Differential Effects of Alkylation of Methionine Residues on the Activities of Pituitary Thyrotropin and Lutropin

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Methionine residues of the α and β subunits of bovine lutropin (LH) and bovine thyrotropin (TSH) have been specifically alkylated with iodoacetic acid. The α subunit has been modified so that two of the four methionines are quantitatively alkylated (residues 8 and 33, in agreement with studies by Cheng, K.-W. (1976) Biochem. J. 159, 71-77). Reassocation of the modified α subunit with unmodified LH-β or thyrotropin (TSH)-β resulted in reconstituted hormones which differed markedly in their respective biological activities. The α-modified TSH was fully active in both radioligand receptor and in vitro assays, while the α-modified LH, because of lowered affinity for receptor, lost approximately 70% of its activity in its radioligand receptor assay. This observation is the first to show that modification of the α subunit leads to a differential loss of activity in one glycoprotein hormone versus another. Circular dichroism studies revealed no changes in conformation; thus, the data strongly support, for LH, a direct interaction of the common subunit with receptor.

Methionine 32 in TSH-β can be modified with retention of full activity under conditions where methionines 8, 9, and 58 are not modified. Previous work on the modification of lysine 42 in LH-β which lies in an analogous domain implicates that residue in receptor interaction (e.g. Liu, W.-K., Yang, K.-P., Nakagawa, Y., and Ward, D. N. (1974) J. Biol. Chem. 249, 5844-5850; Sairam, M. R., and Li, C.-H., (1975) Arch. Biochem. Biophys. 167, 534-539). These results further emphasize the probable importance of this domain in hormone specificity.

The glycoprotein hormones lutropin, follitropin, thyrotropin, and human chorionic gonadotropin are comprised of two dissimilar, noncovalently bound subunits, designated α and β. The α subunit is common to all of these hormones (see Ref. 1 for a review) and has an identical amino acid sequence within a species. The β subunit contains the information for specifying hormonal activity, which is expressed only after association with the α subunit.

Because the α subunit is necessary for activity in each hormone, several different functions have been suggested for it. An interaction of the α subunit with a cell surface component that initiates the hormonal response could explain its conservation among the glycoprotein hormones; however, no data supporting this theory have been forthcoming. It has also been suggested that the α subunit acts as a template, which, when combined with different β subunits, endows them with a unique conformation that results in receptor specific interaction (e.g. Ref. 2). After careful evaluation of the effect of specific protein modifications of the α subunit with respect to conformational changes of the hormone or a reduction of the affinity between subunits, two regions of the α subunit have been implicated as LH 1 receptor-binding domains. One is around the disulfide bond formed between half-cystine residues 11 and 35 (3). Another study has shown that removal of the carboxyl terminus of the α subunit causes loss of receptor-binding activity in both LH and TSH. Conformation of the α subunit, as measured by circular dichroism, was not altered and cross-linking of the subunits showed that loss of activity was not due to a modification-induced dissociation (4). Although these and other data clearly support a role for the α subunit in receptor interaction, they indicate that the COOH-terminal region does not contribute to hormonal specificity, as the ability to bind to both LH and TSH receptors is lost upon modification.

This paper reports the alkylation of methionine residues by iodoacetic acid in the α subunit and in the β subunits of TSH and LH. The modified residues were identified and the effects of modification on recombination with the unmodified counterpart subunit, receptor-binding activity, and conformation were assessed. The modified α subunit was recombined with both LH-β and TSH-β and the activities of the reconstituted hormones were measured in receptor assays.

**EXPERIMENTAL PROCEDURES**

**Materials**—The subunits of LH and TSH were prepared from bovine anterior pituitaries as previously described (5, 6). Iodoacetic acid was obtained from Sigma Chemical Co. and was twice recrystallized from carbon tetrachloride. [1-14C]Iodoacetic acid (287 mCi/mg), Na 14CO3 (16.05 mCi/g), and 32P (0.8 mCi/ml) were obtained from Amersham, Sephadex G-100 and G-25 were obtained from Pharmacia and Bio-Gel P-10 was from Bio-Rad. Trypsin treated with p-toluene-sulfonylphenylalanine chloromethyl ketone was from Worthington Biochemical Corp. and was used without further purification. All other reagents were of the highest possible commercial grade.

**Alkylation of Subunits**—Carboxymethylation of the individual α and β subunits was performed by incubating the protein at a concentration of 1 mg/ml with iodoacetic acid in 0.2 M formic acid at pH 3.0. The reaction was carried out for 18 h at 37 °C in the dark, as described by Vithayathil and Richards (7). Under these conditions, the reaction of iodoacetic acid with amino acid side chains is specific for methionine residues. The reaction was stopped by gel filtration on a Sephadex G-25 column (1.5 × 92 cm) in 0.1 M acetic acid to separate protein from excess iodoacetic acid. The subunit was recovered by lyophilization. The molar excess of iodoacetic acid used was based on the number of methionine residues per subunit, 4 residues in α and 11 in β.

1 The abbreviations used are: LH, lutropin; TSH, thyrotropin; hCG, human chorionic gonadotropin; α and β, the subunits of the glycoprotein hormones; Cm-methionine, carboxymethylated methionine.
The recrystallization of iodinated methionine residues modified in a given experiment was obtained from amino acid analysis following performic acid oxidation of the protein. Performic acid oxidation (8) converts only unmodified methionine to methionine sulfoxide and the number of modified residues can be obtained by the difference.

Recombination of Subunits—Modified subunits were recombined with their native counterpart subunits by dissolving 1 mg in 1 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 2.6 mM EDTA and 0.02% azide. The solution was incubated at 37 °C for 48 h and then gel filtered through a Sephadex G-100 column (1.4 x 200 cm) equilibrated in 1% ammonium bicarbonate to separate whole hormone from unrecombined subunits and to determine the extent of recombination.

Hormone Assays—The receptor-binding activity of LH derivatives was determined by a radioligand receptor assay (9) with crude rat testicular membrane fractions and 125I-labeled bovine LH as the labeled ligand. The receptor-binding activity of TSH derivatives to crude bovine thyroid membrane fractions was determined in a radioligand receptor assay using 125I-labeled bovine TSH as labeled ligand (10). Radiiodination of hormone was performed using the lactoperoxidase procedure of Torell and Johnson (11).

Biological activity of bovine TSH was assayed by measurement of the uptake of 125I into thyroids of day old chicks (12).

Identification of Modified Methionine Residues—After alkylation of methionine in LH-α or TSH-β with 1-14C-labeled iodoacetic acid, the modified subunit was reduced with dithioerythritol and the cysteines were carboxymethylated with unlabeled iodoacetic acid in 0.5 M Tris-HCl, pH 8.5, containing 2.6 mM EDTA (13). No carboxymethylated residues are obtained under these conditions. The reduced S-carboxymethyl derivative was then digested with trypsin at an enzyme/substrate ratio of 1:50 (w/w) in 0.5% ammonium bicarbonate. The tryptic peptides were separated on a Bio-Gel P-10 column (1 x 125 cm) equilibrated in 0.5% ammonium bicarbonate as before (14, 15). The eluates were monitored at 220 nm and the elution position of 14C-labeled peptides was determined by counting 100-μl aliquots of 0.75-ml fractions in 6 ml of scintillation fluid (Research Products International 5aR05) in a Beckman LS-3133T counter. Fractions containing labeled peptides were pooled and lyophilized. Twenty μg of each labeled peptide was dissolved in 30% acetic acid and counted in 6 ml of fluor to determine the incorporation of 14C-jodoacetic acid/μg of peptide. Counting efficiency was determined by the addition of 1.875 x 10^6 Bq of 14C tolueno isomer standard (New England Nuclear) to each sample.

The exact specific activity of the 14C-jodoacetic acid was determined prior to use by carboxymethylating reduced LH-α which incorporates 10 mol of iodoacetic acid/mol of protein. The number of becuqerels per mol of protein was then used to calculate the specific activity of the 14C-jodoacetic acid. The measured specific activity was approximately 10% of the specific activity of the iodine-131. One hundred μg of each labeled peptide was hydrolyzed and subjected to amino acid analysis for peptide composition and to determine accurately the nanomoles of peptide per μg for calculations of specific activity from the incorporation of 14C per μg of peptide. Identification of the labeled peptides was based upon their compositions and positions of elution from the Bio-Gel P-10 column (14, 15). The extent of modification of the methionine residues was based upon the specific activity of the isolated peptides. The amino acid analyses were carried out on a Beckman model 120 amino acid analyzer with an expanded scale. CD measurements were made with a Beckman CD spectrophotometer as previously described (4).

RESULTS

Effect of Iodoacetic Acid Concentration on the Extent of Carboxymethylation of the Methionines in α and β Subunits—The number of Cm-methionine residues in LH-α, LH-β, and TSH-β after alkylation with a 3.5- or a 14-fold molar excess of iodoacetic acid is shown in Table I. The values for LH-α and LH-β essentially agree with those of Cheng (16), although we found somewhat higher ratios of iodoacetic acid were required to achieve comparable degrees of alkylation. The recrystallization of iodoacetic acid immediately prior to the reaction was very important as small amounts of iodide present in the reagent appeared to decrease the extent of alkylation of methionine residues.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Cm-methionine residues/subunit</th>
<th>No. of methionines in subunit</th>
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<tbody>
<tr>
<td><strong>LH-α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5 molar excess</td>
<td>1.8</td>
<td>2</td>
</tr>
<tr>
<td>14 molar excess</td>
<td>3.4</td>
<td>4</td>
</tr>
<tr>
<td><strong>TSH-β</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5 molar excess</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>14 molar excess</td>
<td>1.7</td>
<td>4</td>
</tr>
<tr>
<td><strong>LH-β</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5 molar excess</td>
<td>1.4</td>
<td>N.D.*</td>
</tr>
<tr>
<td>14 molar excess</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

* Studies by Cheng (16) show that 2.3 methionines are alkylated at a 10 molar excess of iodoacetic acid and 2.7 methionines alkylated at a 20 molar excess.

In contrast to LH-β, 3 of the methionine residues of TSH-β appeared less accessible for alkylation. At a 3.5-fold molar excess of iodoacetic acid, one methionine of TSH-β is alkylated; however, increasing the molar excess to 14 resulted in alkylation of an average of only 0.7 additional residue. As the reaction is at pH 3 on the isolated β subunit, this effect is less likely to be due to the burying of 3 methionine residues in TSH-β than to local environments unfavorable to alkylation surrounding the methionine residues. The net charge of a methionine residue does not change after carboxymethylation, but a dipolar structure is introduced (17). Neighboring charged residues may be unfavorable to the formation of such a dipole.

Recombination of Modified Subunits—Carboxymethylated subunits were recombined with their native counterpart subunits and reconstituted hormone was separated from subunits by gel filtration (Fig. 1). The extent of recombination of the modified subunits was determined by comparing the areas of peaks corresponding to whole hormone and subunits. The data show that the amount of hormone formed from a modified and native subunit in a 48-h period decreased as the extent of alkylation of methionines increased in the modified subunit. The fractions containing whole hormone and unrecombined subunits were pooled and, in each case, the number of modified methionines determined. Within experimental error, the extent of methionine alkylation in the reconstituted hormone and in the unrecombined subunits was identical. These results suggest that methionine modification of a particular subunit of aT1 does not change the rate of association with its counterpart subunit. They further suggest modification does not result in a mixed population of alkylated and nonalkylated proteins, because if it did, an unequal distribution of modified subunits would be observed between the two peaks. Amino acid analysis, however, is not sufficiently accurate to detect a fractional difference in the number of modified residues in each peak. In the LH and TSH instances in which analysis gave a nonintegral number of alkylated methionines, there must have been some molecules with one more methionine modified than others.

Identification of Alkylated Methionine Residues in LH-α and TSH-β—The labeled alkylated methionines in LH-α (1.8 residues modified) and in TSH-β (1 residue modified) were located by examination of tryptic peptides whose fragmentations are shown in Fig. 2, A and B. For LH-α (Fig. 2A), the majority of the 14C label was distributed into two peaks. The first peak was identified as αT1 which contains methionine 8 and the second peak contains both αT1a and αT3a. αT1a is the same peptide as αT1 except for truncation at its NH2 terminus (18). Peptide αT3 also contains one methionine, residue 33. The specific activity calculated for αT1 indicated 1 mol of...
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unit preparation (1.8 methionine residues alkylated) reassociated with native TSH-β to generate a hormone with a relative potency of 100% that of the TSH formed by recombining native α and β subunits. However, when the same preparation of alkylated α subunit was reassociated with LH-

...iodoacetic acid was incorporated/mol of peptide and for αT3, 1.4 mol of iodoacetic acid were found incorporated/mol of peptide. The higher ratio for αT3 was probably due to contamination of the αT3 peak with αT2, αT2b, and αT6 which also contained small amounts of radioactivity. These results showed the majority of label to be incorporated into αT1 and αT3, with quantitative modification of methionine 8 and 33, as was reported by Cheng (19).

Fractionation of labeled TSH-β peptides (Fig. 2B) shows greater than 98% of the radioactivity incorporated in TSH-β T4, thus demonstrating specific modification of methionine 32.

Radioligand Receptor and Biological Assay of Modified LH and TSH—The results of radioligand receptor assays for the TSH and LH derivatives are presented in Fig. 3 with approximate relative potencies given in Table II. The α-sub...
β, the resulting hormone had a relative potency of only 25-30% that of LH formed from LH-α and β. Reconstituted hormone formed from either native LH-β or TSH-β with an α subunit preparation containing 3.4 alkylated methionines led to a substantial decrease in the ability of both derivatized hormones to bind to their receptors (5 and 15% retained, respectively).

An LH-β preparation (1.4 methionines alkylated) reassocia-
ted with LH-α to form a derivative with 15-20% of the activity of the recombinant of native subunits, a value slightly lower than that found by Cheng (16).

The derivative formed with native α and TSH-β with one
methionine alkylated had 100% of the receptor-binding activity of the recombinant of undervarized TSH subunits; however, further alkylation of TSH-β (1.7 methionines alkylated) generated a hormone with a relative potency of only 27%. The two fully active derivatives of TSH, one formed from a modified α subunit and the other from a modified β subunit, were found to be active in the assay of uptake of 32P into the chick thyroids. Derivatives with decreased receptor-binding activities were not characterized further in bioassays.

**Table II**

*Receptor-binding activity of methionine-modified derivatives of lutropin and thyrotropin*

<table>
<thead>
<tr>
<th>Purified reassociated subunits</th>
<th>Radioligand receptor assay-relative potency *</th>
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<tbody>
<tr>
<td>LH-α + LH-β</td>
<td>%</td>
</tr>
<tr>
<td>1.8 Cm-Met α + LH-β</td>
<td>100</td>
</tr>
<tr>
<td>3.4 Cm-Met α + LH-β</td>
<td>6</td>
</tr>
<tr>
<td>α + 1.4 Cm-Met LH-β</td>
<td>17</td>
</tr>
<tr>
<td>LH-α + TSH-β</td>
<td>100</td>
</tr>
<tr>
<td>1.8 Cm-Met α + TSH-β</td>
<td>100</td>
</tr>
<tr>
<td>3.4 Cm-Met α + TSH-β</td>
<td>15</td>
</tr>
<tr>
<td>α + 1.0 Cm-Met TSH-β</td>
<td>100</td>
</tr>
<tr>
<td>α + 1.7 Cm-Met TSH-β</td>
<td>27</td>
</tr>
</tbody>
</table>

* Relative potencies were calculated on the basis of the amount of a modified sample required for 50% displacement of specifically bound label relative to the required amount of hormone reassociated from unmodified subunits.

**FIG. 4.** Circular dichroism of LH with methionine-modified α subunit. ———, Bovine LH; - - - - α (1.8 methionines alkylated) + LH-β.

**FIG. 5.** Circular dichroism of TSH with methionine-modified α and β subunits. ———, Bovine TSH; - - - - α (1.8 methionines alkylated) + TSH-β; - - - - α + TSH-β (1.0 methionine alkylated).

**DISCUSSION**

A function of the α subunit in direct recognition of receptor sites has been suggested by other protein modification studies that result in hormone derivatives with low activity in radioligand receptor assays. A model of hormone receptor interaction that accounts for the presence of a common subunit in the glycoprotein hormones is one in which sites are present in all glycoprotein hormone receptors that are specific for binding to the α subunit. Such sites might be important in increasing the affinity of an αβ complex for receptor or might be effector sites at which α initiates a hormonal response. For example, there appears to be a site common to both LH and TSH receptors (4, 21-23) which interacts with the carboxyterminal pentapeptide of an α subunit when it is combined with a β subunit.

Our data now define a separate domain of the α subunit

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1 In this experiment in which the Ks was determined, the relative potency of the modified LH was also found to be 50% that of recombinant LH. This increase in relative potency may reflect a weighing error or difference in moisture content of this preparation of the derivatives.
which differs functionally from that described above. Its role appears to be one of facilitating discrimination between the two types of receptors for hormone binding. Methionines 8 and 32 are likely to be situated near each other and to constitute a single domain of three-dimensional structure because a disulfide bond bridges half-cystines 11 and 35. Interestingly, reduction of the 11–35 bond and subsequent carboxymethylation of the half-cystine residues also result in a loss of binding of the reconstituted, modified hormone to LH receptors.

In TSH-β, the alkylation of methionine 32 does not lead to any loss in relative potency of reconstituted hormone in radioligand receptor assays. This result suggests that this region of TSH-β is not involved in receptor binding. However, we cannot exclude the possibility that methionine 32 is important for receptor binding and that the particular chemical modification made does not perturb the interaction of this residue with the receptor. We feel this is unlikely as the hydrophobic character of the methionine residue is altered considerably upon alkylation.

Methionine 32 of TSH-β and methionine 41 of LH-β lie, when the half-cystines in the linear sequences are aligned, in analogous regions of their respective subunits and are separated by only 1 amino acid residue. Despite their close proximity in the sequence, TSH-β methionine 32 is quantitatively modified at a 3.5-fold molar excess of iodoacetic acid, yet LH-β methionine 41 is not significantly alkylated at this concentration of reagent (19). The positive charge on the adjacent lysine residue 42 in LH-β may inhibit the formation of an alkylated methionine and account for this observation.

The region of the LH-β subunit containing lysine 42 is implicated in LH receptor interaction by modification studies. Chemical modifications of the two lysines in LH-β (lysines 20 and 42) that result in the introduction of a negative charge at these residues cause the reconstituted hormone to lose receptor-binding activity. However, in porcine LH-β, a substitution of aspartate, an acidic residue, for lysine at position 20 occurs. Therefore, the introduction of a negative charge at lysine 42 alone is implicated in the decrease in receptor interaction of the modified hormone. It is interesting to compare this result with our modification of methionine 32 in TSH-β in which no receptor-binding activity is lost. Together these results indicate that this region also may have functional importance in conferring hormonal specificity to LH and TSH.

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REFERENCES