Thyrotrohin binding glycoprotein
isolated from bovine thyroid

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Abstract. A butanol-water extraction of bovine thyroid plasma membranes was used to solubilize a thyrotrophin receptor. This method was found to solubilize approximately 12% of membrane proteins and 40% of the binding capacity of thyroid membranes for $^{125}$I TSH. Thyrotrophin binding proteins were further purified 10 times over the butanol-water extract and 70 times over the plasma membranes by means of chromatography on DEAE-cellulose and AcA-54 Ultrogel columns. Purified fractions were found to be glycoproteins containing galactose, mannose, galactosamine, glucosamine and sialic acid. A minute amount (6.0 μg per sample) of two glycoprotein fractions obtained after chromatography on AcA-54 Ultrogel caused about 50% inhibition of $^{125}$I TSH binding to thyroid plasma membranes. This inhibition was due to specific interaction between thyrotrophin and isolated glycoproteins.

The nature of the thyroid plasma membrane thyrotrophin receptor remains to be established. Although several detergents have been used to solubilize TSH binding proteins from thyroid membrane (Nussey & Mehdi 1974; Tate et al. 1975a, b; Dawes et al. 1978; Czarnocka et al. 1979) further purification and characterization were complicated either by the presence of a detergent in the solubilizate or by changes in the conformation of the putative receptor after its dissociation from the membrane or membrane components. To avoid interaction between detergents and solubilized proteins, butanol-water extraction of membrane proteins was used in this study. Extracted proteins were purified by chromatography on DEAE-cellulose and on AcA-54 Ultrogel.

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Materials and Methods

Freshly obtained bovine thyroids (2 kg) were minced and homogenized in a blender with 8 l of 10 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA. After filtration through cheese cloth the homogenate was centrifuged at 10 000 × g for 15 min. The pellet was washed with 2 l of the same buffer and centrifuged. The crude membranes were resuspended in 6 l of Tris-HCl buffer and extracted with 4 l of butanol overnight at 4°C. The extract was centrifuged at 2000 × g for 30 min and the water phase collected. The thick interphase layer was washed with 3 l of water and the water phase again collected by centrifugation at 2000 × g for 30 min. Both water phases were pooled, concentrated by ultrafiltration through a Visking tube (0.9 cm in diameter) and dialysed overnight against 3 l of 1% butanol in water at 4°C. After dialysis the extract was centrifuged at 10 000 × g for 20 min.

The supernatant was applied to a DEAE-cellulose column 3.5 × 40.0 cm and eluted with 1 l of 0.1 M ammonium acetate followed by 1 l of 0.25 M ammonium acetate (F-3) and 1 l of 0.5 M ammonium acetate solution (F-4); all solutions contained 1% butanol. Fractions eluted with each solvent were pooled, concentrated by ultrafiltration and dialysed for 48 h against 3 l of 1% butanol in water. The fraction eluted with 0.1 M ammonium acetate was enriched with methanol and chloroform to obtain a 1:2:1, chloroform/methanol/water solvent. This mixture was left for 1 h at 4°C and then centrifuged at 10 000 × g for 20 min. The soluble (F-1) and insoluble fractions (F-2) were collected, organic solvents were evaporated in a rotary evaporator and the volume was made up to 100 ml with 1% butanol in water. The concentrations of proteins, hexose, sialic acid and thyroglobulin in all 4 fractions were estimated as was their activity in inhibiting $^{125}$I TSH binding to thyroid plasma membranes. Fractions F-1 and F-4 were purified further by chromatography on AcA-54 Ultrogel. Twenty
mg of protein from each of the two fractions (F-1 and F-4) was applied to a 1.5 × 60 cm column and eluted with 10 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA. 1.5 ml fractions were collected and their activity in inhibition of [125I]TSH binding to thyroid plasma membranes estimated. Active fractions were pooled and concentrated, and their chemical composition and activity inhibition of thyrotrophin binding were evaluated.

Crude bovine thyroid plasma membranes were prepared by a slightly modified method of Amir et al. (1973). Thyroid glands were cut into small pieces and homogenized in a blender with 50 columns of 1 mM NaHCO3, pH 7.4 for 1 min. After filtration through four layers of cheesecloth, the homogenate was centrifuged for 10 min at 500 × g, the pellet was discarded and the crude membrane preparation was collected after centrifugation of the supernatant at 10 000 × g for 10 min. Thyroid plasma membranes were purified further by a discontinuous sucrose gradient ultracentrifugation as described by Feni et al. (1978). Membranes separated between 37% and 41% sucrose after 3 h centrifugation at 105,000 × g in a Spincod-Beckman ultracentrifuge model L5-40 with SW-27 rotor were collected, washed with 10 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA and stored at −20°C before use.

Highly purified bovine TSH (gift of dr. J. Pierce, University of California, Los Angeles, USA) was labelled with [125I] (Amersham, England) with use of iodogen (1,3,4,6-tetrachloro 3/a,6/a, diphenyl glycoluril) as described by Fraker & Speck (1978). Labelled thyrotrophin was purified by the receptor method of Dawes et al. (1978) and stored at −20°C for up to four weeks. Binding of [125I]TSH to plasma membranes was studied as described by Tate et al. (1975a,b). Briefly, thyroid plasma membranes (20 μg of protein) were suspended in 0.1 ml of cold 25 mM Tris-acetate buffer pH 6.0 containing 0.1% BSA and 1 mM EDTA and incubated in an ice-water bath for 30 min with [125I]TSH (about 10 000 cpm). Next 0.5 ml of cold buffer without BSA was added and the samples were mixed and centrifuged for 10 min at 10 000 × g. The supernatant was discarded and the radioactivity in the pellet counted in a 1270 Rack-Gamma Wallac-LKB counter. The displacement of [125I]TSH binding to thyroid plasma membranes by unlabelled thyrotrophin (Ambinon-Organon) was carried out with every membrane and label preparation. The inhibition of [125I]TSH binding to thyroid membranes was also investigated in the presence of a given quantity of glycoprotein fractions dissolved in the same buffer. Every estimation was done in triplicate.

Analytical methods. Sialic acid was estimated as described by Svennerholm (1957), hexoses by the method of Dubois et al. (1956), sphingosine by the method of Lauter & Trams (1962), hexosamine as described by Rondle & Morgan (1955), fatty acids by the method of Pinelli (1973) protein concentration by the method of Lowry et al. (1951), and thyroglobulin by the method of Kielczynski (in press).

Acid hydrolysis for hexosamine estimation was carried out in 4 N HCl for 18 h at 100°C; 10% destruction of aminosugars during hydrolysis was accounted for. The hydrolysis of sugars for paper chromatography analysis was carried out in 2 N HCl for 4 h at 100°C. When hydrolysis was completed the samples were dried in a vacuum dessicator under P2O5 and NaOH pellets. Me-

![Fig. 1.](image_url)

[125I]TSH binding curve to thyroid plasma membranes before and after extration with n-butanol.

- - After extraction. - - Before extraction.
Table 1.
Chemical composition of fractions obtained after chromatography on DEAE-cellulose column.

<table>
<thead>
<tr>
<th>Fraction eluted</th>
<th>Protein (mg)</th>
<th>Hexose (mg)</th>
<th>Sialic acid (mg)</th>
<th>Activity* (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M ammonium acetate, soluble in chloroform:methanol:water 1:2:1 solvent (F-1)</td>
<td>427</td>
<td>121</td>
<td>22.5</td>
<td>49</td>
</tr>
<tr>
<td>0.1 M ammonium acetate, insoluble in chloroform:methanol:water 1:2:1 solvent (F-2)</td>
<td>99</td>
<td>32</td>
<td>6.0</td>
<td>&gt;500</td>
</tr>
<tr>
<td>0.25 M ammonium acetate (F-3)</td>
<td>145</td>
<td>34</td>
<td>10.7</td>
<td>&gt;500</td>
</tr>
<tr>
<td>0.5 M ammonium acetate (F-4)</td>
<td>413</td>
<td>127</td>
<td>13.5</td>
<td>18</td>
</tr>
</tbody>
</table>

* Activity expressed as µg of protein necessary to obtain 50% inhibition of [125I]TSH binding to thyroid plasma membranes.

Table 2.
Chemical composition of fractions obtained after chromatography on AcA-54 ultrogel column.

<table>
<thead>
<tr>
<th>Fraction applied</th>
<th>Tubes No. collected after chromatography pooled and analyzed</th>
<th>Protein (%)</th>
<th>Hexose (%)</th>
<th>Hexosamine (%)</th>
<th>Sialic acid (%)</th>
<th>Sphingosine (%)</th>
<th>Fatty acids (%)</th>
<th>Activity* (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-I</td>
<td>28–48</td>
<td>37.0</td>
<td>28.4</td>
<td>22.0</td>
<td>10.0</td>
<td>0.53</td>
<td>2.0</td>
<td>5.7</td>
</tr>
<tr>
<td>F-I</td>
<td>49–75</td>
<td>45.8</td>
<td>27.5</td>
<td>18.0</td>
<td>8.5</td>
<td>0.17</td>
<td>0.1</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>F-4</td>
<td>25–55</td>
<td>45.0</td>
<td>26.2</td>
<td>25.9</td>
<td>2.3</td>
<td>0.1</td>
<td>–</td>
<td>6.7</td>
</tr>
<tr>
<td>F-4</td>
<td>60–80</td>
<td>66.0</td>
<td>17.7</td>
<td>8.5</td>
<td>7.8</td>
<td>0.1</td>
<td>–</td>
<td>&gt;100.0</td>
</tr>
</tbody>
</table>

* Activity expressed as µg of protein necessary to obtain 50% inhibition of [125I]TSH binding to thyroid plasma membranes.

thanolysis for sphingosine and fatty acid estimation was performed in 1 N methanol-HCl at 80°C for 18 h. Recovery of fatty acids after methanolysis was by extraction with n-heptan. Descending paper chromatography of sugars was carried out on Whatman No 1 paper in the upper phase of ethyl acetate: pyridine: water, 2:1:2 v/v/solvent. Sugar spots were visualized by the silver nitrate sodium hydroxide method of Travelyan et al. (1950).

Results

Crude plasma membranes obtained from 2 kg of bovine thyroid glands contained 10 400 mg of protein. 1456 mg of protein was found to be present in the water phase after n-butanol extraction of the crude membranes fraction showing that approximately 14% of membrane proteins were solubilized by this method. The comparison of [125I]TSH binding to crude membranes and to solubilized proteins (expressed as a per-cent of the hormone bound by mg of protein) revealed that about 40% of thyrotrophin binding capacity was extracted from membranes and was present in water (Fig. 1).

Four different fractions were separated after chromatography of the water-butanol extract on a DEAE-cellulose column. Some fractions did not contain measurable amounts of thyroglobulin. Their chemical composition is shown in Table 1. Two fractions, one eluted with 0.1 M ammonium acetate and soluble in chloroform: methanol: water, 1:2:1 v/v, and the other eluted with 0.5 M ammonium acetate, were found to be active in inhibiting [125I]TSH binding to thyroid membra-
Fig. 2.
Effect of glycoprotein fractions on $[^{125}\text{I}]$TSH binding to thyroid plasma membranes. •—• Fraction 1. ■■■ Fraction 4. Δ·Δ Cold TSH (Ambinon).

Fig. 3.
Effect of pre-incubation of thyroid plasma membranes with glycoprotein fraction on $[^{125}\text{I}]$TSH binding.
— Thyroid membranes. —— Thyroid membranes pre-incubated with fraction 1. ···· Thyroid membranes pre-incubated with fraction 4. ··· ··· Non-specific binding to thyroid membranes in the presence of 10$^5$ μIU of cold TSH.

nes. Both these fractions are heterogenous mixtures of glycoproteins containing a high amount of sialic acid. In addition, the fraction soluble in chloroform: methanol: water contains 2% of fatty acids. Two others fractions (F-2 and F-3) did not influence $[^{125}\text{I}]$TSH binding to plasma membranes, although they were shown also to contain large amounts of sugars and sialic acid. The active fractions (F-1 and F-4) were purified further by chromatography on an Ultrogel AcA-54 column. Neither fractions sedimented after centrifugation at 105,000 x g for 1 h. Components active in inhibi-
tion of $[\text{125I}]$TSH binding to thyroid plasma membranes migrated on the above column as proteins of an apparent molecular weight close to 70 000 daltons.

As shown in Table 2 both components are glycoproteins containing galactose, mannose, glucosamine, galactosamine and sialic acid. In addition, small amounts of lipids were found to be present in fraction 1 (2% of fatty acid and 0.5% of sphingosine). Fig. 2 demonstrates that glycoproteins with the molecular weight of about 70 000 daltons isolated after chromatography of fraction F-1 or fraction F-4 produced 50% inhibition of $[\text{125I}]$TSH binding to plasma membranes at low protein concentrations (5.7 and 6.7 μg, sample respectively).

It was of interest that glycoproteins isolated from fraction 1 at a concentration below 2.0 μg of protein sample increased binding of labelled thyrotrophin to membrane by approximately 10%. To assess the possible mechanism of action of active glycoproteins the purified thyroid plasma membranes were pre-incubated with the above components for 1 h at 37°C. The glycoproteins were then washed out and the binding of $[\text{125I}]$TSH to membranes was examined. As shown in Fig. 3 the binding patterns of TSH to membranes were not altered by pre-incubated of membranes with either fraction F-1 or fraction F-4. On the contrary, incubation of glycoprotein fractions with $[\text{125I}]$TSH and chromatography of these mixtures on AcA-54 Ultrogel column revealed that the elution pattern of TSH was significantly changed (Fig. 4). While almost 90% of the hormone (when applied alone) migrated as a protein of apparent molecular weight close to 30 000 daltons, the same hormone after pre-incubation with glycoproteins present either in fraction 1 or 4 was eluted at the void volume of the column as a protein of apparent molecular weight greater than 80 000 daltons. This effect could not be produced when the isolated glycoproteins were incubated with iodinated LH or FSH instead of $[\text{125I}]$TSH. These experiments have shown that TSH was bound by the glycoproteins under study and migrated as a high molecular weight complex during chromatography on a AcA-54 column.

Discussion

In a previous paper (Czarnocka et al. 1979) we showed that although Triton X-100 can be employed to solubilize TSH binding proteins from thyroid plasma membranes the presence of a detergent affects both the assay and further purification procedures.

Other investigators (Manley et al. 1974; Tate et al. 1975b; Dawes et al. 1978), have pointed out these difficulties. In this study butanol-water extraction was used successfully and the results show clearly that TSH binding proteins are solubilized preferentially by this method. Butanol extraction has been used to solubilize membrane components, since Morton (1950) used the procedure to solubilize succinate dehydrogenase from mitochondrial membrane. Maddy (1964) reported that 90% of erythrocyte membrane proteins can be solubilized by butanol-water extraction and the procedure has been used by several laboratories to solubilize components of erythrocyte membranes (Whittemore et
al. 1964; Gardas 1976). Purifications of solubilized proteins resulted in the separation of two glycoproteins which bind TSH at a low concentration.

The presence of TSH binding activity in two different glycoprotein fractions, one hydrophobic and soluble in organic solvent (F-1), and the other forming an acidic glycoprotein fraction (F-4) eluted from a DEAE-cellulose column with 0.5 m ammonium acetate, does not necessarily indicate heterogeneity of TSH binding proteins in the thyroid membranes. It is likely that the thyrotrophin receptor forms complexes with other glycoproteins of thyroid membranes during the extraction procedure and co-purifies with them. There are reports (Omodeo-Sale et al. 1978) that some phospholipids inhibit thyrotrophin binding to thyroid plasma membrane by interaction with the membrane itself. To eliminate this possibility isolated glycoproteins were pre-incubated with plasma membranes at 37°C for 1 h and [125]TSH binding was estimated after washing.

We found no significant changes in [125]TSH binding to membrane after incubation with glycoprotein fractions and we have concluded from these experiments that the inhibition of thyrotrophin binding to thyroid plasma membranes results from a direct interaction of isolated glycoproteins with thyrotrophin. After incubation with active glycoprotein fraction 125I labelled thyrotrophin migrates as a high molecular weight complex during chromatography on AcA-54 Ultrogel column.

This experiment, and the lack of reaction of the glycoproteins with FSH-125I and LH-125I, are further indications that the isolated glycoproteins might bind thyrotrophin specifically and migrate together during gel filtration. Czarnocka et al. (1979) reported TSH receptor preparations of molecular weight 30,000 and 130,000, Dawes et al. (1978) found a TSH receptor of a molecular weight 50,000, Tate et al. (1975a,b) found TSH receptor activity in proteins with a molecular weights from 15,000 to 286,000 and Manley et al. (1974) isolated receptor active proteins with molecular weights of 150,000 and 500,000. The apparent differences in the estimated molecular weight of isolated thyrotrophin binding proteins might reflect different conditions used in the preparation procedure and it is possible that the TSH receptor may form complexes or aggregates with other membrane components. Preliminary experiments with labelled glycoproteins suggest that our preparation is a mixture of different glycoproteins and further efforts to isolate the thyrotropin binding entity are under way.

References


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