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

EFFECTS ON THE BINDING ACTIVITY WITH RECEPTOR SITES AND INTERACTIONS BETWEEN SUBUNITS

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The reaction of iodoacetic acid with bovine lutropin (luteinizing hormone) at pH 3.0 was specific for methionine residues; it was slow and reached its equilibrium after 12h at 37°C. The number of modified methionine residues increased proportionately with the amount of the alkylating reagent in the reaction mixture. In the presence of a 20-fold molar excess of iodoacetic acid with respect to methionine, essentially all methionine residues in both subunits of bovine lutropin were carboxymethylated. Studies of various recombinations of modified and native α and β subunits showed that methionine residues in bovine lutropin were not essential for interactions between subunits. Various recombinants were characterized by polyacrylamide-gel electrophoresis and gel filtration on Sephadex G-100. Immunological cross-reactivity by radioimmunoassay of the recombinants of modified α and β subunits was relatively similar to that of the native subunits. However, the biological activity measured by receptor-site binding of the recombinants of α and β chains with a total of three alkylated methionine residues was less that 5% of the activity of native lutropin. It is noteworthy that recombinants of a modified subunit and a native counterpart subunit regenerated 20–30% of biological activity. These findings suggested that at least 1–2 methionine residues in each subunit are involved in the hormone–receptor interaction for bovine lutropin.

The pituitary glycoprotein hormones, lutropin (luteinizing hormone), follitropin (follicle-stimulating hormone) and thyrotropin (thyroid-stimulating hormone), have each been shown to consist of two dissimilar polypeptide chains (Papkoff & Samy, 1967; Pierce *et al.*, 1971*b*; Saxena & Rathnam, 1971). The common subunit, designated α, has an identical amino acid sequence within a species (Liao & Pierce, 1970; Papkoff *et al.*, 1971; Pierce *et al.*, 1971*a*; Shome & Parlow, 1974*a*,*b*; Rathnam & Saxena, 1975). The hormone-specific β subunits within a species can be aligned so that about 50% of their sequences are homologous (Pierce, 1971).

Recently, several studies have been reported on the determination of molecular regions responsible for subunit–subunit interaction and for expression of hormonal activity. Effects of modification of tyrosines of lutropin and thyrotropin (Cheng & Pierce, 1972; Sairam *et al.*, 1972; Yang & Ward, 1972; Combarnous & Maghuin-Rogister, 1974), of lysine residues of lutropin (Ascoli & Puett, 1974; de la Llosa *et al.*, 1974; Liu *et al.*, 1974) and of carboxylic acid groups of lutropin (Combarnous & Hennen, 1974; Faith & Pierce, 1975) have been described. The present report describes studies on the effects of modification of the α and β subunits of bovine lutropin by reaction with iodoacetic acid at low pH for the carboxymethylation of specific methionine residues.

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. J. G. Pierce, UCLA, Los Angeles, CA, U.S.A. This material was obtained from side fractions resulting from the chromatography of pituitary concentrates used in the isolation of thyrotropin (Liao *et al.*, 1969) and was further purified by chromatography on CM-cellulose (Ward *et al.*, 1967) and finally passed through a column of Sephadex G-100 in 1% NH_4HCO_3 to remove small amounts of aggregates and free subunits. Iodoacetic acid was purchased from Eastman Chemical Co.,

Rochester, NY, U.S.A., and was twice recrystallized from light petroleum (b.p. 66–75°C) before use. Na^{125}I (carrier-free) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. Sephadex G-100 was obtained from Pharmacia (Canada) Ltd., Dorval, Que., Canada. All other reagents and chemicals were reagent grade.

Carboxymethylation, isolation of carboxymethylated products and separation of subunits

Carboxymethylation of methionine residues with iodoacetic acid was carried out in 0.2M-formic acid at pH3.0, adjusted with 1M-NaOH as described by Vithayathil & Richards (1960). The reaction was carried out at 37°C in the dark for 12h or as specified. It should be pointed out that under these experimental conditions bovine lutropin was largely dissociated into its α and β subunits in the reaction mixture. The protein concentration in the reaction mixture was 10mg/ml for bovine lutropin for the carboxymethylation of α and β subunits simultaneously in the presence of each other, and 5mg/ml for its individual α or β subunit for the carboxymethylation of a subunit in the absence of its counterpart subunit. At the end of the reaction, the mixture was placed immediately on a column of Sephadex G-25 in 0.1M-acetic acid in the dark to remove low-molecular-weight material (e.g. excess of iodoacetic acid), thus terminating the reaction. The recovered carboxymethylated protein was freeze-dried.

The modified carboxymethylated α and β subunits, which were carboxymethylated individually in the absence of their counterpart subunits, were further purified on a column (1.4cm×200cm) of Sephadex G-100 in 0.5% NH_4HCO_3. Separation of modified α and β subunits from CM-lutropin (carboxymethyllutropin), which was alkylated in the form of a mixture of dissociated subunits, was accomplished by counter-current distribution in the system of 40% (w/v) $(NH_4)_2SO_4$/0.15M-*p*-toluenesulphonic acid/propanol/ethanol (60:60:27:33, by vol.) as described by Liao *et al.* (1969). Fractions containing the carboxymethylated α or β chains were pooled, desalted and further purified by gel filtration on a column (1.4cm×200cm) of Sephadex G-100 in 0.5% NH_4HCO_3. Thus these subunits were carboxymethylated in the presence of their counterpart subunits.

The molar excess of iodoacetic acid used was based on molecules of methionine residues in the protein: four residues of methionine in the α subunit and three in the β chain (Pierce *et al.*, 1971*a*; Liu *et al.*, 1972*a*,*b*).

Determination of carboxymethylated methionine residues

Unmodified methionine in the protein was determined as methionine sulphone after performic acid oxidation (Hirs, 1956) followed by acid hydrolysis by the method of Neumann *et al.* (1962) and Vithayathil & Richards (1960). The number of CM-methionine residues was obtained by subtracting the methionine sulphone residues from the total number of methionine residues in the protein molecule before carboxymethylation.

Recombination of subunits

Studies on recombination of subunits were carried out as previously described (Cheng & Pierce, 1972). Combinations of native or modified subunits were mixed, 50μg each of the α and β subunits being used in 100μl of 0.012M-sodium glycinate, pH9.5, and incubated overnight at 37°C. Samples from these incubation mixtures were characterized by polyacrylamide-gel electrophoresis and by radioligand-receptor assay. In several experiments, recombinations of modified and native subunits were also studied by gel filtration on Sephadex G-100 and a specific radioimmunoassay for bovine lutropin.

Radioligand-receptor assay for lutropin

^{125}I-labelling of bovine lutropin (5μg) was carried out by the chloramine-T method (Hunter & Greenwood, 1962) with 0.5mCi of ^{125}I. The ^{125}I-labelled hormone was separated from damaged aggregates and free ^{125}I by gel filtration on Sephadex G-100, being eluted with 25mM-Tris/HCl at pH7.2. The specific radioactivity of ^{125}I-labelled bovine lutropin was 20–60μCi/μg. The radioligand-receptor assay for lutropin was developed with partially purified plasma membranes from bovine corpus luteum (Gospodarowicz, 1973). The procedure of the assay system was identical with that of the radioligand-receptor assay for follitropin reported by Cheng (1975).

Radioimmunoassay for bovine lutropin

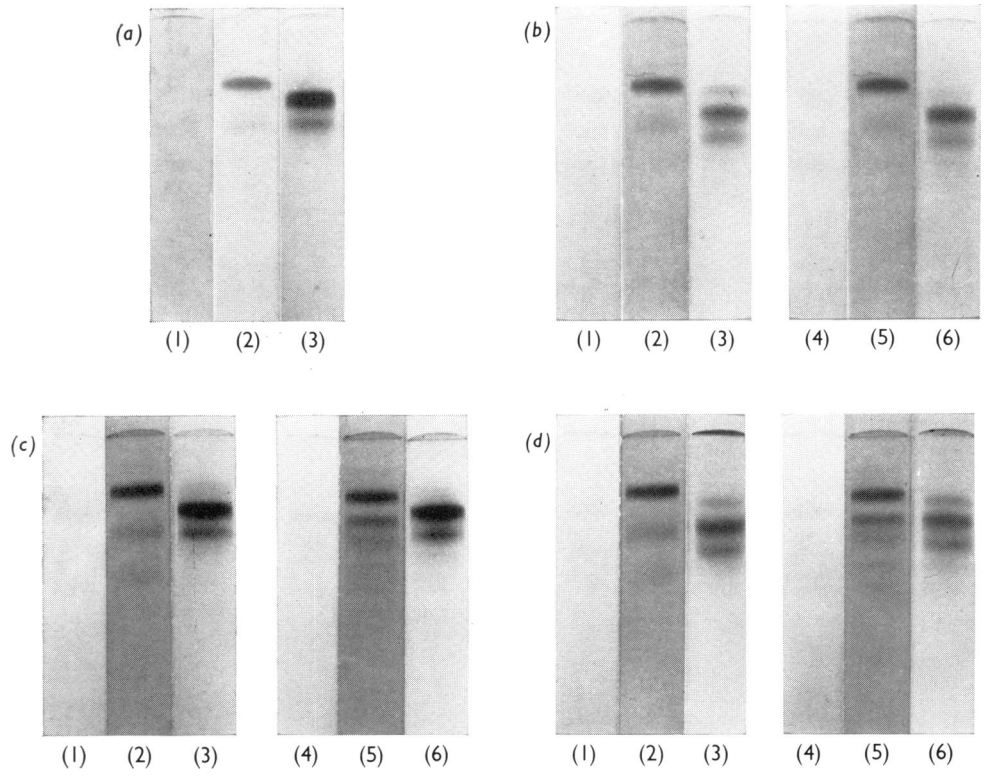
Specific rabbit anti-(bovine lutropin) serum was a gift from Dr. J. G. Pierce. The antiserum was used at a dilution of 1:20000, and a double antibody assay system was used.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out as described by Davis (1964) and Hjerten *et al.* (1965). Gels of 7.5% (w/v) polyacrylamide and at pH8.9 were used. Sodium glycinate buffer (0.012M at pH9.5) was used as electrode buffer. Protein samples of 50 or 100μg were introduced in 40% sucrose solution, and electrophoresis was carried out at 2mA/tube for 1½h. The gels were stained with 1% Amido Black 10B solution in 7% (v/v) acetic acid.

Determination of protein

The dry weights of the hormone or its subunits, obtained by use of a Cahn model 4100 electrobalance, are in these studies quoted as the amounts of proteins used.



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of various recombinations of lutropin subunits

(*a*) Recombinations of native α and β subunits: (1) native α subunit; (2) native β subunit; (3) native α subunit (1) plus native β subunit (2). (*b*) Recombinations of carboxymethylated α subunit and native β subunit: (1) α subunit modified with a 5-fold molar excess of iodoacetic acid; (2) native β subunit; (3) modified α subunit (1) plus native β subunit (2); (4) α subunit modified with a 20-fold molar excess of iodoacetic acid; (5) native β subunit; (6) modified α subunit (4) plus native β subunit (5). (*c*) Recombinations of native α subunit and carboxymethylated β subunit: (1) native α subunit; (2) β-subunit modified with a 5-fold molar excess of iodoacetic acid; (3) native α subunit (1) plus modified β subunit (2); (4) native α subunit; (5) β subunit modified with a 20-fold molar excess of iodoacetic acid; (6) native α subunit (4) plus modified β subunit (5). (*d*) Recombinations of carboxymethylated α and β subunits: (1) α subunit modified with a 5-fold molar excess of iodoacetic acid; (2) β subunit modified with a 5-fold molar excess of iodoacetic acid; (3) modified α (1) plus modified β (2) subunits; (4) α subunit modified with a 20-fold molar excess of iodoacetic acid; (5) β subunit modified with a 20-fold molar excess of iodoacetic acid; (6) modified α (4) plus modified β (5) subunits.

(*facing p.* 72)

Results

Time-course studies for carboxymethylation

The reaction of bovine lutropin with a 50-fold molar excess of iodoacetic acid over methionine residues in 0.2M-formic acid, pH3.0, at 37°C was a slow process. Table 1 shows the number of methionine residues modified under these conditions at different time-intervals of incubation. Under these experimental conditions, carboxymethylation of methionine reached its equilibrium after 8–12h of incubation, and with a 50-fold molar excess of iodoacetic acid over methionine residues, essentially all the methionine residues in both subunits of bovine lutropin were modified at the completion of the reaction (Table 1).

Effects of different molar excesses of iodoacetic acid on the degree of carboxymethylation

Table 2 depicts the number of CM-methionine residues in bovine lutropin and its α and β subunits after alkylations with 0.5, 1, 2, 5, 10, 20 and 50 molar excesses of iodoacetic acid for 12h. The number of CM-methionine residues increased proportionately to the amount of molar excess of reagent in the reaction mixture, and in the presence of a 20-fold molar excess of iodoacetic acid essentially all the methionine residues in lutropin and its subunits were modified. As expected, the sum of CM-methionine residues in the α and β chains was very similar to that of lutropin when alkylated with the same amounts of reagent (Table 2), since lutropin was largely dissociated into its subunits in the reaction mixture.

The amino acid compositions of CM-lutropin and the α and β subunits thereof after treatment with a 10-fold molar excess of iodoacetic acid under these experimental conditions were identical with the unmodified proteins, except for methionine residues as shown in Table 3. No side reaction modifying other amino acids, such as histidine, was observed.

Characterizations of various recombinations of native and individually modified α and β subunits

Plate 1 shows the electrophoretic patterns of various recombinations of native and individually carboxymethylated α and β subunits in the absence of their counterpart subunits. Electrophoretically, only very slight changes were observed between α and β chains carboxymethylated with a 5- or 20-fold molar excess of reagent and native α and β subunits (Fig. 1). Each carboxymethylated α or β chain recombined with its modified or native counterpart subunit (Plates 1*b*, 1*c* and 1*d*). The fact that recombinations occurred with α and β subunits carboxymethylated with a 20-fold molar excess of reagent, in which all methionine residues were modified (Table 2), indicated that methionines in bovine lutropin were not essential for interactions between subunits. The criterion used to detect significant recombination by gel electrophoresis was a marked difference in the protein pattern given by a particular combination of subunits, as compared with the sum of the patterns given by the appropriate individual subunits.

The recombination of partially carboxymethylated bovine lutropin α and β subunits was also examined by the gel-filtration method. After incubation at 37°C of 2.0mg each of carboxymethylated (5-fold molar excess) α and β chains in 1.0ml of 0.012M-sodium glycinate buffer, pH9.5, overnight, the reaction

Table 1. *Number of carboxymethylated methionine residues in bovine lutropin after alkylation with a 50-fold molar excess of iodoacetic acid over methionine residues at different time-intervals*

Results are expressed as carboxymethylated methionine residues per molecule (see the Materials and Methods section). The number of methionine residues in native lutropin from the reported sequence is seven (Liu *et al.*, 1972*a*,*b*).

Time-intervals (h) ...	CM-methionine residues/molecule					
	2	4	8	12	16	24
Expt. 1	4.1	5.5	6.3	6.5	6.6	6.8
Expt. 2	4.3	5.2	6.0	6.7	6.8	6.6

Table 2. *Number of carboxymethylated methionine residues in modified bovine lutropin and its α and β subunits after alkylation with different molar excesses of iodoacetic acid for 12h*

Values of methionine residues for native lutropin and its α and β subunits from reported sequences are seven, four and three respectively (Liu *et al.*, 1972*a*,*b*). Results are expressed as carboxymethylated methionine residues per molecule (see the Materials and Methods section). Values are means of two separate experiments.

Protein / Molar excess of iodoacetic acid ...	CM-methionine residues/molecule						
	0.5	1	2	5	10	20	50
Lutropin	0.5	0.7	2.9	4.7	6.0	6.5	6.7
α subunit	0.2	0.2	1.8	2.7	3.6	3.8	3.8
β subunit	0.2	0.4	1.2	1.8	2.3	2.7	2.8

Table 3. *Comparison of amino acid compositions of native bovine lutropin and its subunits and their carboxymethylated products with 10-fold molar excess of iodoacetic acid*

The results for native lutropin and its subunits are from reported sequences (Liu *et al.*, 1972*a*,*b*). Proteins were hydrolysed with 6M-HCl for 22h in evacuated sealed tubes at 110°C. Residues of cysteic acid and methionine sulphone were obtained after performic acid oxidation. Results are expressed in residues per molecule. Values are from single analyses; similar values except for methionine sulphone were obtained by analysis of different preparations of lutropin and its α and β subunits with different molar excesses of iodoacetic acid.

	Residues/molecule					
Amino acid	Lutropin	CM-lutropin	α subunit	CM-α subunit	β subunit	CM-β subunit
Lysine	12	11.6	10	10.2	2	2.1
Histidine	6	5.6	3	2.8	3	2.7
Arginine	11	10.2	3	3.2	8	7.7
Aspartic acid	11	11.2	6	5.7	5	4.8
Threonine	16	15.6	9	8.4	7	6.7
Serine	14	13.7	6	5.5	8	7.7
Glutamic acid	14	14.3	8	8.2	6	6.1
Proline	27	27.2	7	6.6	20	19.4
Glycine	11	10.7	4	3.7	7	6.8
Alanine	15	14.8	7	7.1	8	7.9
Cysteic acid	22	21.5	10	10.5	12	12.4
Valine	13	12.6	5	4.8	8	7.5
Methionine sulphone	7	1.0	4	0.4	3	0.7
Isoleucine	7	6.5	2	2.0	5	4.5
Leucine	14	13.6	2	1.9	12	11.6
Tyrosine	7	6.7	5	4.7	2	1.7
Phenylalanine	8	7.7	5	5.0	3	2.8

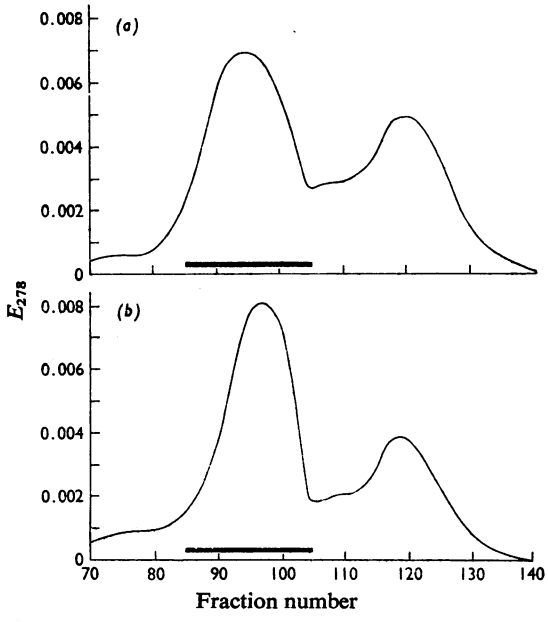


Fig. 1. *Elution patterns after gel filtration on Sephadex G-100 of recombinations of (a) α and β subunits both carboxymethylated with a 5-fold molar excess of iodoacetic acid and (b) native α and β subunits*

A column (1.4cm×200cm) of Sephadex G-100 in 0.5% NH_4HCO_3 was used. The bars indicate recombined material having elution volumes identical with that of intact lutropin.

mixture was then fractionated by gel filtration on Sephadex G-100 in 0.5% $(NH_4)_2SO_4$. Fig. 1 depicts the comparative elution patterns of a recombinant of native α and β subunits, and that of the recombination product of modified α and β subunits. The elution patterns were very similar and the major protein peak in both cases had an elution volume identical with that of intact lutropin.

The recombinants of native and modified α and β subunits were further characterized by a specific radioimmunoassay. Fig. 2 shows the dilution curves of the recombinants of native and partially (5-fold molar excess) and completely (20-fold molar excess) carboxymethylated α and β subunits in a specific radioimmunoassay for bovine lutropin. All the dilution curves of the recombinants of native and modified α and β subunits were parallel to the curve of standard bovine lutropin. The immunological cross-reactivity of the recombinants of native, partially (5-fold molar excess) and completely (20-fold molar excess) carboxymethylated subunits was relatively similar, being 78, 71 and 67% respectively of that of the native intact lutropin (Fig. 2).

Biological activity of the recombinants of partially carboxymethylated and native α and β subunits was assessed by a radioligand-receptor assay. The dilution curves of the various recombinations of partially carboxymethylated or native α and β subunits were all parallel to the curve of standard bovine lutropin as shown in Fig. 3. After incubating overnight a mixture of equal amounts of native α and β subunits, about

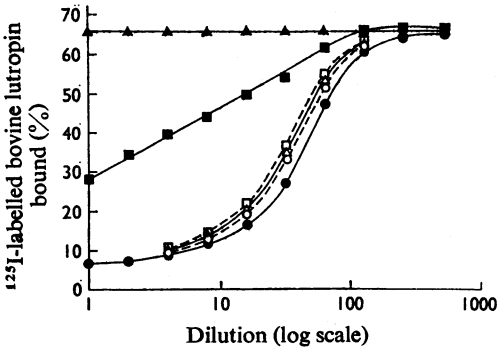


Fig. 2. *Dilution curves of radioimmunoassay for intact lutropin and recombinants of native and carboxymethylated α and β subunits*

Original protein concentration of samples was 100ng/ml. ●, Native lutropin; ▲, α subunit; ■, β subunit; recombinants of native α and β subunits (○), of α and β subunits carboxymethylated with a 5-fold molar excess of iodoacetic acid (△) and of α and β subunits carboxymethylated with a 20-fold molar excess of iodoacetic acid (□).

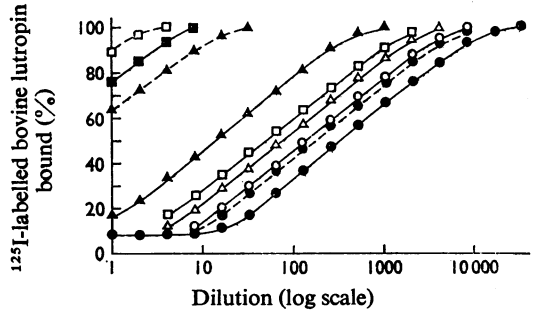


Fig. 3. *Dilution curves of the radioligand-receptor assay for various recombinations of native and carboxymethylated α and β subunits*

Original protein concentration of samples was 10000ng/ml. Native lutropin (●——●), α subunit (□----□) and β subunit (▲----▲); recombination of native α and β subunits (●----●); recombinations of α and β subunits both similarly carboxymethylated with 1-fold (○——○), 2-fold (▲——▲) and 5-fold (■——■) molar excesses of iodoacetic acid; recombinations of a native α and β subunit with a counterpart β or α subunit carboxymethylated with 2-fold (△——△) and 5-fold (□——□) molar excesses of iodoacetic acid.

60% of the total lutropin activity was regenerated as measured by the radioligand-receptor assay (Fig. 3). Similar activity was regenerated in the recombinant of alkylated α and β subunits with only 1 molar excess of the reagent. The biological activities of recombinants of the correspondingly modified α and β chains with 2- or 5-fold molar excesses of iodoacetic acid were observed to be only 3–5% and less than 0.5% respectively of the standard lutropin (Fig. 3). However, when any of these partially modified α or β subunits was incubated with a native counterpart α or β chain, 20–30% activity of the standard lutropin or approx. 50% of the activity of the recombinant of native subunits was regenerated, as measured by the radioligand-receptor assay (Fig. 3).

Characterizations of various recombinations of modified α and β subunits from CM-lutropin

Preparations of modified α and β subunits, carboxymethylated with 2- and 5-fold molar excesses of iodoacetic acid in the presence of their counterpart subunits, were obtained from CM-lutropin after counter-current distribution and gel filtration on Sephadex G-100 as described in the Materials and Methods section. These partially carboxymethylated α and β chains were also characterized with various recombinations of native and modified α and β subunits by polyacrylamide-gel electrophoresis (Plate 1), and radioimmunoassay for interactions between subunits (Fig. 2) and radioligand-receptor assay for receptor-site-binding activity (Fig. 3) as described above. Almost identical results were obtained for the various recombinations of α and β subunits whether they were modified in the presence of their counterpart subunits (as obtained from alkylated lutropin) or modified individually in the absence of their counterpart subunits.

Discussion

Alkylation of methionine residues has been demonstrated to be specific at pH2.5–3.5 with pancreatic ribonuclease (Goren & Barnard, 1970; Gundlach *et al.*, 1959; Neumann *et al.*, 1962). At higher pH, alkylation of histine and lysine was also observed (Goren & Barnard, 1970). Specific modification of methionine residues in bovine growth hormone with iodoacetic acid at pH3.5 has also been reported (Wallis, 1972). Carboxymethylation of bovine lutropin and its subunits with iodoacetic acid in 0.2M-formic acid, pH3.0, at 37°C was shown to be specific for methionine residues (Table 3). However, under these experimental conditions, bovine lutropin was largely dissociated into its α and β subunits in the reaction mixture. The dissociation of sheep lutropin has been demonstrated to be 50% at pH4.0, and the dissociation was reversible (de la Llosa & Jutisz, 1969). The reaction of iodoacetic acid with methionine in bovine lutropin was a slow process, and took approx. 12h at 37°C to reach its equlibrium (Table 1). The degree of carboxymethlation of methionine residues increased proportionately to the amount of the alkylating reagent in the reaction mixture, and at a concentration of a 20-fold molar excess of iodo-

acetic acid, essentially all methionine residues in both α and β subunits of bovine lutropin were carboxymethylated (Table 2).

These studies demonstrated that methionine residues in bovine lutropin were not essential for interactions between α and β subunits to form hybrid molecules. With all methionine residues carboxymethylated [20-fold molar excess; four in the α chain and three in the β chain (Table 2)], interactions between these modified subunits to form recombinants were obvious, as shown by polyacrylamide-gel electrophoresis (Plates 1*b*, 1*c* and 1*d*). The immunological cross-reactivity of these recombinants of modified α and β subunits was relatively similar to those formed from native subunits (Fig. 2). These findings indicated that the seven methionine residues in bovine lutropin were unlikely to be significantly involved in the immunological determinant sites of the molecule.

It is now generally observed that recombination of subunits of glycoprotein hormones regenerates approx. 50–70% of the original hormone activity (de la Llosa & Jutisz, 1969; Gospodarowicz, 1971; Pierce *et al.*, 1971*a*). Our present studies on biological activity of various recombinations of native subunits of lutropin as measured by the radioligand-receptor assay (Fig. 4) were in good agreement with previous studies by bioassay *in vivo* (Cheng & Pierce, 1972; de la Llosa & Jutisz, 1969; Pierce *et al.*, 1971*a*) and by the radioligand-receptor assay (Faith & Pierce, 1975; Liu *et al.*, 1974). The biological activity of bovine lutropin decreased to less than 5% of the original activity with approx. three methionine residues (a total of seven residues) carboxymethylated with a 2-fold molar excess of alkylating reagent (Table 2 and Fig. 3) and the lutropin molecule was largely inactivated (less than 0.5%) when more than three methionine residues were alkylated with a 5-fold molar excess of reagent (Table 2 and Fig. 3).

Another interesting observation was that when a partially modified α or β chain (5-fold molar excess), of which the recombinant was largely inactive, was incubated with an unmodified native counterpart subunit, a recombinant of substantial receptor-site-binding activity (40–50% of native subunit recombinants) was regenerated (Fig. 3). These observations indicate that for optimal binding of bovine lutropin to its receptor sites, at least one or two methionine residues in each α and β chain are essential for the interaction. The following paper (Cheng, 1976) demonstrates that two methionine residues, one each in the α and β subunits, are involved in the receptor-site-binding activity of the bovine lutropin molecule.

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References

Ascoli, M. & Puett, D. (1974) *Biochim. Biophys. Acta* **371**, 203–210
Cheng, K.-W. (1975) *J. Clin. Endocrinol. Metab.* **41**, 581–589
Cheng, K.-W. (1976) *Biochem. J.* **159**, 79–87
Cheng, K.-W. & Pierce, J. G. (1972) *J. Biol. Chem.* **247**, 7163–7172
Combarnous, Y. & Hennen, G. (1974) *FEBS Lett.* **44**, 224–228
Combarnous, Y. & Maghuin-Rogister, G. (1974) *Eur. J. Biochem.* **42**, 13–19
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
de la Llosa, P. & Jutisz, M. (1969) *Biochim. Biophys. Acta* **181**, 426–436
de la Llosa, P., Durosay, M., Tetrim-Clary, C. & Jutisz, M. (1974) *Biochim. Biophys. Acta* **342**, 97–104
Faith, M. R. & Pierce, J. G. (1975) *J. Biol. Chem.* **250**, 6923–6929
Goren, H. J. & Barnard, E. A. (1970) *Biochemistry* **9**, 959–973
Gospodarowicz, D. (1971) *Endocrinology* **89**, 669–678
Gospodarowicz, D. (1973) *J. Biol. Chem.* **248**, 5042–5049
Gundlach, H. G., Moore, J. & Stein, W. H. (1959) *J. Biol. Chem.* **234**, 1761–1764
Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621
Hjerten, S., Jerstedt, S. & Tiselius, A. (1965) *Anal. Biochem.* **11**, 219–223
Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
Liao, T. H. & Pierce, J. G. (1970) *J. Biol. Chem.* **245**, 3275–3281
Liao, T. H., Hennen, G., Howard, S. M., Shome, B. & Pierce, J. G. (1969) *J. Biol. Chem.* **244**, 6458–6467
Liu, W. K., Nahm, H. S., Sweeney, C. M., Lamkin, W. M., Baker, H. N. & Ward, D. N. (1972*a*) *J. Biol. Chem.* **247**, 4351–4364
Liu, W. K., Nahm, H. S., Sweeney, C. M., Holcomb, G. M. & Ward, D. N. (1972*b*) *J. Biol. Chem.* **247**, 4365–4381
Liu, W. K., Yang, K. P., Nakagawa, Y. & Ward, D. N. (1974) *J. Biol. Chem.* **249**, 5544–5550
Neumann, N. P., Moore, S. & Stein, W. H. (1962) *Biochemistry* **1**, 68–75
Papkoff, H. & Samy, T. S. A. (1967) *Biochim. Biophys. Acta* **147**, 175–177
Papkoff, H., Sairman, M. R. & Li, C. H. (1971) *J. Am. Chem. Soc.* **93**, 1531–1532
Pierce, J. G. (1971) *Endocrinology* **89**, 1331–1334
Pierce, J. G., Liao, T. H., Howard, S. M., Shome, B. & Cornell, J. S. (1971*a*) *Recent Prog. Hormone Res.* **27**, 165–212
Pierce, J. G., Laio, T. H., Carlsen, R. B. & Reimo, T. (1971*b*) *J. Biol. Chem.* **246**, 866–872
Rathnam, P. & Saxena, B. B. (1975) *J. Biol. Chem.* **250**, 6735–6746

Sairam, M. R., Papkoff, H. & Li, C. H. (1972) *Biochim. Biophys. Acta* **278**, 421–432
Saxena, B. B. & Rathnam, P. (1971) *J. Biol. Chem.* **246**, 3549–3554
Shome, B. & Parlow, A. F. (1974*a*) *J. Clin. Endocrinol. Metab.* **39**, 199–202
Shome, B. & Parlow, A. F. (1974*b*) *J. Clin. Endocrinol. Metab.* **39**, 203–205
Vithayathil, P. J. & Richards, F. M. (1960) *J. Biol. Chem.* **235**, 2343–2351
Wallis, M. (1972) *FEBS Lett.* **21**, 118–122
Ward, D. N., Adams-Moyne, M., Ray, N., Balke, D. E., Coffey, J. & Showalter, M. (1967) *Gen. Comp. Endocrinol.* **8**, 44–53
Yang, K. P. & Ward, D. N. (1972) *Endocrinology* **91**, 317–320