Carboxymethylation of Methionine Residues in Bovine Pituitary Luteinizing Hormone and its Subunits

LOCATION OF SPECIFICALLY MODIFIED METHIONINE RESIDUES

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Bovine lutropin (luteinizing hormone) was carboxymethylated at pH 3.0 for 12 h at 37°C with iodoacetic acid for specific modification of methionine residues. To facilitate the location of preferentially modified methionine residues, iodo[¹⁴C]acetic acid was added as tracer. The α and β subunits of bovine lutropin were carboxymethylated with a 2- or 5-fold molar excess of iodoacetic acid either in the presence or absence of their counterpart subunits. The modified subunits were separated and isolated by counter-current distribution followed by gel filtration on Sephadex G-100. To locate the modified methionines, the isolated α or β chain was reduced, *S*-carboxymethylated and subjected to tryptic hydrolysis. The tryptic peptides were fractionated by gel filtration on Bio-Gel P-10. From analyses of the purified ¹⁴C-labelled tryptic peptides, it was observed that methionine-8 and -33 in bovine lutropin α chain and methionine-52 in the β chain were preferentially modified. Similar results were obtained when isolated α and β subunits were individually carboxymethylated in the absence of their counterpart subunit under identical conditions. The fact that a recombinant of native human lutropin α chain, in which a valine residue is present in the position corresponding to methionine-8 of bovine lutropin α chain, and carboxymethylated bovine lutropin β chain regenerated a substantial amount of receptor-site-binding activity indicated that methionine-8 in bovine α chain was biologically not essential. These studies showed clearly that both methionine-33 in the α chain and methionine-52 in the β subunit were involved for optimum binding between bovine lutropin and its receptors for expression of hormonal activity.

Pituitary lutropin (luteinizing hormone), a glycoprotein hormone, has been established to consist of two dissimilar subunits (Papkoff & Samy, 1967; Lamkin *et al.*, 1970; Pierce *et al.*, 1971*a*). The α subunit is the 'common' or 'non-specific' subunit, of which the primary amino acid sequence is identical with the α subunit of other pituitary glycoprotein hormones within the same species (Liao & Pierce, 1971; Pierce *et al.*, 1971*b*; Liu *et al.*, 1972*a*), and the β subunit is the 'hormone-specific' subunit (Liao & Pierce, 1970; Pierce *et al.*, 1971*a*). The primary amino acid sequence of both α and β subunits of bovine lutropin has been determined and demonstrated to be identical with the subunits of ovine lutropin (Maghni-Rogister & Dockier, 1971; Pierce *et al.*, 1971*b*; Liu *et al.*, 1972*a,b*; Sairam *et al.*, 1972*a,c*).

In the preceding paper (Cheng, 1976), the effects of carboxymethylation on bovine lutropin and its α and β subunits have been described. Methionine residues in both subunits of bovine lutropin were not essential for interactions between subunits; and at least one to two residues of methionine in each α and β subunit were demonstrated to be essential

for maximum interaction between lutropin and its receptors (Cheng, 1976). The present report describes my studies on locating the specifically modified methionine residues, which are essential for receptor-binding activity, in the α and β chains of bovine lutropin after carboxymethylation with 2- and 5-fold molar excesses of iodoacetic acid.

Materials and Methods

Materials

Highly purified bovine lutropin (potency, 2.0-2.5 times the LH-S1 standard of the National Institutes of Health, Bethesda, MD, U.S.A.) and its α and β subunits were gifts from Dr. John G. Pierce, UCLA, Los Angeles, CA, U.S.A. Purified human lutropin α and β subunits were gifts from Dr. A. F. Parlow, Harbor General Hospital, Torrance, CA, U.S.A. Iodoacetic acid was purchased from Eastman Chemical Co., Rochester, NY, U.S.A., and was recrystallized twice before use. Na¹²⁵I (carrier-free) and iodo[¹⁴C]acetic acid (specific radioactivity = 12.09 mCi/mmol) were obtained from New England

Nuclear Corp., Boston, MA, U.S.A. Bio-Gel P-10 was obtained from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont., Canada. All other reagents and chemicals were reagent grade.

Carboxymethylation of bovine lutropin α and β subunits, and isolation of carboxymethylated products

The procedure for carboxymethylation of methionine residues with iodoacetic acid was carried out in 0.2M-formic acid at pH 3.0, adjusted with 1M-NaOH, at 37°C in the dark for 12h as described in the preceding paper (Cheng, 1976).

To facilitate the location of the CM-methionine (carboxymethyl-methionine) residues, ^{14}C -labelled iodoacetic acid was added as tracer to the unlabelled iodoacetic acid for carboxymethylation of bovine lutropin and its subunits. Two batches of 30mg each of lutropin, which was largely dissociated into its subunits under these experimental conditions, were treated with a 2- or 5-fold molar excess of iodoacetic acid for the carboxymethylation of its α and β subunits simultaneously in the presence of each other. Other batches of 15mg each of individual α and β chains were carboxymethylated similarly with a 2- or 5-fold molar excess of iodoacetic acid in the absence of their counterpart subunits. For lutropin, 0.8mg (0.05mCi), and for individual subunits 0.4mg (0.025mCi), of iodo[^{14}C]acetic acid was added to the unlabelled alkylating reagent to give a final concentration of 2- or 5-fold molar excess of iodoacetic acid. The protein concentration in the reaction mixture was 10mg/ml for lutropin and 5mg/ml for individual subunits. The protein concentration was determined as in Cheng (1976). The molar excess of iodoacetic acid used was based on molecules of methionine residues in the molecule. At the end of the reaction, the carboxymethylated protein was desalted on a column of Sephadex G-25 in 0.1M-acetic acid.

Separation of the partially carboxymethylated α and β subunits from CM-lutropin, which was largely dissociated into subunits in the reaction mixture, was accomplished by counter-current distribution in the system described by Liao *et al.* (1969), and the separated individual subunits were further purified by gel filtration on Sephadex G-100 in 0.5% NH_4HCO_3 (Cheng, 1976).

Determination of carboxymethylated methionine residues

The number of carboxymethylated methionine residues was obtained by subtracting the methionine sulphone residues, which were formed from unmodified methionine after performic acid oxidation, from the total number of methionine residues in the protein molecule before carboxymethylation as described previously (Cheng, 1976).

Preparation and analysis of tryptic peptides

The disulphide bonds of the purified ^{14}C -labelled carboxymethylated α or β subunit were reduced with 2-mercaptoethanol and alkylated with unlabelled iodoacetic acid as described by Crestfield *et al.* (1963) in 0.5M-Tris/HCl buffer, pH 8.5, containing 0.2% EDTA and 8M-urea. Under these conditions, no carboxymethylation of methionine residues was observed. The reduced *S*-carboxymethyl derivative formed was then hydrolysed by trypsin at an enzyme/substrate ratio of 1:50 in 0.5% NH_4HCO_3 , pH 8.0. The digestion was carried out at 38°C for 4h. The tryptic hydrolysate was subjected to gel filtration on a column (2.5cm $\times$ 200cm) of Bio-Gel P-10 in 1% NH_4HCO_3 , and the eluates were monitored at 220 and 278nm to locate the tryptic peptides. The distribution of ^{14}C -labelled peptides was obtained by counting 20 μl of each fraction in 10ml of Bray's solution (Bray, 1960) with a Packard model 3375 Tri-Carb liquid-scintillation spectrometer. The appropriate fractions were pooled and freeze-dried.

The two ^{14}C -labelled tryptic peptides from α chain (Fig. 2) obtained after gel filtration on Bio-Gel P-10 were pure by analyses (Pierce *et al.*, 1971b), but the labelled peptide from the β chain (Fig. 3) was slightly contaminated and was further purified by paper chromatography in 3-methylbutan-1-ol/pyridine/water (7:7:6, by vol.). The purified ^{14}C -labelled tryptic peptides were analysed for amino acid composition after acid hydrolysis as described by Neumann *et al.* (1962). From this and the reported primary amino acid sequence of bovine lutropin α and β chains (Pierce *et al.*, 1971a; Liu *et al.*, 1972a,b), the positions of the tryptic peptides containing the modified methionine residues, were located. The amino acid sequence of the ^{14}C -labelled tryptic peptide from the β chain was examined further by identifying the first two *N*-terminal amino acids by performing two steps of the subtractive Edman degradation (Konigsberg, 1967) in which formation and extraction of the anilinothiazolinone were carried out as described by Gray (1967a,b).

Gel filtration on Bio-Gel P-10

Tryptic peptides were fractionated on a column (2.5cm $\times$ 200cm) of Bio-Gel P-10 in 1% NH_4HCO_3 as described by Pierce *et al.* (1971b).

Recombinations of native human and bovine lutropin α chains with native and modified bovine lutropin β chain

Various recombinations of native human and bovine α chain with native and carboxymethylated bovine β subunit were carried out by incubating overnight at 37°C with 50 μg each of the subunits in

100 μ l of 0.012M-sodium glycinate buffer, pH9.5, as described previously (Cheng, 1976).

Radioligand-receptor assay for lutropin

Recombinants of human and bovine α subunits with carboxymethylated bovine β chain were assessed by specific radioligand-receptor assay for lutropin receptor-site-binding activity as described in the preceding paper (Cheng, 1976).

Polyacrylamide-gel electrophoresis

Various recombinations of human and bovine α chain with native and carboxymethylated bovine β subunit were also monitored by 7.5% (w/v) polyacrylamide-gel electrophoresis as described previously (Cheng, 1976).

Results

Preparation and isolation of 14 C-labelled carboxymethylated bovine lutropin α and β subunits

To facilitate the location of carboxymethylated methionine residues, iodo[14 C]acetic acid was added as tracer to the reaction mixture for alkylation of lutropin and its subunits. Two batches of 30mg each of bovine lutropin were carboxymethylated with a 2- or 5-fold molar excess of iodoacetic acid. Since lutropin was largely dissociated into its subunits in the reaction mixture, the α and β subunits were carboxymethylated simultaneously in the presence of each other. After desalting on a column of Sephadex G-25 the freeze-dried 14 C-labelled α and β chains from CM-lutropin were separated by the method of counter-current distribution. Fig. 1 depicts the distribution of protein and 14 C radioactivity after counter-current distribution of the 14 C-labelled lutropin, alkylated with a 2-fold molar excess of iodoacetic acid. Radioactivity was found in both α and β chains. Similar results were obtained with a preparation of 14 C-labelled lutropin carboxymethylated with a 5-fold molar excess of reagent. Appropriate fractions of α and β subunits were pooled, desalted and freeze-dried. These separated 14 C-labelled carboxymethylated α and β chains (2- and 5-fold molar excess), which were carboxymethylated in the presence of their counterpart subunits, were further purified by gel filtration on Sephadex G-100 in 0.5% NH_4HCO_3 .

Another two batches of 15mg each of individual bovine lutropin α and β subunits were carboxymethylated similarly with 2- and 5-fold molar excesses of iodoacetic acid. At the end of the reaction, the protein was desalted by gel filtration on Sephadex G-25. The 14 C-labelled carboxymethylated α and β subunits, which were alkylated in the absence of their

counterpart subunits, were also further purified by gel filtration on Sephadex G-100 in 0.5% NH_4HCO_3 .

Characterizations of the various preparations of 14 C-labelled α and β subunits

These different preparations of 14 C-labelled bovine lutropin α and β chains, which were carboxymethylated with a 2- or 5-fold molar excess of iodoacetic acid in either the presence or absence of their counterpart subunits, were characterized by amino acid analysis, polyacrylamide-gel electrophoresis, radioimmunoassay and radioligand-receptor assay as reported in the preceding paper (Cheng, 1976). No side modification of other amino acids was

Fig. 1. Separation by counter-current distribution of 14 C-labelled α and β subunits from bovine lutropin carboxymethylated with a 2-fold molar excess of iodoacetic acid

Twenty transfers were carried out manually with 2.5ml each of upper and lower phases to separate 30mg of 14 C-labelled CM-lutropin. The bars indicate recovered α and β subunits for further studies. —, E_{284} ; ···, 14 C radioactivity.

Fig. 2. Gel-filtration pattern on Bio-Gel P-10 of 15 mg each of a tryptic digest of reduced *S*-carboxymethylated ^{14}C -labelled bovine lutropin α chain, modified with (a) 2-fold and (b) 5-fold molar excess of iodoacetic acid

A column (2.5 cm $\times$ 200 cm) of Bio-Gel P-10 (200–400 mesh) in 1% NH_4HCO_3 was used. Elution was carried out at room temperature (25°C). Fractions A and B were pooled and freeze-dried for further characterization. $\cdots$, ^{14}C radioactivity; —, E_{220} ; ----, E_{278} .

observed, and the recombinants of modified α and β subunits were about 70% active immunologically, but had very low biological activity (Cheng, 1976).

Location of carboxymethylated methionine residues

The disulphide bonds of the ^{14}C -labelled modified α and β chains were reduced and *S*-carboxymethylated with unlabelled iodoacetic acid, and the proteins were then subjected to tryptic hydrolysis. The tryptic digest was fractionated by gel filtration on Bio-Gel P-10 exactly as described for native bovine lutropin α chain (Pierce *et al.*, 1971b). The elution profiles for tryptic digests of ^{14}C -labelled carboxymethylated chain with 2- and 5-fold molar excesses of reagent are shown in Fig. 2 (a and b). These elution profiles for modified α chain were almost identical with that reported for native α chain (Pierce *et al.*, 1971b).

Two major peaks of ^{14}C radioactivity were observed, which were coincident with two peptide peaks, designated A and B (Fig. 2). By comparison with data on bovine lutropin α chain reported previously by Pierce *et al.* (1971b), peaks A and B were peptides T-1 (α) and T-3 (α) respectively. Peptide T-1 (α) was the *N*-terminal peptide consisting of amino acid residues 1–15 with a methionine residue at position 8 (Liu *et al.*, 1972a), $\text{NH}_2\text{-Phe-Pro-Asn-Gly-Glx-Phe-Thr-Met-Gln-Gly-Cys-Pro-Glx-Cys-Lys-}$, and peptide T-3 (α) was reported to have 19 amino acid residues of positions 21–39 with a methionine at position 33 (Liu *et al.*, 1972a), $\text{Tyr-Phe-Ser-Lys-Pro-Asx-Ala-Pro-Ile-Tyr-Glx-Cys-Met-Gly-Cys-Cys-Phe-Ser-Arg-}$. These conclusions were confirmed by amino acid analysis of the material recovered from fractions A and B (Fig. 2) as shown in Table 1. With a 2-fold molar excess of iodoacetic acid,

Table 1. *Amino acid composition of peptide fractions A and B (Fig. 2) containing [¹⁴C]methionine residues obtained from tryptic digest of α chain carboxymethylated with a 2- or 5-fold molar excess of iodoacetic acid*

Peptides were hydrolysed with 6M-HCl for 22h in evacuated sealed tubes at 110°C. Values are expressed in residues per molecule, and calculations were made on the basis of the numbers of residues known to be present from a reported sequence as shown in parentheses (Liu *et al.*, 1972a). Residues of cysteic acid and methionine sulphone were obtained after performic acid oxidation. Values are from single analyses; similar values were obtained by analysis of preparations of α chain similarly carboxymethylated in either the presence or absence of its counterpart β subunit.

Amino acid	Fraction A (T-1) (peptide of residues 1–15)		Fraction B (T-3) (peptide of residues 21–39)	
	2-fold molar excess	5-fold molar excess	2-fold molar excess	5-fold molar excess
Lysine	1.10 (1)	0.97	1.15 (1)	1.02
Histidine				
Arginine			1.03 (1)	1.10
Aspartic acid	1.01 (1)	1.13	1.16 (1)	1.09
Threonine	0.88 (1)	0.83		
Serine			1.75 (2)	1.78
Glutamic acid	2.89 (3)	2.86	1.12 (1)	1.20
Proline	2.10 (2)	1.95	1.88 (2)	1.85
Glycine	1.95 (2)	2.02	1.18 (1)	1.15
Alanine			0.98 (1)	1.02
Cysteic acid	2.35 (2)	2.25	3.20 (3)	3.15
Valine				
Methionine sulphone	0.35 (1)	0.08	0.30 (1)	0.07
Isoleucine			1.10 (1)	0.98
Leucine				
Tyrosine			1.78 (2)	1.82
Phenylalanine	2.08 (2)	1.98	1.95 (2)	2.05

60–70% of total methionine-8 and -33 in the α subunit was carboxymethylated, whereas with a 5-fold molar excess of reagent, over 90% of these two methionine residues were modified (Table 1). The same results were obtained with other preparations of similarly modified α chain, whether it was carboxymethylated in the presence or absence of its counterpart β subunit. It should be pointed out that more radioactivity was incorporated into these methionine residues with a 2-fold molar excess of reagent (Fig. 2a) than with a 5-fold molar excess (Fig. 2b) because less unlabelled iodoacetic acid was used in the former than in the latter to make up the specified molar excess of alkylating reagent (see the Materials and Methods section).

Fig. 3 depicts the elution profiles for tryptic digests after gel filtration on Bio-Gel P-10 of ¹⁴C-labelled β chain carboxymethylated with 2-fold and 5-fold molar excesses of iodoacetic acid. In both cases, only

one major peak of ¹⁴C radioactivity, coincident with a peptide peak, was observed (Figs. 3a and 3b). Two other small peaks of radioactivity eluted earlier were also observed. From sequence data of β chain (Liu *et al.*, 1972b), three methionine residues were located in the primary amino acid sequence of the subunit. Assuming that these radioactivity peaks (two minor and one major, Fig. 3) of tryptic digests after gel filtration were contributed only by carboxymethylated methionine residues in three different tryptic peptides, the major peak of radioactivity (over 80% of the total radioactivity) would be due to a [¹⁴C]methionine residue at position 52 in the sequence of the β chain. After tryptic hydrolysis of the reduced *S*-carboxymethylated β chain, a tryptic peptide of 12 amino acid residues of positions 44–55 would be the smallest peptide containing a methionine, according to the reported amino acid sequence of β chain by Liu *et al.* (1972b): -Val-Leu-Pro-Val-Ile-Leu-Pro-Pro-Met-Pro-Gln-Arg-. When this peptide material was subjected to analysis and was found slightly contaminated with two other peptides, it was purified further by paper chromatography. The amino acid composition of the purified ¹⁴C-labelled peptide is shown in Table 2. To confirm the identity of this tryptic peptide of residues 44–55 containing methionine-52, the first two amino acid residues of the peptide from β chain modified with a 2-fold molar excess of reagent were identified, by the subtractive procedure of Edman degradation, as valine followed by leucine (Table 2). Thus the carboxymethylation of the methionine residue at position 52 of the β subunit was confirmed. With a 2-fold molar excess of iodoacetic acid, 70% of methionine-52 was carboxymethylated, whereas with a 5-fold molar excess of reagent, over 90% of the methionine residue was modified (Table 2). Similar results were obtained with other preparations of similarly modified β chain, whether it was carboxymethylated in the presence or absence of its counterpart α subunit.

Recombinations of human α subunit with native and carboxymethylated bovine β subunits

These findings that recombinations of α and β subunits carboxymethylated with 2- and 5-fold molar excesses of iodoacetic acid had very low biological activity (Cheng, 1976) and that both methionine residues at positions 8 and 33 of the bovine α chain were carboxymethylated very similarly under these conditions (Fig. 2) indicated that possibly both methionine-8 and -33 in the α chain together with methionine-52 in the β chain were involved in the interaction of bovine lutropin and its receptor sites. However, a comparison of the primary amino acid sequence of the *N*-terminal region of bovine or ovine lutropin α chain (Liu *et al.*, 1972a; Pierce *et al.*, 1971b) and human lutropin α chain (Closset

Fig. 3. Gel-filtration pattern on Bio-Gel P-10 of 15 mg each of a tryptic digest of reduced *S*-carboxymethylated ^{14}C -labelled bovine lutropin β chain, modified with (a) 2-fold and (b) 5-fold molar excess of iodoacetic acid

The experimental conditions and symbols were identical with those in Fig. 2.

et al., 1973; Sairam *et al.*, 1972b; Shome & Parlow, 1974) indicated that methionine-8 in bovine lutropin α chain might not be essential for hormone-receptor interactions. At the corresponding position in human α chain, a valine residue is present. The following studies were carried out to demonstrate that only methionine-33 in the α chain and methionine-52 in the β chain were essential for the binding of bovine lutropin to its receptor sites.

Plate 1 shows the electrophoretic protein patterns of various recombinations of native human and bovine lutropin α chain and carboxymethylated (5-fold molar excess) bovine lutropin α chain with native and carboxymethylated (5-fold molar excess) bovine lutropin β chain. From the newly generated electrophoretic protein patterns after incubation, it is obvious that recombinations occurred between native human α chain and native and modified bovine β subunit (Plate 1). Methionine-8 in

bovine α chain was thus not essential for interactions between subunits.

The biological activity of the various recombinants of native human and bovine α chains and modified bovine β subunit was assessed by a specific radioligand-receptor assay for lutropin. Fig. 4 depicts the dilution curves of the various recombinations studied. Negligible receptor-site-binding activity (less than 0.5%) was observed with the recombinant of carboxymethylated bovine α and β chains; however, recombinants of either native human or bovine α chain regenerated 19 and 35% activity respectively of the recombinant of native bovine α and β subunits (Fig. 4). It is worth noting that the recombinant of native human α and β subunits regenerated only 58% of the receptor-binding activity of that of the bovine subunits, whereas the recombinant of human α and bovine β chains regenerated about 70% of the activity (Fig. 4). These observations

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of recombinations of native human lutropin subunits and native and carboxymethylated bovine lutropin subunits

Sample (1) native human α chain; (2) native bovine α chain; (3) bovine α chain carboxymethylated with a 5-fold molar excess of iodoacetic acid; (4) native human β chain; (5) native bovine β chain; (6) bovine β chain carboxymethylated with a 5-fold molar excess of iodoacetic acid; (7) human α chain (1) plus human β chain (4); (8) human α chain (1) plus bovine β chain (5); (9) human α chain (1) plus carboxymethylated bovine chain β (6); (10) bovine α chain (2) plus human β chain (4); (11) bovine α chain (2) plus bovine β chain (5); (12) bovine α chain (2) plus carboxymethylated bovine β chain (6); (13) carboxymethylated bovine α chain (3) and β chain (6).

Table 2. Amino acid composition of purified peptide fraction A (Fig. 3) containing [^{14}C]methionine residues obtained from tryptic digests of β chain carboxymethylated with 2- or 5-fold molar excess of iodoacetic acid

Peptides were hydrolysed with 6M-HCl for 22h in evacuated tubes at 110°C. Values in parentheses are theoretical values from a reported sequence (Liu *et al.*, 1972b). Residues of cysteic acid and methionine sulphone were obtained after performic acid oxidation. Values are from single analyses and expressed in residues per molecule.

Amino acid	2-fold molar excess			5-fold molar excess
	Before Edman degradation	After one step of Edman degradation	After two steps of Edman degradation	
Lysine				
Histidine				
Arginine	0.88 (1)	0.94	0.90	1.12
Aspartic acid				
Threonine				
Serine	Trace			Trace
Glutamic acid	1.22 (1)	1.10	0.98	1.15
Proline	3.78 (4)	3.82	3.78	4.23
Glycine	0.10			0.30
Alanine	Trace			0.10
Cysteic acid				
Valine	1.95 (2)	1.23	1.02	2.05
Methionine sulphone	0.26 (1)	0.31	0.28	0.08
Isoleucine	1.10 (1)	1.21	1.05	1.25
Leucine	2.05 (2)	1.91	1.15	1.97
Tyrosine				
Phenylalanine				

indicated that methionine-8 in bovine lutropin α chain, which is not present in the human α chain, was not essential for the interaction of bovine lutropin with its receptor sites, and that probably other structural differences between the human and bovine lutropin molecules were involved for optimal interactions with the receptor sites. It should be pointed out that in this radioligand-receptor assay ^{125}I -labelled bovine lutropin and partially purified plasma membranes from bovine corpus luteum were used as tracer and receptors respectively.

Discussion

These studies showed clearly that methionine-33 in the α chain together with methionine-52 in the β subunit were essential for interactions between bovine lutropin and its receptors. Both methionine-8 and -33 in bovine α chain were preferentially carboxymethylated under relatively low molar concentrations of iodoacetic acid, and both residues were similarly modified with different molar excesses of

Fig. 4. Dilution curves of radioligand-receptor assay for various recombinations of native human lutropin subunits and native and carboxymethylated bovine lutropin subunits

Original protein concentration of samples was 10000ng/ml. Bovine α chain plus bovine β chain (●—●); human α chain plus bovine β chain (▲—▲); human α chain plus human β chain (□—□); bovine α chain plus carboxymethylated (5-fold molar excess) bovine β chain (○—○); human α chain plus carboxymethylated (5-fold molar excess) bovine β chain (■—■); carboxymethylated (5-fold molar excess) bovine α and β chain (△—△); native human α chain (□—□); native human β chain (○—○).

reagent (Fig. 2). To differentiate the biological significance of methionine-8 and -33, the human α subunit, which has no methionine at its *N*-terminus, was demonstrated to recombine with a carboxymethylated bovine β chain to regenerate biological activity (Plate 1 and Fig. 4). Methionine-8 in bovine α chain was not essential for receptor-site-binding activity. Similar findings have been reported by de la Llosa *et al.* (1972) that treatment of ovine lutropin α chain with CNBr resulted in removal of the *N*-terminal octapeptide, and a recombinant of the treated α chain with native β chain was biologically active. Studies (Pierce *et al.*, 1974) showing that partial reduction and alkylation of two pairs of disulphide bonds at positions 11, 14 and 35, 36 at the *N*-terminal region did not lead to detectable inactivation indicated further that the structural requirements at the *N*-terminus of the α chain cannot be too rigid. On the other hand, removal of a limited number of amino acid residues at the C-terminus rendered the recombinant of this treated α chain and a native β subunit biologically inert (Cheng *et al.*, 1973).

When bovine lutropin β chain was carboxymethylated similarly with 2- and 5-fold molar excesses of iodoacetic acid, mainly methionine-52 was modified (Fig. 3). When this modified β chain was incubated with a native α subunit, a substantial amount of receptor-site-binding activity was regenerated; however, a recombinant of the same β subunit

and a similarly modified α chain had very low activity (Cheng, 1976). These observations are consonant with the findings of de la Llosa *et al.* (1972) that treatment of lutropin β chain with CNBr resulted in the removal of a peptide from residues 42–52, and this treated β chain recombined with native α subunit to give a product with approximately half the activity of a control recombinant. These results indicated that the molecular region from residues 42–52, including methionine-52, was unnecessary for recombination and possibly biological response as well. However, it is demonstrated in this report that methionine-52 in the β chain was essential for optimal interactions between bovine lutropin and its receptors.

Of particular interest in the present studies is the fact that modification of methionine residues in the α or β subunit alone permitted regeneration of the lutropin molecule with a significant amount of biological activity, but recombinants of both modified subunits were practically inactive. In several other studies, modification of the α subunit alone prevented significant recombination. Liu *et al.* (1974) reported that carbamoylation of intact ovine lutropin yielded an inactive product and that recombinants of carbamoylated α chain with native β subunits were active (de la Llosa *et al.*, 1974; Liu *et al.*, 1974). Similar effects on intact lutropin and its subunits on conversion of the carboxyl groups into neutral ones were also reported (Combarous & Hennen, 1974; Faith & Pierce, 1975). The effects of modifications of non-charged residues of lutropin also indicated that functional integrity of the α subunit was more easily disrupted than that of the β chain, as evidenced in previous studies of nitration and iodination of tyrosine residues (Cheng & Pierce, 1972; Combarous & Maghuin-Rogister, 1974). The present studies demonstrated that a biologically active recombinant of modified α chain and native β subunit could be obtained (Plate 1 and Fig. 4).

The studies of Wolff *et al.* (1974) suggested that interaction of α chain with the β subunit of thyrotropin caused conformational changes in the β chain, which resulted in binding to membrane receptor sites and expression of hormonal activity. On the contrary, circular-dichroism studies on native thyrotropin and the carboxypeptidase-treated thyrotropin showed nearly identical spectra; yet the biological activity of the treated hormone was significantly lower (Cheng *et al.*, 1973). Further, the circular-dichroism spectrum of the α chain after modification of carboxyl groups was very similar to that of the native form, and yet an active recombinant was not obtained on incubation with a native β subunit (Faith & Pierce, 1975). Together with the present findings that recombinants of both modified α and β subunits were practically inactive, and yet those with a native counterpart subunit were about half as active,

it would suggest that optimum binding of intact lutropin to its receptor sites probably involves molecular active sites in both α and β subunits for expression of hormonal activity.

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