The disulphide bond structure of thyroid-stimulating hormone β -subunit

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Previously only one of the six disulphide bonds within the β -subunit of bovine thyrotropin (bTSH β) has been unequivocally assigned. In the present investigation, the fluorescent alkylating reagent 5-*N*-[(iodoacetamidoethyl)amino]naphthalene-1-sulphonic acid has been employed as part of a double-alkylation strategy to allow the relative reactivities and the location of the six disulphide bonds of bTSH β , after selective reduction, to be assigned by using reversed-phase HPLC peptide mapping techniques and associated methods of structural analysis. The most reactive disulphide bond was Cys⁸⁸–Cys⁹⁵; the second most reactive group of disulphide bonds involved the half-cystine residues Cys¹⁶, Cys¹⁹, Cys⁶⁷ and Cys¹⁰⁵ with the experimental results consistent with the assignment of disulphide bonds to Cys¹⁶–Cys⁶⁷ and Cys¹⁹–Cys¹⁰⁵. The least reactive group of half-cystine residues consisted of Cys², Cys²⁷, Cys³¹, Cys⁵², Cys⁸³ and

Cys⁸⁵. The isolation, by high-performance ion-exchange chromatography, of a partly reduced bTSH β derivative in which only the half-cystine residues Cys³¹, Cys⁸⁵, Cys⁸⁸ and Cys⁹⁵ were labelled enabled the assignment of a previously uncharacterized disulphide bond to Cys³¹–Cys⁸⁵. The remaining two assignments, Cys²–Cys⁵² and Cys²⁷–Cys⁸³, were made by comparison with the recently published human chorionic gonadotropin crystal structure. The flexibility of the double-labelling approach used in these studies demonstrates that only very small quantities are required for proteins containing an extensive number of half-cystine residues such as TSH β , owing to the combination of the high resolution of the reversed-phase HPLC peptide mapping procedures and the sensitivity of the fluorimetric detection method.

INTRODUCTION

Thyroid-stimulating hormone or thyrotropin (TSH) is a member of the glycoprotein hormone (GPH) family, which also includes chorionic gonadotropin (CG), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones possess similar heterodimeric structures consisting of an α -subunit, common to all members of the GPH family, and a β -subunit unique for each member. Significant amino acid sequence homology exists between the β -subunits for TSH, CG, LH and FSH. On alignment of the amino acid sequences of the β -subunits, the 12 cysteine residues of the β -subunits are completely conserved and located in identical sequence positions [1]. These cysteine residues form six disulphide bridges and hence no free sulphydryl groups are present in the β -subunits of the glycoprotein hormones. The recent elucidation of the X-ray crystallographic structure of human CG (hCG) has enabled the assignment of the disulphide bonds between the half-cystine residues of hCG to be made definitively for the first time [2,3]. Before this development, a number of investigations had addressed the assignment of the disulphide bonds for the GPH β -subunits [see, for example, 4–10]. These studies generally employed one of two strategies, namely extensive cleavage of the hGC β /LH β subunit into disulphide-linked peptides or partial reduction followed by alkylation with a radioactive reagent. Only two of these studies, however, were concordant about the assignment of the six half-cystine pairings of the hGC β -subunit [6,10]. Although these latter assignments for the disulphide structures within hCG β have been accepted for more than a decade as accurate, only four

of the six assignments were in agreement with the recent assignments made from the X-ray crystallographic data [2,3]. A major problem with all of the above earlier disulphide assignment studies has presumably been associated with uncontrollable disulphide bond interchange and/or lack of sensitivity and separation selectivity of the analytical methods to trap and characterize the partly reduced species [11].

Previous studies involving the assignment of disulphide bridges of the TSH β -subunit have resulted in just one unequivocal assignment, namely Cys⁸⁸–Cys⁹⁵ [5,9]. This assignment agrees with the pairing made for the analogous half-cystine residues in hCG β (i.e. Cys⁹³–Cys¹⁰⁰) [2,3]. In the present investigation two alternative experimental approaches have been employed to permit the assignment of the half-cystine residue pairs of bTSH β and to confirm whether identical disulphide bond structures exist between the bTSH β and the other GPH β -subunits, as has long been suspected. The first approach involved the partial reduction of the disulphide bonds followed by alkylation with a fluorescent reagent in place of the traditional [¹⁴C]iodoacetic acid (see, for example, [5,9,10]). This experimental approach had two complementary components, the first of these will be referred to as the ‘forward-labelling’ experiments and examined which disulphide bonds were cleaved by partial reduction of bTSH β , whereas the second component, referred to as the ‘reverse-labelling’ experiments, examined which disulphide bonds remained intact after partial reduction of bTSH β . The second experimental approach involved the chromatographic separation of a partly reduced bTSH β -subunit preparation into fractions containing polypeptides with different numbers of reduced disulphide bonds.

Abbreviations used: b, bovine; CG, chorionic gonadotropin; DTT, dithiothreitol; FSH, follicle-stimulating hormone; GPH, glycoprotein hormone; h, human; HPIEX, high-performance ion-exchange; IAA, iodoacetic acid; 5-I-AEDANS, 5-*N*-[(iodoacetamido-ethyl)amino]naphthalene 1-sulphonic acid; LH, luteinizing hormone; RP-HPLC, reversed-phase HPLC; TFA, trifluoroacetic acid; TSH, thyroid-stimulating hormone.

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The results from these two sets of experiments were then used in the assignment of the disulphide bond structure of the intact TSH β -subunit.

MATERIALS AND METHODS

Purification of the bTSH β -subunit

The source of the bovine TSH was a highly purified preparation isolated in this laboratory from bovine thyroid glands by methods described previously [12]. Highly purified bTSH β -subunit was obtained from this preparation by using a combination of reversed-phase (RP)-HPLC procedures with elution under acidic conditions, followed by high-performance gel-filtration chromatography (HPSEC) on a Superdex 75 column to remove any remaining complementary α -subunit or intact hormone, also as described previously [13].

Forward- and reverse-labelling experiments

Samples of bTSH β [5 mg (equivalent to 0.35 nmol) in NH_4HCO_3] were freeze-dried and resuspended in 50 μl of one of the following buffers: (a) 100 mM Tris/HCl, pH 8.2, containing 2.6 mM EDTA; (b) 100 mM Tris/HCl, pH 7.0, containing 2.6 mM EDTA; (c) 200 mM Tris/HCl, pH 7.0, containing 6 M guanidine hydrochloride and 2 mM EDTA; or (d) 200 mM Tris/HCl, pH 8.0, containing 6 M guanidine hydrochloride and 2 mM EDTA. Dithiothreitol (DTT) at concentrations ranging from a 2-fold molar excess over bTSH β disulphide content (final DTT concentration 39 μM) to a 2000-fold molar excess (final DTT concentration 39 mM) was then added to each sample in 20 μl of the same buffer, and the Eppendorf tube flushed with dry nitrogen gas before sealing. The tubes were then incubated for periods of time ranging from 30 s to 3 h on ice.

After reduction, an aliquot (35 μl) of the reaction mixture was removed, and in the forward-labelling approach the reduced polypeptide was alkylated with the addition of the fluorescent reagent 5-*N*-[(iodoacetamidoethyl)amino]naphthalene 1-sulphonic acid (5-I-AEDANS) (Molecular Probes) (molar ratio 5-I-AEDANS:DTT, 5:1) [14] in 35 μl of 1 M Tris/HCl, pH 7.9. For the reverse-labelling procedure, the remaining 35 μl of the reaction mixture of the reduced polypeptide was alkylated with the addition of iodoacetic acid (IAA) (molar ratio IAA:DTT, 5:1) in 35 μl of 1 M Tris/HCl, pH 7.9. The alkylation reactions were allowed to proceed in the dark for 30 min on ice, followed by 60 min in the dark at room temperature. The protein was then isolated by precipitation upon the addition of 1400 μl of 100 % methanol (final methanol concentration 95 %) prechilled at -20°C . After incubation overnight at -20°C , the samples were centrifuged in an Eppendorf Microfuge for 15 min at 4°C . The supernatant was then removed and the reduced and alkylated bTSH β -subunit washed twice with 1 ml of 100 % methanol prechilled at -20°C .

The forward- and reverse-labelling samples were then resuspended in 200 μl of 200 mM Tris buffer, pH 8.0, containing 6 M guanidine hydrochloride and 2 mM EDTA, and the remaining disulphide bonds fully reduced in the presence of DTT as described previously [13]. The forward-labelled samples were alkylated with non-radioactive iodoacetic acid after full reduction, whereas the reverse-labelled samples were alkylated with 5-I-AEDANS. The alkylation reactions and methanol precipitation steps were performed as described above.

Proteolytic digestions and chromatography of fluorescent peptides

After the methanol precipitation steps, the samples were resuspended in 30 μl of 100 mM NH_4HCO_3 containing 2 mM

CaCl_2 and *N*-tosyl-L-phenylalanine chloromethylketone-treated trypsin (trypsin, EC 3.4.21.4) (Worthington Biochemical Corp.) added at a trypsin-to-bTSH β ratio of 1:20 (w/w) in 10 μl of 1 mM HCl. The samples were digested for 24 h at room temperature with occasional mixing, after which the digestion was stopped by the addition of soybean trypsin inhibitor (Boehringer Mannheim), in 10 μl of 100 mM NH_4HCO_3 , at a 2:1 (w/w) ratio to trypsin. The samples were then freeze-dried, resuspended in 30 μl of fresh 100 mM NH_4HCO_3 and chymotrypsin (EC 3.4.21.1) added in 10 μl of the same buffer at a chymotrypsin-to-TSH β ratio of 1:10 (w/w). After incubation for 20 h at 37°C with occasional mixing, a 10 μl aliquot was removed and diluted with 90 ml of 0.1 % (v/v) phosphoric acid. The aliquot was then injected onto a Bakerbond C_{18} RP-HPLC column (25 cm $\times$ 0.46 cm internal diam., 300 Å pore size, 5 mm particle diameter) pre-equilibrated with 0.1 % (v/v) phosphoric acid at a flow rate of 1 ml/min. The peptides were then eluted with a linear gradient of 0–50 % (v/v) acetonitrile in 0.1 % (v/v) phosphoric acid over 120 min at 1 ml/min. Fluorescent peptides were monitored with a Waters fluorescence detector (model 410AC) fitted with a 340 nm excitation filter and 500 nm emission filter. Data from the detector were collected and analysed with the Maxima (Waters, Millipore) chromatography software package. As the peak at approximately 60 min was the last fluorescent peak to be eluted, data for the first 80 min only are presented in all chromatograms.

Characterization of the fluorescent tryptic/chymotryptic map

The fluorescent peaks of the chromatograms produced after RP-HPLC of the tryptic/chymotryptic digests of reduced and alkylated bTSH β were identified by comparison with a previously characterized map of fluorescently labelled, digested bTSH β . This map was produced by fully reducing a sample (30 μg) of bTSH β as described previously [13] and alkylating the reduced sample with 5-I-AEDANS as described above. The sample was then digested enzymically, the digest mixture separated by RP-HPLC as described above, and the fluorescently labelled peaks collected. These peaks were then rechromatographed on a Bakerbond C_{18} RP-HPLC column (25 cm $\times$ 0.46 cm internal diam., 300 Å pore size, 5 mm particle diameter) pre-equilibrated in 0.1 % (v/v) trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. Elution was performed with a linear gradient of 0–50 % (v/v) acetonitrile in 0.09 % (v/v) TFA over 30 min at 1 ml/min. This rechromatography step was necessary to remove the phosphoric acid in the sample, which otherwise interfered with the subsequent amino acid analysis procedure. The fluorescent peaks were again collected and identified by amino acid analysis by the Picotag method (Millipore) [15], which was performed as previously described [16].

Quantification of results

It was necessary to quantify the degree of labelling of each half-cystine residue after reduction of the disulphide bonds in the forward-labelling experiments. To achieve this the area under each peak in the fluorescent chromatograms was determined and expressed as a fraction of the area of a reference peak within the chromatogram. Conversion to relative peak areas was necessary to enable comparisons between chromatograms to be made without the result being affected by losses of material (e.g. due to the methanol precipitation steps or other handling procedures). The reference peak selected was peak 6 (including its breakdown products, peaks 6A and 6B). This peak contains half-cystines Cys⁸⁸ and Cys⁹⁵, which, as will become apparent, are the half-cystine residues that form a single disulphide bond that was

highly susceptible to reduction and was the first disulphide bond to open. Finally, the relative peak areas were normalized to account for any selective chromatographic losses of particular peptides after digestion.

From the accumulated data derived from all the enzymic mapping experiments, the highest relative peak areas in the RP-HPLC chromatograms for the fluorescently labelled peptides were observed when the reducing conditions involved incubation of bTSH β at pH 8.2 for 3 h in the presence of a 1000-fold molar excess of DTT. Hence the relative peak areas for each fluorescently labelled peptide in this chromatogram became the normalization standard by which the relative peak areas could be normalized for the same peptide in all the other RP-HPLC chromatograms obtained with the experiments involving the other reducing conditions. In this manner the relative extent of cleavage of each disulphide bond could be determined and particular half-cystine residues could be paired by matching the different peptides within the same chromatogram with similar normalized peak areas.

Fractionation of reduced and alkylated bTSH β

Aliquots of bTSH β (15 μ g) were reduced with a 2000-fold molar excess of DTT in 200 mM Tris/HCl, pH 7.0, containing 2.6 mM EDTA for 5 min, under nitrogen, on ice. The reduced bTSH β -subunit was then alkylated with 5-I-AEDANS as described above. After methanol precipitation and washing, the reduced and alkylated sample was resuspended in 200 μ l of 20 mM Tris/HCl, pH 9.0, containing 0.015% (v/v) Brij-35, and applied to a Mono-Q anion exchange column (HR5/5) (Pharmacia, Uppsala, Sweden) pre-equilibrated in the same buffer at a flow rate of 1 ml/min. Elution was then performed with a linear gradient of 0–500 mM NaCl in 20 mM Tris/HCl, pH 9.0, containing 0.015% (v/v) Brij-35, over 45 min. Fluorescent peaks were collected manually, freeze-dried, then fully reduced and alkylated with IAA as previously described. The fully reduced and alkylated fractions were then digested and separated by RP-HPLC as described above for the forward- and reverse-labelled samples.

RESULTS

Characterization of the tryptic/chymotryptic peptide map

Under ideal enzymic digestion and chromatographic resolution conditions, 12 fluorescently labelled peptides containing the 12 half-cystine residues of bTSH β would theoretically be expected in the RP-HPLC profiles of each of the tryptic/chymotryptic maps. Inspection of a typical chromatogram (Figure 1) revealed the presence of nine major fluorescent peaks (designated peaks 1–9 in Figure 1) and two minor peaks, designated peaks 6A and 6B. The amino acid composition and sequence of the peptide(s) within these chromatographic peaks were determined (results not shown) and hence the location of the labelled half-cystine residue(s) on each peptide was identified. Peptide peak 6 in the RP-HPLC chromatogram (Figure 1) was found to correspond to the sequence bTSH β ^{88–101} (peptide β FTC8; see Figure 2 for amino acid sequence), and hence contained the two half-cystine residues Cys⁸⁸ and Cys⁹⁵. The remaining peptide peaks each contained a single half-cystine residue (Figure 1). Therefore a total of 10 of the 12 possible half-cystine containing peptides of bTSH β could be readily resolved and detected with the selected conditions of proteolytic digestion and RP-HPLC separation. The two half-cystine residues not represented in the tryptic/chymotryptic RP-HPLC map, Cys¹⁹ and Cys²⁷, are both located within the tryptic/chymotryptic glycopeptide bTSH β ^{19–30} (Figure

Figure 1 Typical RP-HPLC chromatogram of reduced, fluorescently alkylated and digested bTSH β

Bovine TSH β was fully reduced then alkylated by the addition of 5-I-AEDANS at a 5-fold molar excess over DTT present. The fluorescently labelled bTSH β -subunit preparation was then digested with trypsin followed by chymotrypsin. The derived peptides were separated by RP-HPLC procedures on a Bakerbond C₁₈ column pre-equilibrated in 0.1% (v/v) orthophosphoric acid. Elution was performed with a linear gradient of 0–50% (v/v) acetonitrile in 0.1% (v/v) orthophosphoric acid over 120 min with data collection for the first 80 min. Fluorescent peptides were monitored with a fluorescence detector fitted with a 340 nm excitation filter and 500 nm emission filter, at gain 128. The major peaks have been numbered for subsequent identification by amino acid analysis and sequence assignment.

2). No RP-HPLC conditions were found that enabled the elution of this peptide while maintaining adequate resolution of all the other fluorescently labelled peptides. The intrinsic hydrophobic nature of the glycopeptide bTSH β ^{19–30}, which would be increased by the presence of two fluorescent AEDANS labels, is believed to be responsible for its very strong retention on the RP-HPLC adsorbent at acetonitrile concentrations up to 90% (v/v).

As mentioned above, one of the fluorescent peptides (peptide β FTC8, bTSH β ^{88–101}) contained two half-cystine residues Cys⁸⁸ and Cys⁹⁵, which represent the half-cystine residues of a single disulphide bond [5,9]. The labelling of both the half-cystines in this bTSH β ^{88–101} peptide was confirmed by extensive digestion of the purified peptide with chymotrypsin, for which Tyr⁹² is a potential cleavage site. The products of this further digestion were the peptides corresponding to the peaks labelled 6A (bTSH β Cys⁸⁸–Tyr⁹²) and 6B (bTSH β Ser⁹³–Lys¹⁰¹) as shown in Figure 1.

Figure 2 Sequence of bTSH β

The locations of the fluorescently labelled tryptic/chymotryptic (FTC) peptides are indicated and the positions of the half-cystine residues are circled. The position of the N-linked carbohydrate (CHO) is also shown.

Figure 3 Representative chromatograms demonstrating the susceptibility to reduction of the Cys⁸⁸–Cys⁹⁵ disulphide bond

The RP-HPLC profiles represent the forward-labelling (**a, c** and **e**) and reverse-labelling (**b, d** and **f**) experiments respectively of bTSH β reduced for 30 min with a 128-fold molar excess of DTT at (**a, b**) pH 7.0; (**c, d**) pH 7.0 in the presence of guanidine hydrochloride; and (**e, f**) pH 8.2. After full reduction, tryptic and chymotryptic digestion, the fluorescent peptides were separated by RP-HPLC with elution and detection performed as described in the legend to Figure 1. The asterisk in each chromatographic profile indicates the reference peak, which corresponds to the peptide containing Cys⁸⁸ and Cys⁹⁵. In the reverse-labelling chromatograms (**b, d** and **f**) the expected position of the reference peak is indicated.

Forward- and reverse-labelling experiments

In all of the chromatograms for the forward-labelling experiments in which bTSH β was reduced under relatively mild conditions (i.e. with DTT concentrations of 128-fold molar excess) or for very short reduction times (i.e. less than 2 min), peak 6 was the largest (in peak height and area) of all the chromatographic peaks (e.g. Figures 3a, 3c and 3e). This result indicated that the two half-cystine residues, Cys⁸⁸ and Cys⁹⁵, located within this fluorescent peptide, are involved in the most reactive disulphide bond. The reverse-labelling experiments, in which the disulphide bonds remaining after reduction were examined, confirmed this conclusion as peak 6 was the first peak to disappear from the fluorescent chromatograms in all experiments (Figures 3b, 3d and 3f). All other major peaks were still present at significant levels in the chromatograms from these reverse-labelling experiments, indicating that all of the other disulphide bonds were still intact under these partial reduction conditions. Moreover the conditions for this experiment were chosen so that possible disulphide interchange reactions were minimized: the reduction was performed at neutral pH, with a short (5 min) reaction time and a high concentration of reducing reagent.

The results concerning the other half-cystine residues, summarized in Figure 4, can be divided into two groups according to the apparent reactivities of their constituent disulphide bonds. The first of these groups contained the half-cystine residues Cys¹⁶, Cys⁶⁷ and Cys¹⁰⁵. The second group contained the half-cystine residues Cys², Cys³¹, Cys⁵², Cys⁸³ and Cys⁸⁵. Under a variety of reduction conditions, e.g. pH 8.2 for times ranging from 30 s to 3 h, the reactivities of the disulphide bonds involving the half-cystine residues of both of these groups were very similar (Figure 4, panels A, B and C). However, there were also certain conditions that resulted in significant differences in the reactivities of the disulphide bonds involving the half-cystine residues of these two groups. In particular the first group of half-cystine

residues were significantly more reactive when the reduction was performed at pH 7.0 for 30 min in the presence of a low concentration of DTT (i.e. 16-fold molar excess) (Figure 4, panel D; Figure 5b) and at pH 7.0 in the presence of guanidine hydrochloride at all DTT concentrations tested (Figure 4, panel F; Figure 5a). It can therefore be concluded that the two next most reactive disulphide bonds of bTSH β after the Cys⁸⁸–Cys⁹⁵ bond involved this first group of half-cystine residues whereas the remaining three, and least reactive, disulphide bonds involved the second group of half-cystine residues.

Fractionation of reduced and alkylated bTSH β

In an attempt to isolate populations of bTSH β with different numbers of opened disulphide bridges, bTSH β (which had been partially reduced for 5 min in the presence of a 2000-fold molar excess of DTT at pH 7.0 before alkylation with 5-I-AEDANS) was fractionated by high-performance ion-exchange (HPIEX) chromatography. The reduction conditions were chosen to ensure that all disulphides would be at least partly reduced, and at the same time potential disulphide interchange would be minimized by the selected conditions of neutral pH, short (5 min) reduction time and high concentration of reducing agent. The HPIEX separation of this partly reduced bTSH β preparation yielded several, relatively closely eluting, peaks and peak shoulders, designated fractions 1–7 (Figure 6). After recovery of these fractions, the polypeptides within them were subjected to complete reduction and alkylation with IAA, tryptic/chymotryptic digestion and finally RP-HPLC separation of the derived peptides. It was apparent from these experiments that fractions 1–5 contained bTSH β with just one reduced disulphide bond, namely Cys⁸⁸–Cys⁹⁵, as all of these HPIEX chromatographic fractions generated only the reference fluorescently labelled peptide peak 6 or the reference peak 6 plus its chymotryptic breakdown products, peaks 6A and 6B (Figure 7), after RP-HPLC peptide

Figure 4 Summary of the normalized peak areas for all peaks in all chromatograms produced throughout these investigations

The molar excess of DTT (2–2000-fold) employed for the reduction or the reduction time is indicated on the x-axis. The graphs have been divided horizontally into two groups on the basis of the reactivity of the various disulphide bonds, as discussed in the text. The graphs have been further divided vertically into panels A–G to permit identification of the results of particular experiments. The + above some of the bars indicates that the reference peak was marginally off-scale (by approximately 10%) in these chromatograms and hence the normalized relative peak areas for all of the peaks will be greater than the real relative peak area. Not shown in this figure are the data for peak 6, which as the reference peak always gave a normalized peak area ratio of 1.

mapping. However, the RP-HPLC peptide map chromatograms corresponding to fractions 6 and 7 were found to contain two extra fluorescently labelled peaks in addition to the reference peak 6 (Figure 7). These additional peaks (peaks 1 and 3) corresponded to the peptides containing half-cystine residues Cys⁸⁵ and Cys³¹ respectively. This result provides direct evidence that a disulphide bridge exists between these half-cystine residues.

DISCUSSION

In this investigation the disulphide bridge structure of bTSH β has been examined by two experimental strategies involving selective fluorescent labelling of the individual half-cystine

Figure 5 Representative RP-HPLC profiles for the forward-labelling experiments, demonstrating the susceptibility to reduction of the disulphide bonds involving Cys¹⁶, Cys⁶⁷ and Cys¹⁰⁵

Chromatograms are shown for samples of bTSH β that were reduced for 30 min with a 16-fold molar excess of DTT at (a) pH 7.0 in the presence of guanidine hydrochloride and (b) pH 7.0 in the absence of guanidine hydrochloride. Both samples were then alkylated with 5-I-AEDANS. Digestion, elution and detection are as described in the legend for Figure 1. The reference peak is indicated with an asterisk and the peaks corresponding to the peptides containing Cys¹⁰⁵ (peak 2), Cys¹⁶ (peak 4) and Cys⁶⁷ (peak 5) are also indicated.

Figure 6 Anion-exchange HPLC chromatography of partly reduced and fluorescently alkylated bTSH β

A sample of bTSH β was partly reduced by incubation with a 2000-fold molar excess of DTT over disulphide content for 5 min at pH 7.0, then alkylated with 5-I-AEDANS at a 5-fold molar excess over DTT present. After removal of reactants by methanol precipitation, the partly reduced subunit was freeze-dried then resuspended in 20 mM Tris/HCl, pH 9.0, containing 0.015% (v/v) Brij-35, and applied to a Mono-Q anion exchange column (HR5/5) pre-equilibrated in the same buffer at a flow rate of 1 ml/min. Elution was then performed with a linear gradient of 0–500 mM NaCl in 20 mM Tris/HCl, pH 9.0, containing 0.015% (v/v) Brij-35, over 45 min. Fluorescently labelled peaks were monitored with a fluorescence detector fitted with a 340 nm excitation filter and 500 nm emission filter at gain 16, and the fractions collected manually.

Figure 7 Disulphide bond analysis of polypeptide peaks separated by ion-exchange chromatography

Fluorescent peaks from HPIEX (see Figure 6) were collected, fully reduced and alkylated with IAA, then digested with trypsin followed by chymotrypsin. Fluorescently labelled peptides were then separated by RP-HPLC chromatography, with elution and detection performed as described in the legend to Figure 1. The asterisk in each chromatogram indicates the reference peak.

residues of bTSH β after reduction of their corresponding disulphide bonds. RP-HPLC separation of the fluorescently labelled peptides resulting from proteolytic digestion of the subsequently fully reduced and alkylated bTSH β then enabled rapid assessment of the relative reactivities of the half-cystine residues at particular sequence positions. With the RP-HPLC peptide mapping procedures employed in this study, 10 of the 12 half-cystine residues of bTSH β could be concomitantly resolved in a single chromatographic experiment; the remaining pair of half-cystine residues corresponded to the glycopeptide bTSH β ^{19–30}. The fluorescent-labelling micro-procedure employed with bTSH β and the 5-I-AEDANS reagent [14], when coupled with this RP-HPLC peptide mapping method, permitted routine analysis of as little as 250–500 ng of the reduced and alkylated bTSH β and has a number of advantages over the more traditional methods using ¹⁴C-labelled alkylating reagents such as [¹⁴C]iodoacetic acid. In particular, the use of the fluorescent reagent 5-I-AEDANS enabled direct online monitoring of the extent of incorporation of the fluorescent label into bTSH β at the level of approx. 10 pmol, without the requirement for subsequent scintillation counting or the concomitant and significant losses in

the overall amount of labelled peptides available for structural analysis because of handling limitations.

The use of the partial reduction approach under a variety of experimental conditions, followed by alkylation with the fluorescent reagent, has enabled the half-cystine residues of bTSH β to be classified into three distinct groups according to the reactivities of the corresponding disulphide bonds (Figure 4). The first group, which consisted of the half-cystines Cys⁸⁸ and Cys⁹⁵, was the most readily reduced. Both of these half-cystine residues are located within the same tryptic peptide, obtained after digestion of the reduced and alkylated bTSH β . As the fluorescent peak corresponding to this peptide was the dominant peak in all chromatograms derived from the forward-labelling experiments and the first to disappear from the reverse-labelling experiments, a disulphide bond can be assigned between Cys⁸⁸ and Cys⁹⁵. Other studies have concluded that the same disulphide bond exists between Cys⁸⁸ and Cys⁹⁵ on the basis of partial reduction of bTSH β either at pH 7.0 [5] or pH 8.2 [9]. The possibility that the half-cystines Cys⁸⁸ and Cys⁹⁵ could be linked to the two half-cystine residues, Cys¹⁹ and Cys²⁷, not represented in the chromatogram, can in addition be discounted for the following two reasons. First, an analogous disulphide bridge between the homologous half-cystine residues, Cys⁹³ and Cys¹⁰⁰, in bLH β and hCG β has been assigned after partial reduction [5,10] or cleavage studies [6,17]. Secondly, a corresponding disulphide bond has been confirmed in hCG β (e.g. Cys⁹³–Cys¹⁰⁰) from the recently established X-ray crystal structure [2,3].

The second group of half-cystine residues of bTSH β that can be classified on the basis of their disulphide bond chemical reactivity included Cys¹⁶, Cys⁶⁷ and Cys¹⁰⁵ (Figures 4, 5a and 5b). The disulphide bonds associated with this group of half-cystine residues exhibits greater reactivity than all other half-cystines (except Cys⁸⁸ and Cys⁹⁵) at neutral pH and low reducing reagent concentrations, both in the presence and absence of denaturant (Figure 4, panels D and F). The experimental data suggest that the half-cystine residues Cys¹⁶ and Cys⁶⁷ form one disulphide bridge in bTSH β , consistent with the observations based either on partial reduction [9,10,18] or extensive cleavage [4,6,17] techniques that the analogous half-cystine residues, Cys²³ and Cys⁷², in LH β and hCG β also form a similar disulphide bond. The half-cystine residue Cys¹⁰⁵ must therefore be linked to one of the two half-cystines, Cys¹⁹ or Cys²⁷, that are not represented in the RP-HPLC chromatogram of the tryptic/chymotryptic digest of fluorescently labelled bTSH β because of their presence in the bTSH β ^{19–30} glycopeptide. By comparison with previous disulphide assignments for LH β and hCG β , Cys¹⁰⁵ is most probably linked to Cys¹⁹ because an analogous assignment (Cys²⁶–Cys¹¹⁰) has been made for LH β and hCG β [6,9,10,17]. Of the six β -subunit disulphide bonds, the three described above for LH β and hCG β , Cys⁹³–Cys¹⁰⁰, Cys²³–Cys⁷² and Cys²⁶–Cys¹¹⁰, have historically been the best accepted assignments. The results arising from the present investigation provide for the first time structural evidence that identical assignments can be made for bTSH β . It can be concluded from these results that the disulphide bonds in bTSH β involving Cys¹⁶–Cys⁶⁷ and Cys¹⁹–Cys¹⁰⁵ are more reactive than the remaining three disulphide bonds and hence are probably more accessible to the reducing reagent [19]. The X-ray crystallographic structure for hCG supports this conclusion with both the β -subunit Cys¹⁶–Cys⁶⁷ and Cys²³–Cys⁷² disulphide bonds exposed to the solvent, whereas two of the three remaining disulphide bridges are buried within the cystine knot [2,3].

The least reactive disulphide bonds in bTSH β involved the group of half-cystine residues Cys², Cys³¹, Cys⁵², Cys⁸³ and Cys⁸⁵ (Figure 4). The remaining half-cystine residue, Cys²⁷, which was not represented as an eluting fluorescently labelled peptide in the

RP-HPLC chromatograms, can also be included in this group. Disulphide bond assignments for the analogous half-cystine residues in LH/hCG β are the most contentious in the literature, with a number of studies using different techniques having reached dissimilar conclusions [4,10,17]. As is evident from the results shown in Figure 4, the susceptibility to reduction of these three remaining disulphide bonds in bTSH β was remarkably similar under the experimental conditions employed in this study for the forward-labelling experiments.

However, the results from the second experimental strategy in which partly reduced and alkylated bTSH β was fractionated by HPIEX chromatography (Figure 6) enabled clarification of the assignment of these last three disulphide bonds. Two of the fractions obtained after HPIEX chromatography were found to contain the Cys⁸⁸–Cys⁹⁵ reference peptide 6 and just two other peaks corresponding to peptides with amino acid sequences containing the half-cystine residues Cys³¹ and Cys⁸⁵ (Figure 6, fractions 6 and 7). This result indicates that a disulphide bond exists in bTSH β between these two half-cystine residues. This Cys³¹–Cys⁸⁵ assignment in bTSH β does not, however, agree with the assignment made by Mise and Bahl [10] for the analogous half-cystine residues of hCG β , which previously was the best accepted of the glycoprotein hormone disulphide bond assignments. The results obtained in the present study from the HPIEX fractionation and RP-HPLC peptide-mapping experiments are unambiguous as there were just two peaks, corresponding to the peptides containing Cys³¹ and Cys⁸⁵, present in the RP-HPLC chromatogram other than the reference peak 6. In addition, the X-ray crystal structures of hCG [2,3] and selenomethionyl hCG [20] indicate that Cys³⁸ in the hCG β -subunit is linked via a disulphide bridge to Cys⁹⁰, which is directly analogous and fully consistent with the assignment of the Cys³¹–Cys⁸⁵ disulphide bond in bTSH β identified by the HPIEX fractionation experiments described above. The divergence in the assignment evident between this present study and the results of Mise and Bahl [10] with the hCG β -subunit probably occurred as a result of uncontrollable disulphide interchange in the earlier study due to the reduction's being carried out at alkaline pH (pH 8.2) and for a longer (30 min) time than in the present investigations. These results, taken together with the observations of Lustbader et al. [20] for the hCG β -subunit, suggest that alternative disulphide bonds may exist as intermediates within this cystine core region during the folding and unfolding stages of the glycoprotein hormone subunits. An additional interesting aspect of the HPIEX chromatographic fractions (fractions 6 and 7) of the partly reduced bTSH β , in which the Cys³¹–Cys⁸⁵ disulphide bridge was identified, was that only one other disulphide bond, Cys⁸⁸–Cys⁹⁵, was partly reduced. This indicates that the reduction of the Cys³¹–Cys⁸⁵ disulphide bond is not dependent on the prior reduction of any of the other disulphide bonds.

On the basis of the assignment of the Cys³¹–Cys⁸⁵ disulphide bond derived from the results of the HPIEX fractionation experiment, the remaining disulphide pairings must involve the half-cystine residues Cys², Cys²⁷, Cys⁵² and Cys⁸³. Owing to the very similar reactivities of the disulphide bonds involving these half-cystine residues, no evidence was available from these experiments to permit the direct assignment of particular half-cystine residue pairings. However, it is likely that the assignments of these two disulphide bonds will follow those for the analogous half-cystine residues in hCG derived from its X-ray crystal structure. Hence the remaining two disulphide bond assignments

of bTSH β can provisionally be considered to be Cys²–Cys⁵² and Cys³⁴–Cys⁸⁸. According to the X-ray crystal structure of hCG, the three disulphide bonds that correspond to the three least reactive disulphide bonds in bTSH β would be arranged in a cystine knot structural motif [2,3]. In hCG β -subunit this cystine knot is formed by the linkage of two of the disulphide bonds (Cys³⁴–Cys⁸⁸ and Cys⁸⁸–Cys⁹⁰) to adjacent antiparallel strands, thereby forming a ring through which the third disulphide bridge (Cys⁹–Cys⁵⁷) penetrates. In the free hCG β -subunit, the two bonds Cys³⁴–Cys⁸⁸ and Cys⁹–Cys⁵⁷ are buried within the knot. Because of the high level of amino acid sequence homology between bTSH β and hCG β it is likely that a similar tertiary structure exists for bTSH β ; this would explain the lower reactivity observed for the corresponding disulphide bonds, with the disulphide bond analogous to Cys³⁸–Cys⁹⁰ exposed to the solvent. It has long been suspected that the various glycoprotein hormone β -subunits must possess similar overall conformations to enable them to combine with a common α -subunit, and that these similar conformations result in part from identical disulphide structures made possible by the conserved location of the half-cystine residues. The results presented in the present paper thus provide clarification of the disulphide bond structure within the bTSH β -subunit and reveal additional evidence consistent with the proposal that the TSH β -subunit and the hCG β -subunit possess identical disulphide bond structures.

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