using a flow-rate of 25 ml/h was initially

employed with a 0.25 *M* Tris–HCl buffer. Molarity was increased stepwise to 0.5 and 1.0 *M*, pH being kept constant. Fractions of 5 ml were collected. Radioactivity and absorbance were measured. The fractions with the highest relative specific radioactivity were concentrated by ultrafiltration.

Polyvinyl chloride electrophoresis

Polyvinyl chloride (Pevikon C-870; Stockholms Superfosfat, Stockholm, Sweden) was soaked in Tris–HCl buffer (pH 8.5; 0.1 *M*). The Pevikon material was moulded into a block 14 cm broad × 30 cm long × 0.7 cm thick in an electrophoresis cell. A 0.5-ml dialysed sample from the column chromatography step was applied near the cathode end of the block, as were unfractionated dialysed muscle and liver supernatants and diluted rabbit serum incubated with RT_4. A current of 450 V and 60 mA was switched on and the electrophoresis was run for 17 h. Bromphenol blue was used as a marker to check the movement of serum albumin. The Pevikon block was cut into 1-cm pieces for measurement of radioactivity. The proteins were eluted from the pieces of Pevikon with 5 ml buffer. Pevikon and buffer were separated by centrifugation and the absorbance of the supernatant was measured.

The maximal T_4-binding capacities of the isolated C-TBP were estimated by displacement with increasing amounts of stable T_4 (1.0–20 μg T_4/mg protein). The Pevikon electrophoresis system was used to separate bound from free RT_4. In order to free the C-TBP of RT_4 used as marker during the purification procedures dialysis against bovine serum albumin in Tris–HCl buffer was performed. The concentration of protein was determined by the method of Lowry *et al.*[5].

RESULTS

Muscle

The result of fractionation of muscle C-TBP on Sephadex G-200 is seen in Fig. 1. Most of the RT_4 is in the tail portion of the main protein fraction. On paper chromatography in a one dimensional *n*-butanol–0.5 *N* ammonia–ethanol (5:2:1, by vol.) system the radioactivity after extraction with *n*-butanol was identified as T_4. A second labelled fraction was eluted with low-molecular-weight material. After extraction with butanol and paper chromatography this labelled material was found to consist, in addition to T_4, of radioiodide and an unidentified compound moving close to the triiodothyronine marker. Most of the radioiodide was from the commercial RT_4 preparation, which contained 2–5% radioiodide, but some might have been derived from a very small amount of deiodination of T_4 during processing of the muscle extract. When correcting for radioiodide and the unknown labelled compound, this second C-TBP fraction bound about five times less RT_4 than the first fraction and was not therefore studied further.

Fractionation of the major C-TBP was continued on DEAE-cellulose. A number of protein fractions with very varying affinities for RT_4 were found (Fig. 2). Two of these fractions had much higher relative specific radioactivities than the other fractions. After all the protein had emerged from the column, contaminating radioiodide was eluted and, by increasing the molarity of the buffer, RT_4 attached to the ion exchanger was released.

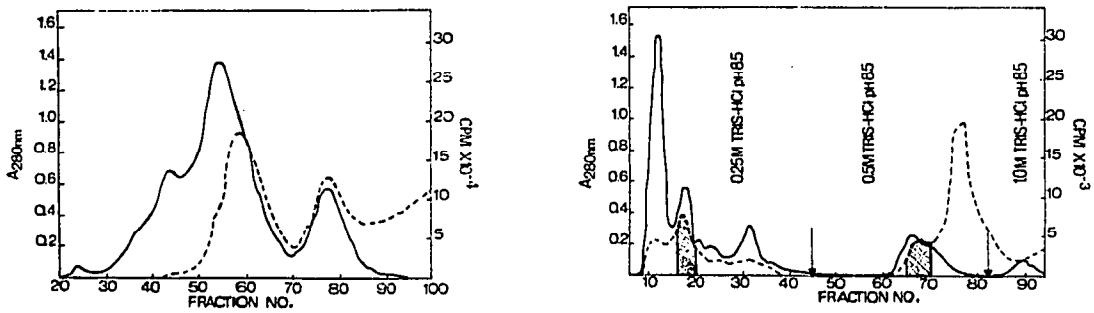


Fig. 1. Gel filtration on a Sephadex G-200 column (70 × 3 cm I.D.) of rabbit skeletal muscle extract incubated with ^{125}I-labelled RT_4. 5-ml fractions were collected .——, Absorbance at 280 nm; – – –, radioactivity.

Fig. 2. DEAE-cellulose column chromatography (17 × 2.5 cm I.D.) of the major T_4-binding fraction obtained by gel filtration of proteins from rabbit muscle. 5-ml fractions were collected. Hatched areas depict radioactive fractions studied by electrophoresis (see Fig. 3). ———, Absorbance at 280 nm; – – – –, radioactivity.

The unfractionated muscle supernatant contained two major and incompletely separated C-TBP fractions on polyvinyl chloride electrophoresis (Fig. 3a). The muscle supernatant accommodated 600 μg T_4 per 100 ml before significant displacements of RT_4 took place. The maximal T_4-binding capacity could not be measured with this technique because of the very large capacity for T_4 found in the supernatant. The results of electrophoresis of the two isolated C-TBP (T_4-1 and T_4-2) are shown in Figs. 3b and c. Yields from nine column chromatographic fractionations of muscle

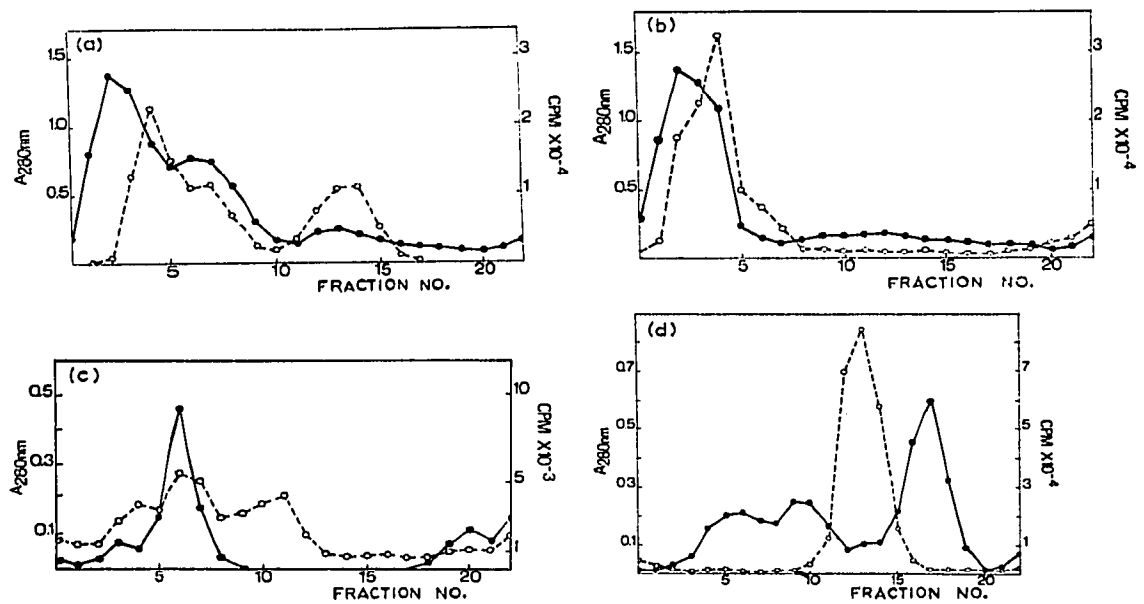


Fig. 3. Polyvinyl chloride electrophoresis of T_4-binding protein fractions in rabbit muscle. (a) Unfractionated muscle extract labelled with RT_4; (b) slow mobility C-TBP obtained from the column chromatography fractionation; (c) fast mobility C-TBP; (d) rabbit serum labelled with RT_4. ——, Absorbance at 280 nm; – – –, radioactivity.

extract were pooled to obtain sufficient material. The curves depicting protein and radioactivity for T_4-1 did not fully coincide. RT_4 added to rabbit serum had a different electrophoretic mobility from either T_4-1 or T_4-2; it moved with the α-globulin fraction containing the T_4-binding globulin (Fig. 3d).

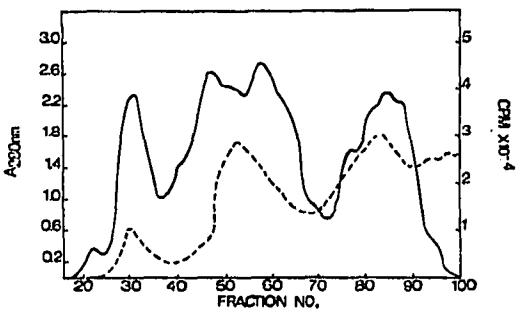
T_4-1 was enriched with increasing quantities of stable T_4 and labelled with RT_4 and thereafter analysed electrophoretically. No loss of radioactivity as free RT_4 was observed until a T_4 concentration of 10.5 μg/mg protein was reached. When adding increments of stable T_4 to T_4-2, apparent loss of label was not detected until 3.5 μg T_4 per mg protein had been added.

Liver

Fractionation of liver C-TBP was started by gel filtration (Fig. 4). Three radioactive fractions were eluted. A high-molecular-weight fraction that was eluted early had a low relative specific radioactivity and was not analysed further. The second radioactive peak had the highest relative specific radioactivity and was collected for more detailed studies. Testing by paper chromatography showed this radioactivity to move in a similar way to marker T_4.

A minute amount of radioactivity moved close to the triiodothyronine marker. Much radioactivity was also eluted with the low-molecular-weight material. On paper chromatography this radioactivity was heterogeneous, consisting, in addition to RT_4, of radioiodide and two unknown labelled compounds, although only in minute amounts. The relative specific radioactivity of this third fraction was thus four to five times lower than that of the second.

On column chromatography (material from seven gel filtration experiments was collected) using DEAE-cellulose a number of protein fractions with highly varying affinities for RT_4 were observed (Fig. 5). The two C-TBP fractions with the clearly highest relative specific radioactivities were studied by electrophoresis. In the unfractionated liver supernatant there were two incompletely separated C-TBP fractions (Fig. 6a). This liver supernatant did not start to displace RT_4 until a T_4



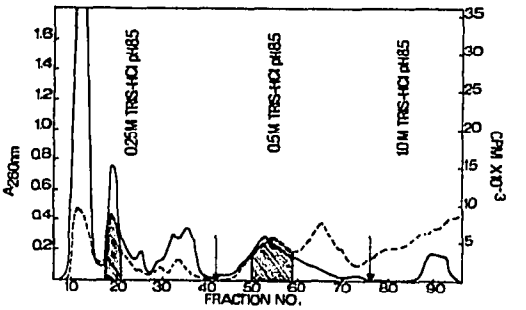


Fig. 4. Gel filtration on a Sephadex G-200 column (70 × 3 cm I.D.) of rabbit liver extract incubated with RT_4. 5-ml fractions were collected. ——, Absorbance at 280 nm; – – –, radioactivity.

Fig. 5. DEAE-cellulose column chromatography (17 × 2.5 cm I.D.) of the major T_4-binding fraction obtained by gel filtration of proteins from rabbit liver. 5-ml fractions were collected. Hatched areas depict radioactive fractions studied by electrophoresis (see Fig. 6). ———, Absorbance at 280 nm; – – – –, radioactivity.

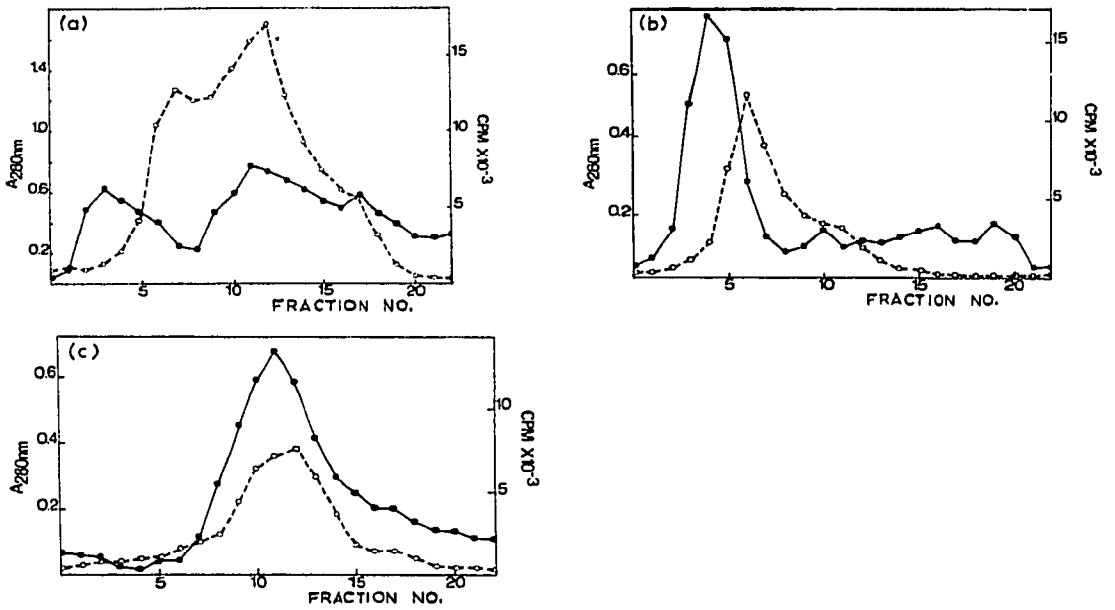


Fig. 6. Polyvinyl chloride electrophoresis of T_4-binding protein fractions in rabbit liver. (a) Unfractionated liver extract labelled with RT_4; (b) slow mobility C-TBP obtained from the column chromatography fractionation; (c) fast mobility C-TBP. ——, Absorbance at 280 nm; – – –, radioactivity.

concentration of 850 μg/100 ml. After purification, electrophoresis revealed that the two C-TBP (T_4-1 and T_4-2) were still not completely homogeneous (Figs. 6b and c). After isolation the T_4-binding capacities of the C-TBP fractions were tested by incremental amounts of stable T_4. The T_4-1 fraction showed loss of label at 5.0 μg T_4/mg protein. For the T_4-2 a higher binding capacity was found. There was no displacement of RT_4 before the T_4 concentration was increased to 8.5 μg/mg protein.

DISCUSSION

A protein fraction capable of binding T_4 was described by Tata[1], but the experiment gave no information about the homogeneity of this fraction. Studies by Manté-Bouscayrol *et al.*[3] on C-TBP in rabbit muscle and brain disclosed two binders in each tissue. We found two C-TBP in unfractionated rabbit muscle. When muscle C-TBP was purified by gel filtration and ion-exchange column chromatography, a higher degree of heterogeneity was found, but two C-TBP fractions had a distinctly higher affinity for T_4 than any other protein fraction. The electrophoretic behaviour of these C-TBP was similar to that already observed in unfractionated supernatant. So far an explanation for the fact that the protein curve and RT_4 peak of both muscle and liver T_4-1 did not fully coincide cannot be given, although the reason may be heterogeneity of this fraction. This phenomenon also occurred when checking with an agarose gel electrophoresis system.

The two liver C-TBP found differed in their electrophoretic mobilities from that of serum TBP. Partial purification of a C-TBP from rat liver was also reported by Hamada *et al.*[2] In rabbit liver, however, we found two main C-TBP.

During the purification procedures much RT_4 was released from C-TBP. This considerable amount of RT_4 eluted as non-protein-bound on gel filtration and ion-exchange chromatography gave the impression that the C-TBP have a rather low affinity for T_4. Some deiodination of RT_4 by both muscle and liver extracts might have occurred because the tissues contain thyroxine-deiodinating activity[6]. However, deiodination cannot have contributed very much to the radioiodide because the protein fractionations were carried out at 4°. Purified commercial preparations of RT_4 and inhibitors of T_4-deiodinase are now being used to clarify this point.

The problem of getting tissue extracts free from any contaminating serum has been thoroughly investigated[3]. The possibility of having interfering serum TBP therefore seems to be minimal. Creese *et al.*[7] found that interstitial fluid from guinea-pig muscle contained all the major protein constituents of serum. This might seem a serious problem to overcome when studying C-TBP, but if there were some serum TBP from the interstitial fluid in the present experiments, it should have moved in a similar way to rabbit serum TBP on electrophoresis and, therefore, differently from the C-TBP.

Displacement studies with increasing amounts of stable T_4 showed very high binding capacities for the unfractionated supernatant of muscle and liver. However, the capacities of the isolated C-TBP varied from 3.5 to 10.5 μg T_4/mg protein. Compared to these figures human T_4-binding globulin and thyroxine-binding prealbumin have higher affinities for T_4 (ref. 8). However, a further increase in the purity of C-TBP may demand a re-evaluation of these figures. Furthermore, determination of the association constants must await further proof of the purity of C-TBP. At present the data on C-TBP agree with the impression that C-TBP have a low affinity but fairly high capacity for T_4.

The biological significance of C-TBP is not known. The possibility that C-TBP regulates T_4 transport from the blood into the cell deserves consideration. C-TBP might also function as a hormone carrier of T_4 to the nucleus. Evidence indicates that the nucleus could be the primary site of action for thyroid hormone[9].

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