Biochem. J. (1985) **225**, 753–760
Printed in Great Britain

Affinity-labelling of the thyrotropin receptor

Characterization of the photoactive ligand

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(*Received 24 August 1984/Accepted 12 October 1984*)

Thyrotropin (TSH) has been coupled to the photoactive heterobifunctional reagent *N*-hydroxysuccinimidyl 4-azidobenzoate (HSAB) and the properties of the product (HSAB-TSH) investigated. Preparations of HSAB-TSH containing two molecules of HSAB per molecule of TSH were used in most experiments and these preparations retained about 40% of the original receptor-binding activity of the TSH. HSAB-TSH could be labelled with ^{125}I and cross-linked to porcine and human TSH receptors. Analysis of the cross-linked complexes indicated that the receptors consisted of two subunits (designated A and B) linked by a disulphide bridge. In the case of the human TSH receptor, the A- and B-subunits had approximate M_r values of 50000 and 30000 respectively, whereas the M_r values for porcine TSH-receptor A- and B- subunits were approx. 45000 and 25000 respectively. Only the A subunit was cross-linked to TSH. Comparison of the effects of trypsin and mercaptoethanol on the TSH–TSH-receptor complexes suggested that the trypsin cleavage point on the A-subunit was at a point close to the disulphide bridge.

TSH is a glycoprotein hormone consisting of two subunits designated α and β, which have M_r 13600 and 14700 respectively (Pierce *et al.*, 1971). The actions of TSH on the thyroid are mediated by a cell-surface receptor, but little is known about the receptor's structure. Recently we reported the cross-linking of ^{125}I-labelled TSH to its receptor with the photoactive heterobifunctional reagent HSAB coupled to TSH (HSAB-TSH) and the homobifunctional reagent DSS (Buckland *et al.*, 1982; Buckland & Rees Smith, 1984). These preliminary studies suggested that the TSH receptor was made up of two non-identical subunits linked by a disulphide bridge. We now describe some of the properties of HSAB-TSH and further studies on the structure of the human and porcine TSH receptors.

Methods

Preparation of TSH

TSH was extracted from bovine pituitaries and purified by previously described procedures (Pierce *et al.*, 1971). The specific activity of the purified preparations was 50–70 MRC (W.H.O.) bovine TSH standard units/mg of protein (Lowry *et al.*, 1951) when measured by TSH-receptor assay (Southgate *et al.*, 1984).

Preparation of HSAB-TSH

Highly purified bovine TSH (200 μg) was dissolved in 200 μl of 150 mM-NaCl/20 mM-$NaHCO_3$, pH 7.7. HSAB (Pierce Chemicals) was dissolved in DMSO to give a solution with a concentration of 20 mM, and two aliquots of 1 μl each were added to the TSH at intervals of 5 min while the mixture was agitated on a rotary shaker at room temperature in the dark. After a further 15 min an excess of glycine (5 μl; 1 M) was added and incubation was continued for 10 min. The free HSAB was separated from HSAB-TSH by column chromatography using a column (150 mm × 9 mm) of Sephadex G-25 in 200 mM-K_2HPO_4 adjusted to pH 7.5 with conc. HCl.

Samples of HSAB-TSH were also prepared with different amounts of HSAB in the reaction mixture.

Preparation of ^{125}I-labelled HSAB-TSH

Highly purified bovine TSH and HSAB-TSH were labelled with ^{125}I to a specific radioactivity of

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

50 μCi/μg by using the Iodogen method (Fraker & Speck, 1978; Rees Smith & Hall, 1981). Free and TSH-bound ^{125}I were separated on a 200ml column of Sephadex G-100, run in 50mM-NaCl/10mM-Tris/HCl, pH7.5 (Tris/NaCl). All manipulations involving HSAB-TSH were carried out in the dark.

TSH-receptor preparations

Crude thyroid membranes were prepared from porcine or human (Graves') tissue and solubilized with 1% Lubrol 12A9 (ICI Organics) in Tris/NaCl (Petersen *et al.*, 1977; Brennen *et al.*, 1980; Rees Smith & Hall, 1981). Detergent-solubilized TSH receptors (containing about 1mg of protein/ml) were purified (approx. 100-fold) by TSH–Sepharose affinity chromatography as previously described (Rickards *et al.*, 1981). The purified receptor preparations contained about 10μg of protein/ml.

Assessment of TSH–TSH-receptor cross-linking

(*1*) *HSAB*. Samples of purified TSH receptors (100μl) were incubated with ^{125}I-labelled HSAB-TSH in Tris/NaCl/bovine serum albumin (100μl; 5000c.p.m.) for 1h at 37°C in the dark. Some samples were photolysed (20min, 5cm below a 50W u.v. lamp, 254nm). NaCl was added to some samples to give a final concentration of 2M to dissociate non-cross-linked ^{125}I-HSAB-TSH from the receptor. The samples were incubated for 10min at room temperature before separation of bound and free ^{125}I-HSAB-TSH by precipitation of the TSH–TSH-receptor complex by addition of poly(ethylene glycol) to 15% (w/v) final concentration, normal IgG (500μg) being used as a co-precipitant (Rees Smith & Hall 1981; Rickards *et al.*, 1981).

(*2*) *DSS*. Samples of purified TSH receptors (100μl) were incubated with ^{125}I-labelled TSH in Tris/NaCl/bovine serum albumin (100μl; 5000c.p.m.) for 1h at 37°C. DSS (Pierce Chemicals) dissolved in DMSO (2μl) was added to some samples to give final DSS concentrations of 1mM, 0.33mM, 0.1mM or 0mM. The samples were incubated at 20°C for 15min, followed by the addition of ammonium acetate (2μl; 1M) and incubated for a further 10min. Samples were treated with NaCl and poly(ethylene glycol) as described above.

Preparation of samples for SDS/polyacrylamide-gel electrophoresis

Affinity-purified porcine or human TSH-receptor preparations were cross-linked to ^{125}I-labelled HSAB-TSH or ^{125}I-labelled TSH as described above. The cross-linked complexes were precipitated (without using IgG as co-precipitant) by addition of poly(ethylene glycol) to a concentration of 25% (w/v) and centrifugation at 14000*g* for 10min at 4°C. The pellets were dissolved in 4% SDS buffer (containing 2% mercaptoethanol in some cases) heated to 100°C for 10min, followed by addition of a 1.5–2-fold molar excess of iodacetamide and heating at 100°C for a further 2min. Finally samples were centrifuged (14000*g*, 15min, room temperature) and applied to the gel.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). Gradient gels of 5–15% or 8–12% (w/v) acrylamide were run at constant voltage (60V) in a Bio-Rad Protean vertical-slab-gel apparatus. Gels were stained with Coomassie Brilliant Blue and dried with a Bio-Rad slab dryer. Autoradiography of dried gels was carried out with Kodak X-OMAT RP film and Cronex lightning plus enhancing screens for 16h at −70°C (Swanstrom & Shank, 1978). Approximate relative intensities were determined by performing scanning densitometry of the autoradiographs.

Trypsin treatment

Cross-linked TSH–TSH-receptor complexes were treated with trypsin after separation of bound and free ^{125}I-labelled TSH or HSAB-TSH by precipitation with poly(ethylene glycol). The pellet was resuspended in 100μl of 1% Lubrol in Tris/NaCl and 1μl of trypsin solution in Tris/NaCl was added to give a final concentration of trypsin varying between 0.00001 and 0.1%. The mixture was incubated for 15min at room temperature; a 1.5-fold excess of trypsin inhibitor was then added and incubation continued for 10min. Samples were prepared for SDS/polyacrylamide-gel electrophoresis by the addition of SDS buffer as described above.

Cross-linking to thyroid membranes

Porcine thyroid membrane suspensions in Tris/NaCl were incubated with an equal volume of ^{125}I-labelled HSAB-TSH in the dark at 37°C for 1h. Samples were photolysed as described above and free and receptor bound ^{125}I-HSAB-TSH separated by centrifugation at 14000*g* for 5min. The pellets were washed (twice) with 1ml of Tris/NaCl and then resuspended in 2M-NaCl/10mM-Tris/HCl, pH7.4 (100μl) for 10min at room temperature to dissociate non-cross-linked TSH. Then 10mM-Tris/HCl, pH7.4 (900μl) was added and the samples pelleted by centrifugation at 14000*g* for 10min. The pellets were then prepared for SDS/polyacrylamide-gel electrophoresis as described above.

Results

HSAB-TSH

HSAB has a molar absorption coefficient ($20900\,M \cdot cm^{-1}$) at 275nm similar to that of TSH ($20100\,M \cdot cm^{-1}$). The number of molecules of HSAB attached to each molecule of TSH could therefore be calculated by comparing the u.v. absorption of HSAB-TSH and TSH. Samples of HSAB-TSH prepared in molar ratios (HSAB/TSH) of 1:1, 2:1, 2.4:1, 5:1 and 7:1 were prepared.

Addition of 2μl of HSAB (20mM) to 200μg of TSH resulted in HSAB-TSH with the ratio 2:1, with about 35% of the HSAB used in the reaction being incorporated into TSH.

Fig. 1 shows the u.v. spectra of TSH, HSAB-TSH (2:1) and HSAB-TSH after photolysis. Preparations of HSAB-TSH in a ratio 2:1 retained approx. 40% of their TSH-receptor-binding activity (Buckland *et al.*, 1982). This binding activity decreased sharply as the ratio of HSAB to TSH increased, but samples with decreased ratio (1:1) did not show significantly greater receptor-binding activity (results not shown).

HSAB-TSH prepared in a ratio of 2:1 was used throughout the experiments described, except when otherwise indicated.

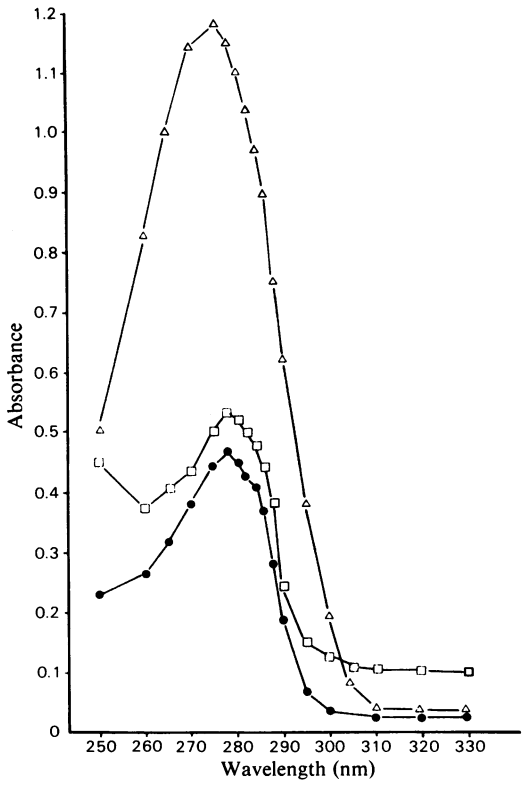

Fig. 1. *U.v. spectrum of TSH and HSAB-TSH*
●, Normal TSH (20μM); △, HSAB-TSH prepared in a ratio of 2 HSAB molecules per molecule of TSH (20μM); □, HSAB-TSH (2:1) after photolysis (20μM).

^{125}I-HSAB-TSH

By using the Iodogen method, ^{125}I was incorporated into HSAB-TSH with high efficiency compared with untreated TSH. In 33 experiments the mean (±S.D.) binding of ^{125}I to HSAB-TSH was 74.7±8.8% compared with 44.8±11.2% for TSH in the same number of experiments. This may have been due to incorporation of ^{125}I into HSAB itself or the influence of HSAB on an adjacent tyrosine residue. The product, ^{125}I-HSAB-TSH gave binding to the TSH receptor of between 17 and 33% (total binding). Non-specific binding was between 3 and 5%. Normal ^{125}I-TSH, which had not been receptor-purified, gave specific binding of about 30% under the same conditions.

Cross-linking efficiency

The addition of NaCl to a final concentration of 2M resulted in almost complete dissociation of TSH–TSH-receptor complexes (Table 1). However, after cross-linking with 1mM-DSS, about 80% of receptor-bound ^{125}I-TSH was undissociable in the presence of 2M-NaCl (Table 1). Lower doses of DSS gave correspondingly lower amounts

Table 1. *Efficiency of TSH cross-linking to porcine TSH receptors using DSS and HSAB*
Affinity-purified detergent-solubilized receptors were incubated with either ^{125}I-TSH or ^{125}I-HSAB-TSH and in some samples cross-linking was then effected by either DSS addition or photolysis (in case of HSAB-TSH). Results are expressed as % total specific binding (B_0) and this was 50% using receptor purified ^{125}I-TSH and 22% using ^{125}I-HSAB-TSH.

Cross-linking method	Treatment of sample	Binding of hormone (B/B_0) (%)
DSS+^{125}I-TSH	Untreated	100
	Photolysed	96
	2M-NaCl	2
	And photolysed	3
	+0.1mM-DSS	20
	+0.33mM-DSS	50
	+1.0mM-DSS	78
^{125}I-HSAB-TSH	Untreated	100
	2M-NaCl	5
	Photolysed	95
	Photolysed +2M-NaCl	55

of cross-linking. Higher doses of DSS did not increase the amount of cross-linking (results not shown). Cross-linking efficiency with ^{125}I-HSAB-TSH was lower, approx. 50% of the bound material being undissociable with 2M-NaCl. Photolysis of samples did not cause significant dissociation of the ^{125}I-HSAB-TSH–TSH-receptor complex (Table 1). Similarly in experiments with ^{125}I-TSH–TSH-receptor complex, photolysis did not significantly effect the total binding, and in the absence of cross-linking there was little or no residual binding of the ^{125}I-TSH after addition of NaCl, whether or not the sample was photolysed.

Analysis of ^{125}I-HSAB-TSH by SDS/polyacrylamide-gel electrophoresis and autoradiography

When not photolysed, ^{125}I-HSAB-TSH gave a pattern of bands similar to that reported for TSH (Liao & Pierce, 1970); two bands corresponding to α- and β-subunits labelled with similar amounts of ^{125}I. When photolysed, however, intact αβ-dimer was present even when samples were heated in 4% SDS solution before analysis.

Analysis of cross-linked complexes by SDS/polyacrylamide-gel electrophoresis and autoradiography

Autoradiographs of cross-linked TSH–TSH-receptor complexes analysed by SDS/polyacrylamide-gel electrophoresis after reduction with 2-mercaptoethanol showed bands corresponding to intact TSH and its subunits (M_r 28000 and 14000 respectively) and two other bands of higher M_r (Figs. 2 and 3). The latter bands differed in M_r by approx. 14000 and were not present under control conditions, i.e. in the absence of receptors or cross-linking reagent, or in the presence of an excess of unlabelled TSH (Buckland *et al.*, 1982).

In the absence of reducing agent the pattern of bands was similar when ^{125}I-HSAB-TSH was used (Fig. 3). However, the two bands due to cross-linked complex were at higher M_r values and were less distinct and blurred into each other. Material was also present at the top of the gel and in the stacking gel. No bands due to cross-linked complex could be seen under non-reducing conditions when DSS was used as cross-linking reagent, but material was present in the stacking gel, indicating aggregation.

When samples were treated with trypsin before analysis, the M_r values of bands due to cross-linked complex were lowered (Figs. 2 and 3, Table 2). Non-reduced samples showed considerably more lowering of M_r than did reduced samples, but three bands were visible, one corresponding to the lower-M_r band before trypsin treatment.

Bands due to DSS-cross-linked complex had an M_r approx. 3000 lower than those produced by

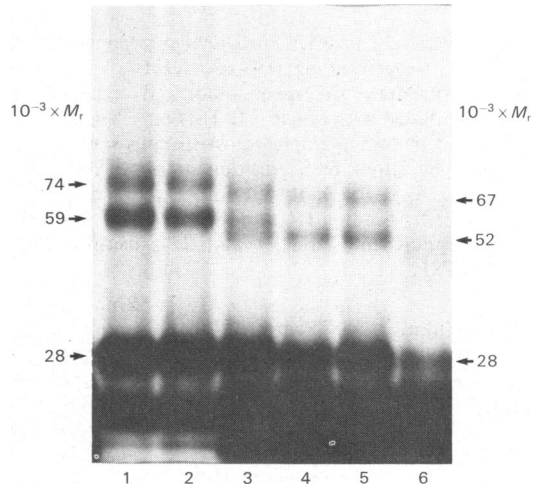

Fig. 2. *Autoradiograph of purified porcine TSH receptors cross-linked to ^{125}I-HSAB-TSH and analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions*

Lane 1, untreated cross-linked complexes; lanes 2–6, complexes treated with 0.00001% (lane 2), 0.0001% (lane 3), 0.001% (lane 4), 0.01% (lane 5) and 0.1% (lane 6) trypsin.

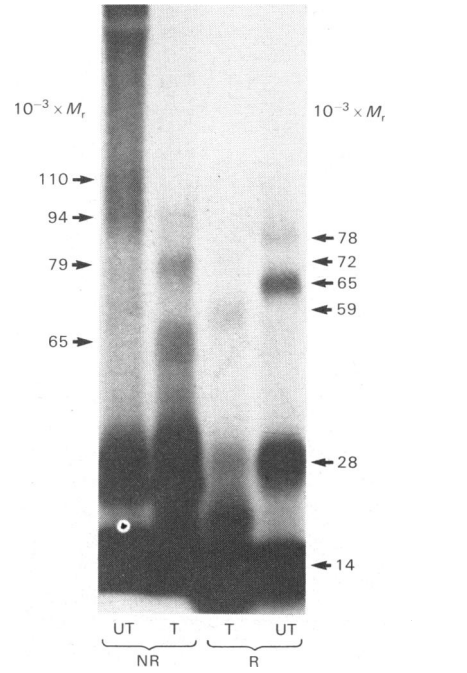

Fig. 3. *Autoradiograph of human soluble TSH receptors cross-linked to ^{125}I-HSAB-TSH analysed by SDS/polyacrylamide-gel electrophoresis, under reducing (R) and non reducing (NR) conditions, untreated (UT) or after 0.01% trypsin treatment (T)*

Table 2. *M_r values of cross-linked TSH–TSH-receptor complexes on SDS/polyacrylamide-gel electrophoresis revealed by autoradiography*

Values shown are the means ± S.D. of several independent measurements. The number of measurements is shown in parentheses. The M_r values of samples were determined by using a standard curve for protein markers of known M_r. Reduced samples were compared with reduced M_r markers, and non-reduced samples were compared with non-reduced M_r markers. The following markers were used (Sigma Chemical Co.): β-lactoglobulin (18400), IgG light chain (23500), ovalbumin (44000), IgG heavy chain (50000), bovine serum albumin (67000), human transferrin (77000), phosphorylase *b* (94000), β-galactosidase (116400) and myosin (205000).

TSH receptor	Type of Cross-linking	M_r of TSH–TSH-receptor complex: Non-trypsin-treated, Reduced	Non-trypsin-treated, Unreduced	Treated with 0.01% trypsin, Reduced	Treated with 0.01% trypsin, Unreduced
Porcine	HSAB	59300 ± 1700 (14) 73900 ± 2000 (14)	100400 ± 1700 (9) 87100 ± 1800 (4)	52100 ± 1600 (11) 66700 ± 900 (11)	58700 ± 1400 (16) 73700 ± 1700 (16) 86700 ± 1100 (13)
	DSS	57400 ± 2300 (5) 70900 ± 2700 (5)		53600 ± 1200 (7) 67400 ± 900 (7)	
Human	HSAB	64600 ± 3200 (5) 78200 ± 2400 (5)	110500 ± 1900 (7) 94200 ± 2200 (3)	59000 ± 2400 (24) 72000 ± 2500 (24)	64700 ± 2300 (20) 78700 ± 1900 (20) 92500 ± 2500 (15)
	DSS	62700 ± 2500 (3) 75800 ± 1000 (3)			

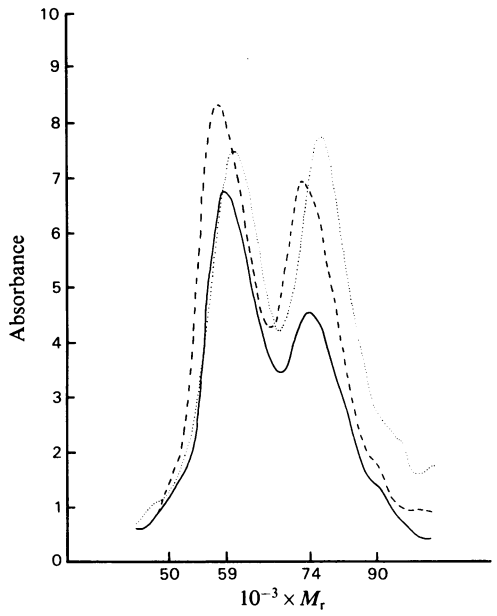

Fig. 4. *Densiometric traces taken from lanes of autoradiograph (SDS/polyacrylamide-gel electrophoresis) of porcine TSH receptors cross-linked to ^{125}I-HSAB-TSH using three different ratios of HSAB to TSH*

Peaks represent amounts of ^{125}I in the protein bands of the electrophoresis gel. The absorbance scale is arbitrary. HSAB/TSH ratio: ——, 1:1; – – –, 2:1; ·····, 2.4:1.

HSAB-TSH. After trypsin treatment, however, this difference was decreased to less than 1000 (Tables 2 and 3).

Variations in the molecular ratio HSAB/TSH

HSAB-TSH prepared in HSAB/TSH ratios of 1:1, 2:1 and 2.4:1 incorporated ^{125}I with high efficiency. All three preparations cross-linked to porcine receptors and resulted in bands at 59000 and 74000 on autoradiographs. However, when analysed by a densiometer, the relative intensities of these bands varied (Fig. 4). It could be seen that, as the ratio of HSAB to TSH increased, the relative intensity of the higher-M_r (74000) band increased compared with that of the lower-M_r (59000) band.

Cross-linking to thyroid membranes

When analysed under reducing conditions, TSH–TSH-receptor cross-linked complexes obtained by using crude porcine thyroid membranes gave the same results as when detergent-solubilized TSH receptors were used (Fig. 5). When analysed under non-reducing conditions however, no bands could be discerned above a high background other than those due to TSH and its subunits.

Discussion

Photolysis of ^{125}I-HSAB-TSH gave a mixture of TSH subunits and intact (internally cross-linked)

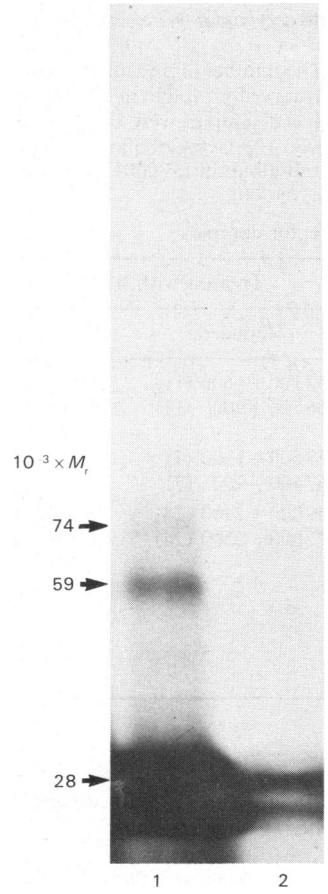

Fig. 5. *Autoradiograph of porcine thyroid membranes cross-linked to ^{125}I-HSAB-TSH and analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions*

Lane 1, untreated cross-linked complexes; lane 2, membranes incubated with an excess of unlabelled TSH before incubation with ^{125}I-HSAB-TSH.

TSH, which were seen as two bands at M_r 14000 (TSH subunits) and 28000 (intact TSH) on autoradiographs after analysis by SDS/polyacrylamide-gel electrophoresis under reducing conditions. When ^{125}I-HSAB-TSH was incubated with receptors before photolysis, analysis showed the presence of these two bands (M_r 14000 and 28000) and two other bands (M_r 59000 and 74000) which we have suggested correspond to a single receptor subunit cross-linked to one TSH subunit or intact TSH ($\alpha\beta$ dimer) (Buckland *et al.*, 1982). Further evidence for this interpretation is provided by the data shown in Fig. 4. This Figure shows that increasing the HSAB/TSH ratio increased the amount of the higher-M_r material relative to the amount of the lower-M_r material. Increasing the HSAB/TSH ratio would be expected to increase the likelihood of more than one cross-linking event occurring per molecule of TSH, thus increasing the likelihood of cross-linking between α-and β-subunits of TSH with the formation of more $\alpha\beta$-dimer cross-linked to the receptor. The receptor subunit cross-linked to TSH under reducing conditions is defined as the A-subunit.

When analysed under non-reducing conditions the cross-linked TSH–TSH-receptor complex ran as two bands with M_r values differing by approx. 14000 (Fig. 3). Their M_r values, however, were 20000–30000 higher than those of bands produced under reducing conditions. This indicated that a second receptor subunit of M_r 20000–30000 was associated with the A-subunit by a disulphide bridge. This subunit is defined as the B-subunit.

Bands produced under non-reducing conditions were broader, less distinct and often blurred into each other. Measured M_r values shown in Table 2 were therefore less accurate and also less reliable than those obtained after reduction, owing to the effects of the remaining disulphide bridges (Griffiths, 1972).

The presence of other material of high M_r on the autoradiographs was probably due to aggregation, possibly resulting from disulphide interchange occurring during heating in SDS, since this material was not seen with reduced or trypsin-treated samples or when samples were not heated in SDS before electrophoresis. When samples were not heated, however, only one band due to cross-linked complex could be seen, since the HSAB-TSH had a tendency to dissociate less under non-reducing conditions than under reducing conditions (Buckland *et al.*, 1982).

The M_r values for the non-reduced complex reported in Table 2 are somewhat lower than originally reported (Buckland *et al.*, 1982). This was partly a result of previous misassignment of M_r values to anomalous bands produced by M_r standards and partly due to the use of improved electrophoresis equipment and techniques.

Analyses, under reducing conditions, of trypsin-treated complexes revealed a single sensitive cleavage point near one end of the receptor A-subunit (Fig. 2). Several observations indicate that the cleavage point is on the receptor and not the TSH. In particular no material remained at the original M_r, although a large proportion of the non-cross-linked HSAB-TSH was unaffected by trypsin (Fig. 2). Also the change in M_r varied between species from 6000 in the case of human thyroid, to 13000 in the case of guinea-pig epididymal fat (Buckland & Rees Smith, 1984), and the difference of 14000 between the two bands remained constant (Table 2).

Analysis, under non-reducing conditions, of

Table 3. *Deduced M_r values for TSH-receptor subunits*
Receptor subunit-A M_r values are derived by subtracting the M_r of intact TSH (M_r 28300) from the highest-M_r band observed under reducing conditions with TSH–TSH-receptor complexes. Receptor subunit-B M_r values are derived by subtracting the M_r value of the highest-M_r band observed, under reducing conditions, with TSH–TSH-receptor complexes, from the corresponding M_r value obtained under non-reducing conditions.

		M_r		
TSH receptor	Type of cross-linking	Subunit A	Subunit B	Subunit A after trypsin treatment
Porcine	HSAB	45600	26500	38400
	DSS	42600		39100
Human	HSAB	49900	32300	43700
	DSS	47500		

trypsin-treated complexes showed a pattern similar to that observed under reducing conditions (Fig. 3). The two major bands that differed in M_r by approx. 15000, however, appeared not only to have lost a peptide of 6000 M_r on trypsin treatment, but also the entire B-subunit. This indicated that the disulphide bridge is located on the M_r-6000 peptide of the A-subunit that was released by trypsin. The additional faint band at M_r 87000 (Fig. 3) probably represented the TSH receptor (A- and B-subunits) cross-linked to one subunit of TSH that has not dissociated after trypsin cleavage. Heating of the sample in SDS. for a greater time decreases the intensity of this band.

Most of the studies described were carried out with affinity-purified TSH receptors and HSAB-TSH. Similar results were obtained with porcine thyroid membranes and HSAB-TSH when samples were reduced before analysis (Fig. 5). In the absence of reduction, however, bands due to ^{125}I-labelled TSH–TSH-receptor complexes could not be discerned.

Affinity-purified TSH receptors could also be cross-linked to ^{125}I-labelled TSH with DSS (Buckland *et al.*, 1982). Under reducing conditions, similar results were obtained to those with HSAB-TSH (Tables 2 and 3), although observed M_r values for the complex were about 3000 lower than with HSAB. After trypsin treatment, however, the observed M_r values for DSS- and HSAB-cross-linked complexes were similar (Tables 2 and 3). This suggested that the differences in M_r in the intact complexes were due to the production of a more compact structure by DSS cross-linking than by HSAB cross-linking.

The derived M_r values for the A- and B-subunits of the human and porcine TSH receptors are shown in Table 3. The human TSH receptor has an A-subunit with an M_r of about 50000, disulphide-linked to a B-subunit with an M_r of about 30000. Similarly the porcine TSH receptor A- and B-subunits have M_r values of about 45000 and about 25000 respectively. A possible structure for the receptors is shown in Fig. 6, but the A- and B-chains may also be associated non-covalently with other subunits.

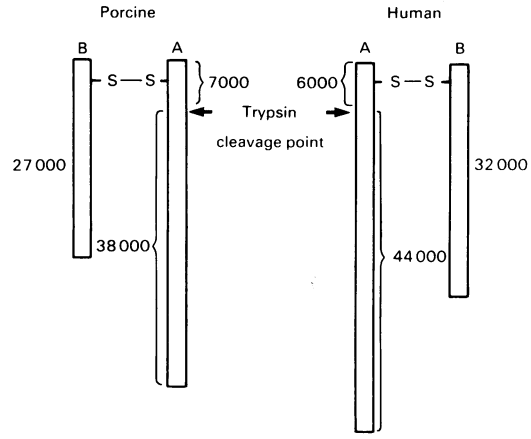

Fig. 6. *Diagrammatic representation of the subunit structure of porcine and human TSH receptor*
M_r values are rounded up to the nearest 1000. The site of the disulphide linkage on the B-subunits is not known at present.

This work was supported by the Medical Research Council, the Welsh Office and R.S.R. Ltd., Cardiff.

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