PARTIAL REDUCTION OF DISULFIDE BONDS IN THE HORMONE-SPECIFIC SUBUNITS OF TSH AND LH*

by

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Received September 5,1975

Summary: Partial reduction at pH 7.0 of the hormone specific (β) subunit of either bovine thyrotropin or luteinizing hormone with dithioerythritol results primarily in the opening of a single disulfide bridge. The partially reduced subunits were alkylated with [1-^{14}C]iodoacetic acid, followed by complete reduction and alkylation with non-radioactive iodoacetic acid. Isolation and degradation of the radioactive tryptic peptides shows that the bond primarily reduced in each β subunit links analogous half-cystine residues in the two sequences (88-95 in TSH-β and 93-100 in LH-β). These results are the first direct evidence of similar disulfide structures in hormone specific subunits of glycoprotein hormones.

The glycoprotein hormones of the anterior pituitary (TSH, LH and follicle stimulating hormone) and of the placenta (human chorionic gonadotropin) share similar structural features. Each consists of two subunits; one, α, is common with major differences in amino acid sequence found only between species. The β subunits each have different sequences but marked similarities are also apparent, for example between TSH-β and LH-β, when sequences are aligned so that their 12 half-cystine residues are in juxtaposition (e.g. 1, 2 (a review), 3, 4). This striking coincidence suggests that the relative positions of the 6 disulfide bonds in each hormone are the same. However, location of these bonds has proven very difficult by the usual methods of

*A brief presentation of some of these data was made at a Ciba Foundation Symposium on "The Peptide Hormones:Molecular and Cellular Aspects", July 1975. Abbreviations used are LH, luteinizing hormone; TSH, thyroid stimulating hormone; DTE, dithioerythritol.

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isolation and analysis of cystine containing peptides, and most peptides have not been obtained by hydrolysis under conditions which preclude disulfide interchange. This communication reports the identification of a disulfide bond in both TSH-β and LH-β after partial reduction at pH 7.0; a procedure found to yield relatively stable intermediates particularly in the case of LH-β.

MATERIALS AND METHODS

The β subunits were isolated as previously described (5,6). Experimental conditions were, in general, those of Cornell and Pierce (7) in studies of the disulfide bonds of the α subunit except that partial reductions were at pH 7.0 rather than 8.5 to minimize possible disulfide interchange. Subunit (3.6×10^{-1} μmoles per ml) was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, containing 1% EDTA and DTE (5×10^{-3} molar, a 2.4 fold excess of DTE to total protein disulfide). After 30 minutes, [1-^{14}C]iodoacetic acid (a 1.25 fold excess over initial -SH present in the DTE)* was added in an equal volume of 0.2 M phosphate buffer, pH 7.2. Alkylation was for 30 minutes at 0° and 30 minutes at room temperature, which was then followed by complete reduction and alkylation (10). Peptides are designated as before (8,9) and were isolated by paper electrophoresis at pH 6.5 (7,8). Specific radioactivity was determined by liquid scintillation counting and amino acid analysis (7) (Beckman 120 analyzer with an expanded scale).

RESULTS AND DISCUSSION

The distribution of label introduced into the tryptic peptides of TSH-β and LH-β is given in Fig. 1a,b. The TSH-β contained 1.1 moles of S-carboxymethyl cysteine per mole, the LH-β 2.1 moles. With TSH-β, approximately 65% of the radioactivity eluted in a single peak (Fraction I) which contained only Peptide βT-12 (residues 88 through 101). Its sequence is in Fig. 2; its analysis and specific radioactivity are in Table I. A partial cleavage of βT-12 (Fraction I) was obtained with chymotrypsin; three radioactive peptides were detected after paper electrophoresis. One was intact βT-12 while analysis of the others (Table I) showed them to be the two expected chymotryptic peptides. Within experimental error, an equal amount of radioactivity was in each chymotryptic peptide (i.e. 30-34% of the total); no more than 16% of the total was found in any other fraction. Thus it is con-

*Use of a 10-fold excess of iodoacetic acid yielded a product containing the same amount of S-carboxymethyl cystine as found with the amount of alkylating agent used in the labeling experiments.

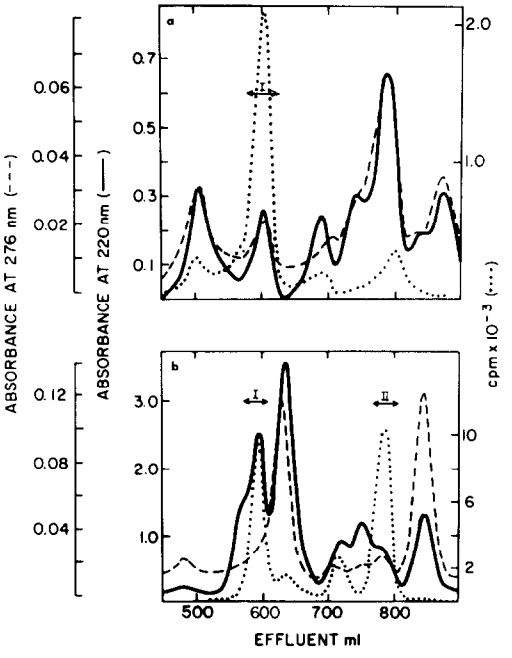

Fig. 1. Gel filtration on Bio-gel P-10 (200-400 mesh) of reduced and carboxymethylated subunits after hydrolysis with trypsin. Columns (2.4 x 200 cm) were run at room temperature with a flow rate of about 20 ml per hour with 0.63 M ammonium bicarbonate as eluting buffer. a, 9 mg of TSH-β partially reduced and labeled as described in the text; b, 50 mg of LH-β partially reduced and labeled as described in the text. Radioactivity is designated by the dotted ..... line.

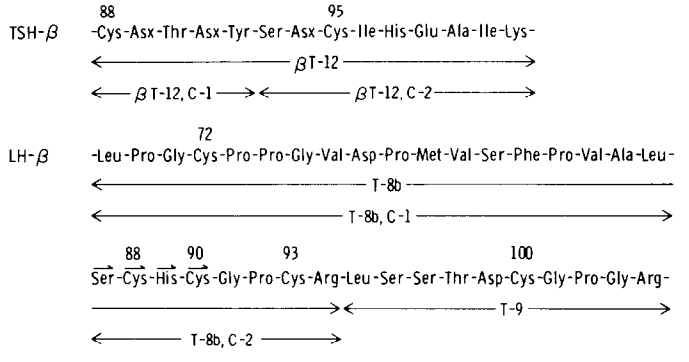

Fig. 2. Amino acid sequences of the regions of TSH-β and LH-β containing the radioactive half-cystine residues.

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cluded that residues 88 and 95 are disulfide bonded in the intact subunit.

Partial reduction of TSH-β with 3.5×10^{-3} M DTE (1.6 M excess over total

Table I

Composition and specific radioactivity of the major radioactive peptides isolated from TSH-β and LH-β after partial reduction

The nanomoles of peptide in each sample were calculated on the basis of the number of nanomoles of each stable residue or in the case of smaller peptides on the average of the number of nanomoles of each residue found. Numbers in parentheses show theoretical number of residues[a].

Residue	TSH βT-12	TSH βT-12, C-1 (Cys 88)	TSH βT-12, C-2 (Cys 95)	LH βT-8b, C-2 (Cys 88, 90, 93)	LH βT-8b, C-2, Ed-4[b] (Cys 93)	LH βT-8b[c] (Cys 72, 88, 90, 93)	LH βT-9 (Cys 100)
Lys	1.1(1)	-	0.7(1)	n.d.[d]	0.1	0.2	-
His	0.8(1)	-	0.8(1)	n.d.(1)	0.4	0.9(1)	-
Arg	-	-	-	n.d.(1)	1.0(1)	1.0(1)	1.0(1)
CM-Cys	1.7(2)	1.0(1)	0.9(1)	2.8(3)	1.0(1)	4.2(4)	1.0(1)
Asp	2.8(3)	2.0(2)	1.1(1)	0.6	0.3	1.1(1)	0.9(1)
Thr	1.0(1)	0.7(1)	-	0.6	0.2	0.2	0.8(1)
Ser	0.9(1)	0.2	0.9(1)	1.1(1)	0.4	1.8(2)	1.5(2)
Glu	1.2(1)	0.3	1.1(1)	0.6	0.3	0.3	-
Pro	-	-	-	1.7(1)	1.2(1)	6.0(6)	1.4(1)
Gly	-	0.3	-[f]	1.0(1)	1.0(1)	2.9(3)	2.1(2)
Ala	0.9(1)	0.1	1.0(1)	0.6	0.3	1.1(1)	-
Val	-	-	-	0.4	0.2	2.8(3)	-
Met	-	-	-	-	-	0.8(1)	-
Ile	1.8(2)	0.3	1.8(2)	0.5	0.1	-	-
Leu	-	-	-	0.5	0.2	2.0(2)	1.0(1)
Tyr	0.9(1)	0.7(1)	-	0.2	-	-	-
Phe	-	-	-	0.2	-	0.9(1)	-
radioactivity, cpm per nmole[e]	5800	3150	2750	4500	3900[c]	5400	4200

[a]A dash indicates less than 0.1 residue was found.

[b]Analyses of Peptide βT-8b, C-2 after 4 steps of Edman degradation.

[c]Separated from the glycopeptide βT-2 by absorption of the latter on Concanavalin A Sepharose.

[d]Not determined.

[e]Radioactivity present in the extracted anilino thiozolinones after steps 2 and 4 of the Edman degradation of βT-8b, C-2 was 400 cpm in each case. The specific radioactivity of the S-carboxymethyl TSH-β (before tryptic digestion) was 8700 cpm per nmole, that of the LH-β was 11,500 cpm.

[f]This fraction was contaminated with glycine, probably from the paper from which it was eluted.

protein disulfide) in 0.1 M Tris-HCl at pH 8.5 for 30 minutes at 0° resulted in a pattern similar to that in Fig. 1a except that a lesser portion of the radioactivity was in Fraction I. Determination of the specific radioactivity of the two chymotryptic peptides again showed an equal distribution of radioactivity between half-cystines 88 and 95.

In LH-β the analogous half-cystines (residues 93 and 100) are in two different peptides, βT-8b (26 residues) and βT-9 (10 residues) (Fig. 2). Fig. 1b shows the elution profile of the tryptic digest of LH-β labeled in the same manner as the TSH-β. Fraction I was shown to contain primarily Peptide βT-8b and glycopeptide, βT-2; the latter, after isolation by paper chromatography, was found to contain essentially no radioactivity. Peptide βT-8b contains four S-carboxymethyl cysteines; two methods were used for the

location of label. A portion of Fraction I was treated with chymotrypsin and a fraction of the digest containing most of the radioactivity was obtained by paper electrophoresis. Its composition showed the fraction to be primarily the carboxyl terminal portion of βT-8b, i.e. (βT-8, C-2, Fig. 2) though some contamination was present (Table I). Four steps of the Edman degradation removed 2 of the 3 half-cystines, 3900 counts remained in the peptide fraction which must contain half-cystine 93 (Table I, Fig. 2). Confirmation of the radioactivity at this position was obtained by passing another portion of Fraction I through a column of Concanavalin A Sepharose (0.4 x 3 cm, equilibrated with 0.01 M sodium phosphate pH 7.5 (containing 0.85% NaCl, 10^{-3} M $CaCl_2$, 10^{-3} M $MnSO_4$, and 10^{-3} M $MgCl_2$) to separate βT-8b from the glycopeptide βT-2. All radioactivity emerged with the void volume; the retained glycopeptide had less than 1% of the radioactivity after its elution with 0.3 M methyl mannoside. The composition of the non-retained fraction is given in Table I; it is that of Peptide βT-8b. After removal of the COOH-terminal arginine (Fig. 2) by treatment with carboxypeptidase B (10), hydrazinolysis was carried out for 40 hours as described by Braun and Schroeder (11)*. After a preliminary purification by paper electrophoresis, the fraction containing the bulk of the radioactivity was placed on the amino acid analyzer and free S-carboxymethyl cysteine was determined. The effluent containing the S-carboxymethyl cysteine was collected and the specific activity was found to be 4300 counts per nmole of S-carboxymethyl cysteine. The same specific radioactivity was found in Peptide βT-9 (Table I). This peptide, containing only one half-cystine was purified from Fraction II (Fig. 1b) by paper electrophoresis. Thus the data, which show that about 80% of the counts of Peptide βT-8b were in half-cystine 93 and equivalent amount to be at position 100 in Peptide βT-9, strongly indicate a disulfide bond between positions 93 and 100, which is analogous to that found in TSH-β.

*A newly opened bottle of hydrazine was used rather than redistilled material.

While limited to one bond, the data offer direct chemical evidence concerning a disulfide placement in these hormones and provide evidence that the tertiary structures of the hormone specific subunits are very similar, as well as supporting the homology apparent in their linear sequences. While it is presumably the similarities in tertiary structure of β subunits which allow them to recombine with the common α subunit even to the extent of interhormone-interspecies hybrids (e.g. 1,12) additional direct evidence is needed. It should be noted that the circular dichroism spectra of TSH-β and LH-β also are very similar in many features (10).

At pH 7.0 a ten fold increase in DTE concentration or the inclusion of 8 M urea did not give significant additional reduction of LH-β over a 2 hour period (a maximum of 2.7 residues of S-carboxymethyl cysteine were found) thus showing marked resistance to further reduction at pH 7.0. That only 1.1 residues of S-carboxymethyl cysteine were found with TSH-β and that appreciable amounts of label were introduced into positions other than 88 and 95 suggests that the reduction of this bond facilitates additional disulfide bond reduction in TSH-β more readily than in LH-β.

By reaction at pH 7.0 it was hoped to prevent disulfide interchange. Were the labeling pattern to reflect interchange, it would have to have proceeded with high specificity to near completion in spite of low concentrations of thiol anions at pH 7.0 (13). It would also require that the new disulfide bond formed by interchange be more difficult to reduce than that originally present. Such events would allow alkylation of the two thiol anions remaining on the protein which were originally not bonded together. The same sequence of events would also have to be postulated for both β subunits despite differences in their primary structures. In a preliminary report, Ward et al. (14) describe successive treatment of ovine LH-β with cyanogen bromide, pepsin and thermolysin to obtain cystine containing peptides whose composition indicated bonds between positions 23-72 and 26-110. Partial acid hydrolysis of a second fraction from the above degradations led to the tentative conclu-

sion (as emphasized by Ward et al. (14)) of bonds between positions 9-88, 34-93, 38-100, and 57-90. Two of the latter are incompatible with the present data, but disulfide interchange is certainly not precluded during partial acid hydrolysis.

The course of reduction of LH-β was also examined by polyacrylamide gel electrophoresis at pH 9.5 (10). Three major components were seen after the 30 minute reduction and subsequent alkylation. One component with intermediate mobility contained 70-80% of the radioactivity and was shown, after excision from the gel and amino acid analysis, to contain two residues of S-carboxymethyl cysteine as did the component with the least mobility (10-15% of the radioactivity). The third component, which had the greatest mobility, probably represents material with more than one bond reduced. Experiments are in progress to separate components in which different numbers of disulfide bonds have been reduced and determine the locations of these bonds.

Acknowledgments - Supported in part by USPHS Grants CA 2290 and AM 18005 and Training Grant GM 00364. Appreciation is expressed to Miss Tiiu Reimo for technical assistance.

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