Analysis of thyrotropin receptors by photoaffinity labelling

Orientation of receptor subunits in the cell membrane

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Porcine thyrotropin (TSH) receptors have been purified by Sepharose–TSH affinity chromatography and crosslinked to a ^{125}I -labelled photoactive derivative (*N*-hydroxysuccinimidyl 4-azidobenzoate; HSAB) of TSH (^{125}I -HSAB-TSH). Purification of the crosslinked complexes on Sephacryl S-300 followed by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate showed that the receptor contained two subunits. One subunit (A) with M_r 45000 was crosslinked to TSH and the other (B) subunit, M_r 25000, was linked to the A subunit by a disulphide bridge(s). Other, as yet unidentified, subunits may have been non-covalently associated with the A and B subunits. Analysis of reduced and non-reduced crosslinked TSH receptor– ^{125}I -HSAB-TSH on Sephacryl S-300 in the presence and absence of detergent indicated that the A subunit was a hydrophilic peptide. This was confirmed in studies of the release into aqueous solution by reducing agent treatment of ^{125}I -HSAB-TSH crosslinked to the TSH receptor A subunit in thyroid membranes. Similar results were obtained with TSH receptors in human thyroid and guinea pig fat cell membranes. These studies suggest that the hydrophilic A subunit of the receptor forms a binding site for TSH on the outside surface of the cell membrane and that the A subunit is linked to the cell membrane by way of a disulphide bridge to the receptor B subunit.

Recent studies using a photoactive crosslinking reagent coupled to TSH have shown that the porcine TSH receptor contains two subunits linked by one or more disulphide bridges (Buckland *et al.*, 1982; Buckland & Rees Smith, 1984). The A subunit, which crosslinks to TSH, has an M_r of approx. 45000 and the B subunit, which does not crosslink to TSH, has an M_r of approx. 25000. TSH receptors in different species have similar structures, with small differences in the M_r values of the A and B subunits (Buckland *et al.*, 1982; Buckland & Rees Smith, 1984).

We now describe a more detailed analysis of the TSH receptor using affinity labelling with particular emphasis on the orientation of the two subunits in the cell membrane.

Abbreviations used: TSH, thyrotropin; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; DSS, disuccinimidyl suberate; SDS, sodium dodecyl sulphate.

Methods

TSH preparations

TSH was prepared from bovine pituitaries by using previously described procedures (Pierce *et al.*, 1971). The purified material contained 65–75 units (MRC bovine TSH standard)/mg of protein (Lowry *et al.*, 1951) as judged by TSH receptor assay (Southgate *et al.*, 1984). Purified TSH was coupled to the photoactive crosslinking reagent HSAB (Pierce Chemicals) in the ratio of 2 molecules of HSAB/molecule of TSH as described previously (Buckland *et al.*, 1982). TSH or HSAB-TSH were labelled with ^{125}I to a specific activity of $50\mu\text{Ci}/\mu\text{g}$ by using the Iodogen method (Fraker & Speck, 1978; Rees Smith & Hall, 1981), purified on a column (2.6 cm $\times$ 35 cm) of Sephadex G-100 in 50 mM-NaCl/10 mM-Tris/HCl, pH 7.5 (Tris/NaCl) and stored at -70°C for up to 2 weeks.

TSH receptor preparations

Crude membrane fractions were prepared from homogenates of human (Graves') thyroid, porcine thyroid or guinea pig epididymal fat pads by differential centrifugation (Rees Smith & Hall, 1981; Teng *et al.*, 1975). Solubilization of the membranes was carried out by homogenization in Lubrol 12A9 [ICI Organics; 1% (w/v) in Tris/NaCl] containing 15 mM-iodoacetamide and 0.1 mM-phenylmethanesulphonyl fluoride (Sigma), followed by centrifugation at 100000g for 2 h at 4°C (Rees Smith & Hall, 1981). In some experiments, solubilized porcine thyroid membranes were purified by affinity chromatography on Sepharose-TSH (Rickards *et al.*, 1981). A weight of 1 g of porcine or human thyroid gave a crude membrane preparation which contained about 50 mg of protein (Lowry *et al.*, 1951), and 1 g of guinea pig fat gave a preparation which contained about 2 mg of protein. Non-

purified solubilized membrane preparations contained about 1 mg of protein/ml and affinity-purified porcine TSH receptors contained about 10 µg of protein/ml. The TSH binding activities of the various preparations were determined by receptor assay (Rees Smith & Hall, 1981).

Gel electrophoresis

Polyacrylamide-gel electrophoresis in 0.1% sodium dodecyl sulphate was carried out using a modification of the method of Laemmli (1970) with 8–12% or 5–12% gradient gels. Samples were diluted with an equal volume of 4% SDS with or without reducing agent (2% mercaptoethanol or 20 mM-dithiothreitol) and centrifuged (14000g, 2 min) before application to the gel. After electrophoresis, the gels were fixed and stained in Coomassie Blue solution and dried. Autoradiography was carried out at -70°C using Kodak X-OMAT RP film and Cronex Lightning Plus enhancing screens (Dupont). The following M_r standards were run on each gel: thyroglobulin (320000), β -galactosidase (116000), phosphorylase b (94000), transferrin (77000), bovine serum albumin (67000), immunoglobulin G and its com-

Fig. 1. Affinity-purified porcine TSH receptors (5 ml) crosslinked to ^{125}I -HSAB-TSH (5 ml; 40×10^3 c.p.s.) and chromatographed on Sepharose S-300.

See the text for experimental details. Fractions were pooled as indicated, concentrated to 5 ml and rerun.

Fig. 2. Affinity-purified porcine TSH receptors crosslinked to ^{125}I -HSAB-TSH (complex) analysed by SDS/polyacrylamide-gel electrophoresis.

Crosslinked complexes were purified by gel filtration as shown in Fig. 1 and treated with or without reducing agent and with or without heating (100°C, 5 min).

ponent chains (150000; 50000 and 23000, respectively), ovalbumin (44000) and TSH (28000). Analyses under reducing and non-reducing conditions used reduced and non-reduced standard preparations respectively.

Crosslinking

Affinity-purified TSH receptors (1–5 ml) were incubated in the dark at room temperature with an equal volume of ^{125}I -HSAB-TSH [(6–10) $\times 10^3$ c.p.s./ml] for 3 h. The material was then photolysed for 20 min at 5 cm from a 50 W 254 nm u.v. lamp and run (15 ml/h) at 4°C on a column (3 cm $\times$ 35 cm) of Sephacryl S-300 in Tris/NaCl containing 0.1% Lubrol (Fig. 1). The peak containing crosslinked complex was collected (Fig. 1), concentrated about 10-fold by ultrafiltration (Amicon

YM-10 membrane) and stored at 0°C for up to 4 weeks.

In experiments with membranes, suspensions (material from 1 g of starting tissue/ml of Tris/NaCl) were preincubated with unlabelled TSH (0–0.5 units/ml) for 10 min at room temperature followed by incubation with an equal volume of ^{125}I -HSAB-TSH for 3 h at room temperature and photolysed. After washing three times by sedimentation (14000g, 5 min) and resuspension in Tris/NaCl, suspensions (material from 1 g of tissue in 200 μl of Tris/NaCl) of the membranes cross-linked to ^{125}I -HSAB-TSH were adjusted to 10 mM-dithiothreitol and incubated for 1 h at room temperature. After centrifugation at 100000g for 1 h at 4°C the supernatants were treated with a 5-fold molar excess of iodoacetamide in 2M-Tris/HCl,

Fig. 3. Analysis of reduced (10 mM-dithiothreitol) and non-reduced crosslinked TSH-TSH-receptor complexes on Sephacryl S-300 with and without detergent

(a) Affinity-purified porcine TSH receptors crosslinked to ^{125}I -HSAB-TSH were run on Sephacryl S-300 in 0.1% Lubrol before and after reduction. Also shown is chromatography of Lubrol micelles labelled with [^3H]oleic acid (see the text for experimental details). (b) Affinity-purified porcine TSH receptors crosslinked to ^{125}I -HSAB-TSH were reduced and run on Sephacryl S-300 in Tris/NaCl in the absence of detergent.

pH 8.2, and either analysed directly by SDS/polyacrylamide-gel electrophoresis or run on Sephacryl S-300 in Tris/NaCl.

In some experiments membranes were preincubated (15 min at room temperature) with 200 μ l of normal human serum (pool of six donors) or serum from a Graves' patient with high levels of autoantibody to the TSH receptor (as measured by the method of Southgate *et al.*, 1984).

Additional crosslinking with DSS

In some experiments, S-300-purified crosslinked complexes were treated with DSS (10 mM in dimethyl sulphoxide) so that the final DSS concentration was 0, 0.5 or 1 mM. After incubation for 15 min at room temperature, aqueous 1 M-ammonium acetate was added to give a 200-fold molar excess over DSS. Samples were then analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography.

Labelling of Lubrol micelles with ^3H

[^3H]Oleic acid in toluene (1 μCi ; Amersham) was added to 1% Lubrol in Tris/NaCl (1 ml) in a glass container and mixed thoroughly. Incorporation of the [^3H]oleic acid into the Lubrol micelle was virtually complete (see Fig. 3).

Results

Analysis by SDS/polyacrylamide-gel electrophoresis of affinity-purified porcine TSH receptors crosslinked to ^{125}I -HSAB-TSH and purified on Sephacryl S-300 (Fig. 1) is shown in Fig. 2. Under reducing conditions bands corresponding to intact TSH (28 000) and TSH crosslinked to the A subunit of the TSH receptor (about 75 000) were observed. Heating of the samples prior to electrophoresis caused the A subunit-TSH complex to dissociate into two bands differing in M_r by about 15 000. Under non-reducing conditions TSH crosslinked to intact TSH receptor (A and B subunits) was observed at about 100 000 and free TSH at 28 000. Heating the non-reduced samples prior to electrophoresis caused the M_r 100 000 band to show some dissociation into slightly lower M_r material. The diffuse background at higher M_r was less evident in reduced and non-reduced samples which had been heated.

Additional crosslinking of the TSH receptor crosslinked to ^{125}I -HSAB-TSH using DSS did not change the M_r of the complex when run under reducing conditions, but there was an increase in diffuse background at higher M_r (results not shown). Similarly, the mobility of the M_r 100 000 band observed under non-reducing conditions was not influenced by DSS but there was considerable

increase in diffuse background at higher M_r (results not shown).

Analysis of crosslinked complexes which had been reduced with 10 mM-dithiothreitol on Sephacryl S-300 in detergent (0.1% Lubrol in Tris/NaCl) or aqueous (Tris/NaCl) buffer is shown in Fig. 3. In Lubrol buffer, the crosslinked complex was resolved into three peaks (Fig. 3a) with K_{av} values (Laurent & Killander, 1964) of 0.14, 0.32 and 0.5. Non-reduced complex was eluted as a peak with K_{av} = 0.14 (Fig. 1), TSH with K_{av} = 0.5 and Lubrol micelles with K_{av} = 0.275 (Fig. 3a).

Analysis of the three peaks by SDS/polyacrylamide-gel electrophoresis showed that the K_{av} = 0.32 component contained the A subunit of the TSH receptor crosslinked to ^{125}I -labelled HSAB-TSH (M_r about 75 000) (Fig. 4). The K_{av} = 0.14 peak did not give a clear band and the K_{av} = 0.5 peak corresponded to TSH (28 000).

When chromatography was carried out in aqueous buffer, only two peaks were observed (Fig. 3b) and SDS/polyacrylamide-gel electro-

Fig. 4. Analysis of Sephacryl S-300 fractions from Fig. 3(a) by SDS/polyacrylamide-gel electrophoresis I, fractions 37-41; II, fractions 44-48; III, fractions 51-57.

Fig. 5. Analysis of Sephacryl S-300 fractions 1 and 2 from Fig. 3(b) by SDS/polyacrylamide-gel electrophoresis. Unfractionated crosslinked complex is labelled 'Complex'.

phoresis indicated that they corresponded to the A subunit of the receptor cross-linked to TSH (M_r about 75000) and free TSH respectively (Fig. 5).

In a separate series of experiments, thyroid or fat cell membranes were crosslinked to ^{125}I -HSAB-TSH, treated with 10 mM-dithiothreitol and centrifuged at 100000g for 1 h at 4°C. Analysis of the supernatants by SDS/polyacrylamide-gel electrophoresis (Fig. 6) indicated that reduction caused the release of the TSH receptor A subunit cross-linked to ^{125}I -HSAB-TSH in the case of both porcine and human thyroid membranes. Human material showed a slightly higher M_r value. No complex was evident in the absence of dithiothreitol (Fig. 6) and release of the A-subunit-TSH complex was inhibited by preincubation of membranes with unlabelled TSH. Preincubation of membranes with serum containing high levels of autoantibodies to the TSH receptor also inhibited release of the A subunit-TSH receptor complex, and the results of such an experiment with fat cell membranes are shown in Fig. 7. Similar results were obtained when human and porcine thyroid membranes were preincubated with Graves' serum (results not shown).

Chromatography on Sephacryl S-300 in Tris/NaCl of the labelled material released from por-

Fig. 6. Release by dithiothreitol (10 mM) treatment of ^{125}I -HSAB-TSH crosslinked to the TSH receptor A subunit in porcine and human thyroid membranes and effect of preincubation with unlabelled TSH.

See the text for experimental details. Sample buffer for SDS/polyacrylamide-gel did not contain mercaptoethanol. The centre lane contained ^{125}I -IgG and other M_r markers and the material in each of the other lanes was derived from the same amount of thyroid membranes (500 μl) and ^{125}I -labelled HSAB-TSH (500 μl). Unlabelled TSH inhibited binding of labelled TSH to the receptor prior to crosslinking and treatment with dithiothreitol. Consequently the amount of non-crosslinked material (M_r 28000 band) was lower in this lane. However the same amount of TSH receptor was present.

cine thyroid membranes by dithiothreitol treatment is shown in Fig. 8. Analysis by SDS/polyacrylamide-gel electrophoresis showed that the shoulder on the leading edge of the major peak (TSH) contained the A subunit of the receptor crosslinked to TSH (Fig. 9). Similar results were obtained with human thyroid and guinea pig fat cell membranes (results not shown).

Discussion

Partial purification of crosslinked ^{125}I -HSAB-TSH-affinity-purified TSH receptor complexes on Sephacryl S-300 prior to analysis by SDS/polyacrylamide-gel electrophoresis and autoradiography gave clearer bands than in previous studies which did not employ the gel-filtration step (Buckland *et al.*, 1982; Buckland & Rees Smith, 1984). Under reducing conditions, the unheated crosslinked complex ran as a single band (M_r about 75000) characteristic of the receptor A subunit (about 45000) combined with intact TSH (28000).

Fig. 7. Release by dithiothreitol (10 mM) treatment of ^{125}I -HSAB-TSH crosslinked to the TSH receptor A subunit in guinea pig fat membranes and effect of preincubation with Graves' serum containing high levels of autoantibody to the TSH receptor

Sample buffer for the SDS/polyacrylamide-gel electrophoresis did not contain mercaptoethanol. The material in each of the lanes was derived from the same amount of guinea pig fat membranes (500 μl) and ^{125}I -labelled HSAB-TSH (500 μl). The Graves' sera inhibited binding of labelled TSH to the receptor prior to crosslinking and treatment with dithiothreitol. Consequently the amount of non-crosslinked material (M_r 28000 band) was lower in this lane. However the same amount of TSH receptor A subunit was present.

On heating in SDS prior to SDS/polyacrylamide-gel electrophoresis, however, a band lower in M_r by about 15000 was also evident, suggesting that some dissociation of TSH subunits (Pierce *et al.*, 1971) in the crosslinked complex had occurred. It would appear therefore that the reduced complexes contained (i) TSH receptors crosslinked to TSH with its α and β subunits also crosslinked and therefore undissociated by heating in SDS and (ii) TSH receptors crosslinked to TSH with its α and β subunits not crosslinked and therefore dissociated by heating in SDS.

Non-reduced crosslinked complexes ran as a single band with an M_r of approx. 100000, charac-

Fig. 8. Release by dithiothreitol treatment of ^{125}I -HSAB-TSH crosslinked to the TSH receptor A subunit in porcine thyroid membranes: analysis of released material by gel filtration on Sephacryl S-300 in Tris/NaCl

Fractions shown were pooled and rerun on the same column (see the text for experimental details).

teristic of a complex containing the A (about 45000) and B (about 25000) subunits of the receptor linked by a disulphide bridge with the A subunit crosslinked to intact TSH. Heated, non-reduced samples showed some evidence of dissociation of TSH subunits but this was not as marked as when analysis was done under reducing conditions. Heating also reduced the amount of aggregated material and diffuse background at higher M_r (Fig. 2).

As the analysis described above was performed in the presence of SDS, other receptor subunits

Fig. 9. Analysis by SDS/polyacrylamide-gel electrophoresis of Sephacryl S-300 fractions from Fig. 8. (a) Analysis of fractions from run A in Fig. 8. I, fractions 31–38; II, fractions 39–46; III, fractions 47–58; IV, fractions 62–64. (b) Analysis of fractions 45–60 (A subunit) from run B in Fig. 8. The reduced IgG marker from the same gel is also shown.

may well have been associated non-covalently with the A and B subunits, and some attempt to assess this possibility was made by adding DSS to cross-linked TSH receptor– ^{125}I -HSAB-TSH complexes. The formation of bands at higher M_r was not evident, but the additional subunits may have been inaccessible to crosslinking with DSS.

When TSH receptors or TSH receptors cross-linked to ^{125}I -HSAB-TSH were solubilized by treatment with non-ionic detergents such as Lubrol, they would tend to become associated with large micelles (Tanford & Reynolds, 1976) and the micelles would be expected to make an important contribution to the low K_{av} observed on gel filtration of non-reduced crosslinked complexes (Fig. 3a). However, analysis of reduced crosslinked complexes by gel filtration on Sephacryl S-300 in 0.1% Lubrol followed by SDS/polyacrylamide-gel electrophoresis showed that under these conditions, the receptor A subunit crosslinked to labelled TSH was eluted from the column with a higher K_{av} than the non-reduced complex or the Lubrol micelles alone (Fig. 3a).

This indicated that the receptor A subunit–TSH complex had a smaller Stokes' radius than the Lubrol micelle and was probably not therefore associated with the micelle. Consequently, the A subunit of the TSH receptor appeared to be a hydrophilic protein and support for this hypothesis

was obtained from analysis of reduced crosslinked complexes on Sephacryl S-300 in the absence of detergent (Fig. 3b).

As the non-reduced crosslinked complex was probably an amphiphilic protein associated with a Lubrol micelle, these observations suggested that the A subunit was linked to the micelle by way of the B subunit. This would imply that the B subunit itself was amphiphilic or that it was linked non-covalently to an as yet unidentified amphiphilic receptor subunit. If this model is correct it should be possible to release the A subunit from particulate porcine thyroid membrane preparations by treatment with reducing agent. The data shown in Fig. 6 indicate that when thyroid membranes crosslinked to ^{125}I -HSAB-TSH were treated with 10mM-dithiothreitol in the absence of detergent, the TSH receptor A subunit crosslinked to labelled TSH was released. Similar results were obtained with TSH receptors in particulate preparations of human thyroid and guinea pig fat cells (Fig. 7), indicating that the TSH receptor A subunit was also hydrophilic in these preparations. Chromatography of material released from the membranes on Sephacryl S-300, as shown in Figs. 8 and 9, confirmed these observations and emphasized the hydrophilic nature of the receptor A subunit.

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