Photo-affinity labelling of the thyrotropin receptor

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1. INTRODUCTION

The thyrotropin (TSH) receptor is an important but poorly characterised component of the thyroid cell surface [1] and in this paper we describe an analysis of the receptor using affinity labelling [2]. Detergent solubilised porcine and human TSH receptors have been partially purified by Sepharose–TSH affinity chromatography [3] and then covalently crosslinked to ^{125}I-labelled TSH. Analysis of the TSH–TSH receptor complexes by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) suggests that both porcine and human TSH receptors contain a similar basic unit of two peptide chains linked by one or more disulphide bridges. These basic units bind one molecule of TSH and have relative molecular masses (M_r) of 87 000 in the case of the porcine TSH receptor and 100 000 in the case of the human TSH receptor.

2. MATERIALS AND METHODS

2.1. *Preparation of ^{125}I-labelled HSAB–TSH*

TSH was extracted from bovine pituitaries and purified by conventional procedures [4,5]. The specific activity of the purified hormone was 50–60 MRC units per mg as judged by receptor assay [3,5] and bioassay [6]. *N*-Hydroxysuccinimidyl-4-azidobenzoate (HSAB) (Pierce Chemicals; 10 mM in dimethyl sulphoxide) was added (4 × 1 μl) to 200 μg of TSH (in 200 μl of 150 mM NaCl, 20 mM $NaHCO_3$, pH 7.7) in the dark at room temperature over a period of 25 min. 10 μl of 1 M glycine was then added and after a further 10 min at room temperature, the TSH bound and free HSAB were separated on a column of Sephadex G-25 run in 0.1 M Na_2HPO_4, pH 7.5. Comparison of the UV spectra of HSAB, TSH and HSAB–TSH indicated that about 2 molecules of HSAB were incorporated into each molecule of TSH. The distribution of the two molecules of HSAB between the two subunits of TSH was investigated by reacting TSH with the Bolton–Hunter reagent [7] which would be expected to couple to the same amino groups as HSAB. Analysis of TSH labelled in this way on SDS–PAGE followed by autoradiography indicated that both α and β subunits contained similar amounts of label. HSAB–TSH was found to have a specific activity of about 25 MRC units per mg of protein as judged by both receptor and bioassays. Labelling of TSH and HSAB–TSH with ^{125}I (1 atom per molecule) was carried out using the iodogen method followed by chromatography on Sephadex G-100 [5].

2.2. *TSH receptor preparations*

Porcine and human TSH receptors were solubilised and purified by Sepharose–TSH affinity chromatography [3]. IgG was prepared from normal human or Graves' sera by ammonium sulphate precipitation and gel filtration on Sephacryl S-300. The ability of unlabelled TSH or Graves' IgG to inhibit ^{125}I-labelled TSH or ^{125}I-labelled HSAB–TSH binding to affinity purified receptors was investigated as described previously [3,5].

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2.3. *Preparation and analysis of crosslinked TSH–TSH receptor complexes*

Affinity purified receptors (200 μl) were incubated with or without unlabelled TSH (10 μl) for 15 min at room temperature followed by addition of ^{125}I-labelled HSAB–TSH (200 μl) and incubation at room temperature for a further 3 h in the dark. The mixture was then photolysed by placing 5 cm from a 40 watt UV light for 20 min. Crosslinking with disuccinimidyl suberate (DSS) was performed under similar conditions except that ^{125}I-labelled TSH was used and after 3 h at room temperature 4 μl of 0.1 M DSS in dimethyl sulphoxide was added followed by 4 μl of 1 M ammonium acetate 15 min later. Crosslinked TSH–TSH receptor complexes were precipitated by addition of polyethylene glycol (M_r = 4000) to a final concentration of 25% and suspended in 4% SDS. In some cases, 2-mercaptoethanol was added to a final concentration of 0.1 M and after heating at 90°C for 5 min, iodoacetamide was added to a final concentration of 0.15 M. The samples were centrifuged (14 000 × *g* for 20 min) and subjected to gradient (5–12%) SDS–PAGE [8] together with the following M_r markers: β-lactoglobulin (18 400), IgG light chain (23 500), TSH (28 300), ovalbumin (44 000), IgG heavy chain (50 000), bovine serum albumin (67 000), transferrin (77 000), phosphorylase B (94 000), β-galactosidase (116 400), myosin (205 000) and thyroglobulin (320 000 in SDS). M_r values were determined using reduced standards for reduced samples and non-reduced standards for non-reduced samples. After staining (Coomassie Blue) and drying, autoradiography was carried out for 16 h at −70°C using Kodak X-OMAT RP X-ray film and CRONEX lighting-plus enhancing screens (Dupont).

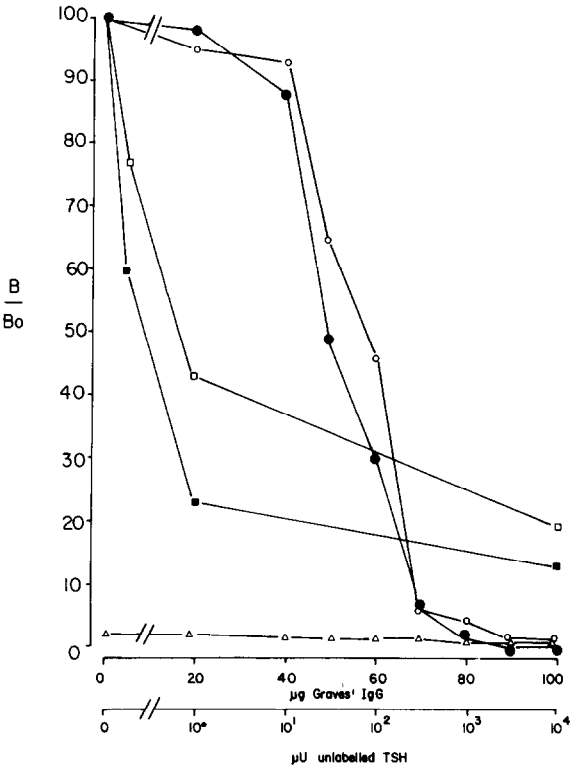

Fig.1. Inhibition of ^{125}I-labelled TSH binding to affinity purified porcine TSH receptors by unlabelled TSH = ○ and Graves' IgG = □. Inhibition of ^{125}I-labelled HSAB–TSH binding by unlabelled TSH = • and Graves' IgG = ■. Effect of unlabelled TSH on pre-photolysed ^{125}I-labelled HSAB–TSH binding = △. % Label specifically bound in the presence or absence of unlabelled TSH or Graves' IgG = B and B_0 respectively. In the case of ^{125}I-labelled TSH, 30–35% was bound in the absence of unlabelled TSH or Graves' IgG (total binding) and this was reduced to about 5% in the presence of 10 mU unlabelled TSH (non-specific binding). With ^{125}I-labelled HSAB–TSH total and non-specific binding were 20–25% and 5% respectively.

3. RESULTS

^{125}I-labelled HSAB–TSH showed similar receptor binding properties to ^{125}I-labelled TSH with both unlabelled TSH and Graves' IgG effectively inhibiting the binding of both label preparations to affinity purified TSH receptors (fig.1).

In preliminary experiments, ^{125}I-labelled HSAB–TSH was incubated with thyroid membrane preparations or non-purified detergent solubilised receptors, photolysed and the complexes formed analysed by SDS–PAGE followed by autoradiography. However the autoradiograph backgrounds were high and it was difficult to discern distinct bands which might have corresponded to crosslinked TSH–TSH receptor complexes. In subsequent experiments therefore, affinity purified receptors were used and with these preparations covalent crosslinking of labelled TSH to the receptor was clearly evident (fig.2). In particular, the ^{125}I in the TSH–porcine TSH receptor complexes

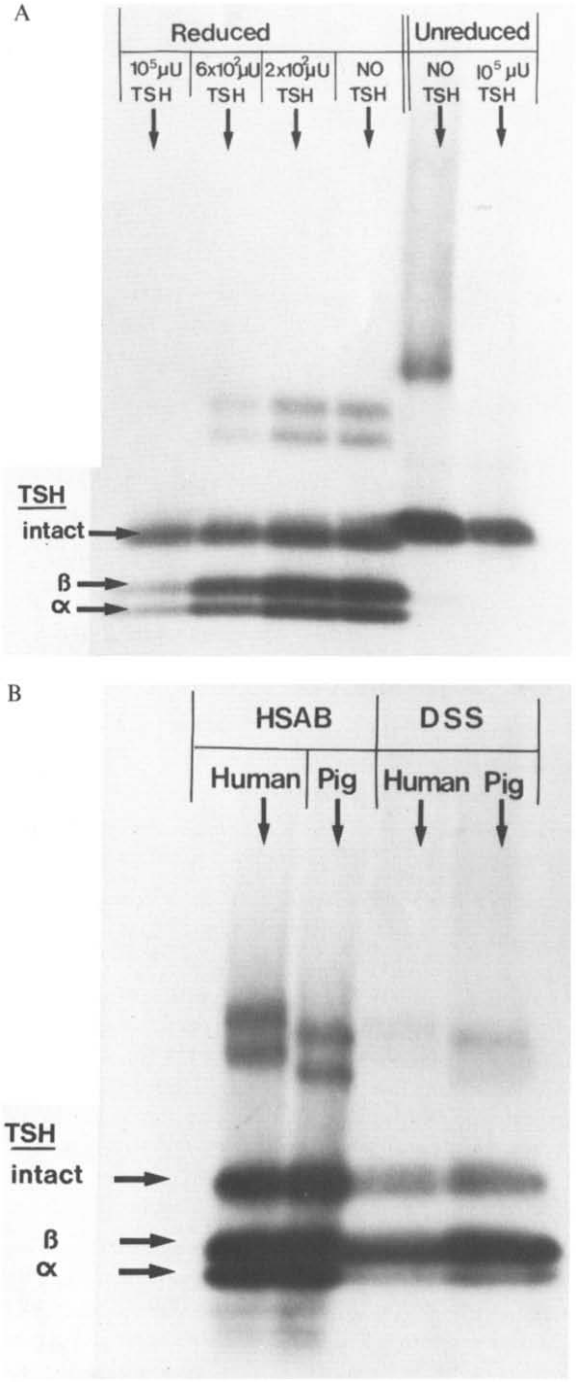

Fig.2. (A) A comparison of reduced and unreduced crosslinked complexes of ^{125}I-labelled HSAB–TSH and porcine TSH receptors. The amounts of unlabelled TSH included in the incubations are shown at the top of the autoradiograph. (B) A comparison of reduced HSAB or DSS crosslinked complexes obtained using porcine and human TSH receptors. The data shown are typical of several separate experiments (see table 1).

was associated (in the absence of reducing agent) with a band of M_r = 115 000 and a band characteristic of intact TSH (M_r = 28 000). The M_r = 115 000 band was not observed in the following control experiments:

(a) When an excess (10 mU) of unlabelled TSH was added to receptor preparations prior to incubation with ^{125}I-labelled HSAB–TSH and photolysis (fig.2).

(b) When labelled HSAB–TSH was incubated with heat inactivated receptors or detergent only prior to photolysis.

(c) When mixtures of ^{125}I-labelled HSAB–TSH and receptors were not photolysed prior to SDS–PAGE (dark control).

(d) When ^{125}I-labelled TSH was used in place of ^{125}I-labelled HSAB–TSH.

Consequently the M_r = 115 000 band appeared to represent a component of TSH specifically bound to a component of the porcine TSH receptor.

Reduction of photolysed TSH–porcine TSH receptor complexes prior to SDS–PAGE changed the distribution of ^{125}I on the gels. The M_r = 115 000 band was not evident and instead, two labelled components M_r = 73 000 (slow) and 59 000 (fast) (table 1 and fig.2) were observed together with bands characteristic of intact TSH and the α and β subunits (M_r = 13 600 and 14 700 respectively) [4]. The M_r = 73 000 and 59 000 bands appeared therefore to be specific components of the reduced TSH–TSH receptor complex.

As an alternative approach to the use of ^{125}I-labelled HSAB–TSH, TSH was labelled with ^{125}I only, incubated with affinity purified porcine TSH receptors and the complex formed crosslinked with the homobifunctional reagent DSS. Analysis of the reduced complexes by SDS–PAGE followed by autoradiography gave a similar gel pattern to that observed with HSAB–TSH. Two ^{125}I-labelled bands of M_r = 71 000 and 57 000 were apparent together with bands characteristic of intact TSH and TSH subunits (fig.2 and table 1). The formation of the M_r = 71 000 and 57 000 components was completely inhibited by an excess (10 mU) of unlabelled TSH. Reduced, non-crosslinked ^{125}I-labelled TSH was resolved almost completely into α and β subunits on SDS–PAGE (fig.2). Unreduced DSS crosslinked complexes were not readily soluble in 4% SDS even after prolonged heating and

Table 1

Relative molecular mass (M_r) values of crosslinked TSH–TSH receptor complexes observed on SDS–PAGE

Species of TSH receptor	Type of cross-linking	Component of TSH–TSH receptor complex	Number of samples analysed	Mean M_r ($\times 10^{-3}$)	Standard deviation
Reduced samples					
Porcine	HSAB	Fast	14	59.3	1.7
		Slow		73.9	2.0
		Slow–Fast		14.6	1.2
Porcine	DSS	Fast	5	57.4	2.3
		Slow		70.9	2.7
		Slow–Fast		13.6	0.8
Human	HSAB	Fast	4	64.6	3.2
		Slow		78.2	2.4
		Slow–Fast		13.6	1.2
Human	DSS	Fast	3	62.7	2.5
		Slow		75.8	1.0
		Slow–Fast		13.1	1.6
Non-reduced samples					
Porcine	HSAB		10	115	1.8
Human	DSS		6	128	1.8

consequently gel analysis of unreduced samples was not possible.

Parallel experiments to those described for porcine TSH receptors were carried out using affinity purified human (Graves') TSH receptors and crosslinking with both HSAB and DSS. Similar results were obtained to those with porcine receptors except that both reduced and non-reduced bands gave somewhat higher M_r values (table 1, fig.2).

4. DISCUSSION

Interpretation of these results can be most appropriately made by first considering the analysis of reduced TSH–TSH receptor complexes. In the case of crosslinking with HSAB or DSS to human or porcine receptors, the TSH–TSH receptor complex was resolved into 2 bands differing in M_r by a value (14 000), very close to the M_r of a single TSH subunit (table 1). In addition, HSAB–TSH which was not covalently linked to the receptor but had been subject to crosslinking conditions and reduced was resolved into a mixture of intact (internally crosslinked) molecules and free subunits (fig.2). These observations suggest therefore that the higher and lower M_r bands represent, respectively, a component of the TSH receptor linked to an intact molecule of TSH and a single TSH subunit. Based on data with HSAB–TSH, this component of the TSH receptor appears to have an M_r of 45 000 in the case of the porcine thyroid and 50 000 in the case of the human thyroid (after subtraction of the M_r values for intact TSH or a single TSH subunit). The values obtained with DSS for porcine and human TSH receptors were slightly lower being M_r = 43 000 and 48 000 respectively and the reason for this difference is not clear at present. One possibility is that crosslinking with DSS resulted in more compact complexes which would run faster on SDS–PAGE.

When unreduced samples of crosslinked TSH–TSH receptor complexes were analysed by SDS–PAGE, little if any free TSH subunits were evident, the gels principally showing intact TSH and

TSH–TSH receptor complexes. The apparent inability of SDS alone to dissociate TSH (which does not contain intersubunit disulphide bridges) into subunits is consistent with previous studies using guanidine hydrochloride [4]. It seemed likely therefore that the non-reduced TSH–TSH receptor complex observed on the gels contained one molecule of intact TSH (M_r = 28 000), the receptor component with M_r = 45 000 observed in reduced samples and an additional receptor component or TSH molecule. The additional molecule must have an additional M_r of 42 000 (to give a total of 115 000) suggesting that it is a component of the receptor rather than TSH. This M_r = 42 000 component appears to be linked to the M_r = 45 000 component by one or more disulphide bridges. Similarly the human TSH receptor appears to contain a slightly larger unit consisting of two disulphide linked M_r = 50 000 subunits.

Previous studies have indicated that TSH binding by the TSH receptor is reversibly inactivated under conditions of mild reduction [9]. This suggests that the integrity of the inter subunit disulphide bridge(s) is essential for maintaining the structure of the TSH binding site.

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