LUTEINIZING HORMONE DERIVATIVES WITH COVALENTLY-LINKED SUBUNITS

Yves COMBARNOUS and Georges HENNEN

Section d'Endocrinologie, Département de Clinique et de Séméiologie médicales, Institut de Médecine, Université de Liège, B–4000 Liège, Belgium

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1. Introduction

Data on the reversible dissociation of luteinizing hormone into subunits (α and β) as a function of pH strongly suggest that ionic bonds between carboxyl- and amino-groups are involved in the maintenance of the quaternary structure of the hormone [1]. In order to test this possibility, the study of the modification of carboxyl-groups with water-soluble carbodiimide in the absence and in the presence of ammonium chloride was undertaken. When ammonium chloride is present, the reaction leads to the formation of carboxamido-groups [2]. In its absence amino-groups of proteins can react with unstable *O*-acyl isourea intermediates resulting from the derivatization of carboxyl-groups with carbodiimide. This leads to the formation of intramolecular amide bridges provided that carboxyl- and amino-groups are situated close together in the native conformation [3].

2. Materials and methods

Porcine luteinizing hormone (pLH) was purified as previously described [4]. The incubations of the hormone with 1 ethyl, 3 (3 dimethylaminopropyl) cerbodimiimide (EDC) (Ott Chemical Co.) were carried out in water at room temperature for 1 hr [5]. The pH was maintained at 4.75 with the aid of a Radiometer automatic titrator. When present, ammonium chloride (Merck) was at a concentration of 5.5 M. EDC was either 0.1 M or 0.01 M. The hormone was 2 mg per ml when ammonium chloride was present. In the absence of the nucleophile the protein concentration was lowered to 0.1 or 0.5 mg per ml in order to avoid polymerization and to favour intramolecular crosslinking. The reactions were stopped either by deep-freezing followed by lyophilization or by extensive dialysis against 10^{-3} N HCl. In one instance, the reaction was stopped by the addition of sodium acetate to a final concentration of 1 M.

Gel filtrations were on Sephadex G-100 (Pharmacia) columns at 4°C. Amino acid analyses were done after HCl hydrolysis with a Beckman 121 Amino Acid Analyzer [6]. Molecular weight estimations by sodium dodecyl sulfate (SDS)-poly-acrylamide gel electrophoresis were as previously described [7] on 10% polyacrylamide gels. Biological activities were measured by the ovarian ascorbic acid depletion test [8] with NIH-LH S17 as standard.

Immunological activity was determined as previously described [9] by a double antibody radioimmunoassay using pLH ^{131}I as tracer and rabbit pLH antiserum at a final dilution of 1:600 000. Complete dose–response curves were run with 2 replicates at each dose level.

Circular dichroism (CD) spectra were recorded on a JASCO Model ORD/UV-5 spectropolarimeter as previously described [10].

3. Results

Fig. 1 A shows that after amidation of carboxyl-groups of pLH by 0.1 M EDC and 5.5 M ammonium chloride, the elution volume on Sephadex G-100 of the hormone corresponds to that of its subunits ($V_e/V_0 = 2.0$) while the hormone incubated with ammonium chloride alone still exhibits a normal elution volume ($V_e/V_0 = 1.7$).

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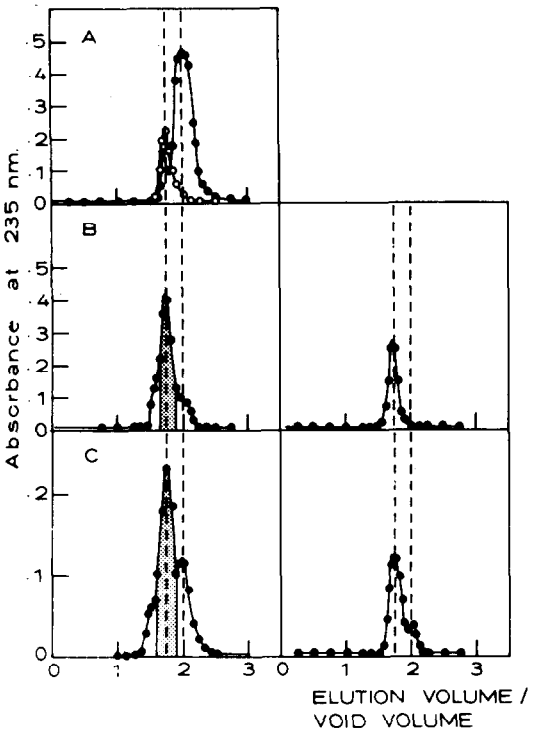

Fig. 1. Gel filtration of porcine LH derivatives. Sephadex G-100 column (1.7 X 95 cm), with 0.05 M ammonium bicarbonate (pH 8.2) buffer. Flow rate 4 ml.hr^{-1}·cm^{-2}. Dotted lines indicate the respective elution positions of native porcine LH (1.7) and of its subunits (2.0). A) porcine LH treated with 0.1 M EDC and 5.5. M NH_4Cl (•—•—•); porcine LH treated by 5.5 M NH_4Cl alone as reference (o—o—o). B) Porcine LH treated with 0.1 M EDC (left); Material from the shaded area was incubated with 8 M urea, 10^{-2} N HCl and rechromatographed on the same column (right). C) Porcine LH treated with 0.01 M EDC (left); Material from the shaded area was incubated with 8 M urea 10^{-2} N HCl and rechromatographed on the same column (right).

pLH treated with 0.1 M EDC alone was dialyzed against 10^{-3}N HCl and lyophilized. On Sephadex G-100 this product exhibits a major peak (shaded on fig, 1B) with an elution volume identical to that of the native hormone. Material from this fraction was incubated overnight in 8 M urea at pH 2.0 and submitted to a second gel filtration on the same column (fig. 1, B right). A single symmetrical peak is ob-

Table 1
Properties of native luteinizing hormone and of its carbodiimide-treated derivatives

Treatments[1]	V_e/V_0	V_e/V_0 after 8 M urea	Molecular weight[3]	Biological activity[4]
None	1.7	2.0	15 700 19 100	100%
5.5 M NH_4Cl	1.7	N.D		N.D
0.1 M EDC + 5.5 M NH_4Cl	2.0	N.D		< 1%
0.1 M EDC	1.7	1.7	27 500	< 1%
0.01 M EDC stopped by dialysis:				
expt 1	1.7	1.7	29 000	100%
expt 2	1.7	1.7		70%
0.01 M EDC stopped by quenching with 1 M sodium acetate	1.7	1.7		< 1%

N.D Not determined.
[1] See Materials and methods.
[2] See fig. 1.
[3] Estimation by sodium dodecyl sulfate-gel electrophoresis.
[4] Percentage of remaining activity.

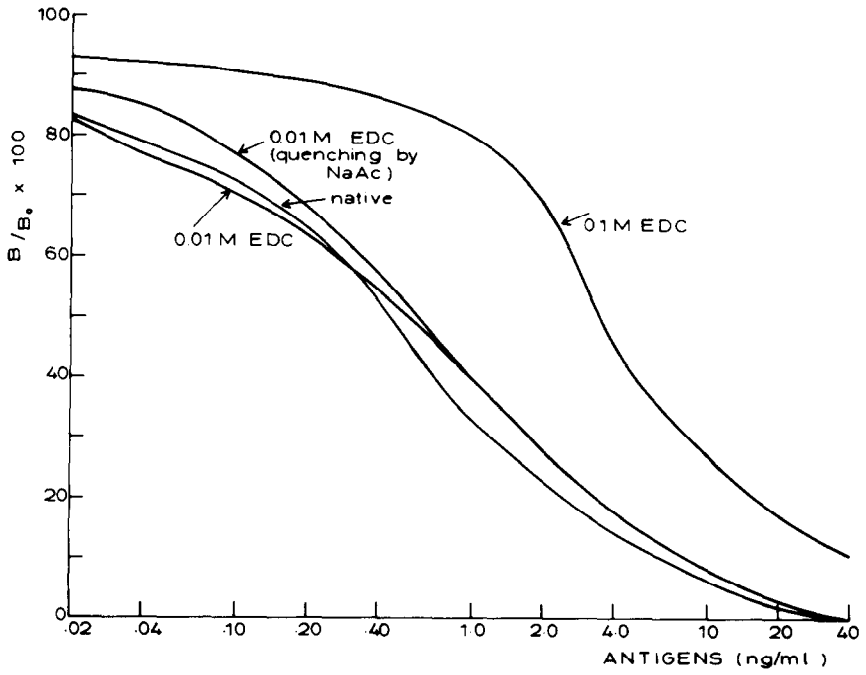

Fig. 2. Radioimmunological activity of porcine LH derivatives. Rabbit pLH-antiserum is used at a final dilution of 1:600 000 with [^{131}I]pLH as tracer. Duplicates were made at each dose level.

tained which is still eluted at the elution volume of the native hormone.

Similar results were obtained with porcine LH treated with 0.01 M EDC except that the proportion of material eluted at the position of the subunits is greater with consequently less material recovered in front of the major peak. In both cases the yield of protein with the the elution volume of native LH after the second run on Sephadex G-100 (fig. 1B and C) amounted of 45–60% of starting material.

The amino acid composition of EDC-treated hormones is identical to that of native LH. In particular the tyrosine content is unaffected by the treatment. As the *O*-aryl isourea derivative of tyrosine is stable to acid hydrolysis [11] it appears that the tyrosine residues of the hormone had not been modified by EDC.

Estimations of molecular weight and biological activities of LH derivatives are shown in table 1.

It is shown in fig. 2 that the immunological dose–response curves obtained with the native porcine hormone and the materials treated with 0.01 M EDC are similar while the 0.1 M EDC-treated hormone exhibits a significantly different pattern, with a five-fold decreased affinity to pLH specific antibodies.

In the 320–240 nm region, native hormone, 0.1 M EDC-treated and 0.01 M EDC-treated LH exhibit exactly the same CD spectrum with a broad negative maximum ($[\theta]_M = -33 \times 10^3$ deg. cm^2 dmol^{-1}) at 280 nm [10]. In contrast, 0.1 M EDC-treated LH differs from native and 0.01 M EDC-treated hormones, in the 200–250 nm region of its CD spectrum. The ellipticity of this derivative at 212 nm is greatly decreased compared to native LH [10] while an important negative band appears below 210 nm. As the dynode voltage rose to exceedingly high values (> 700 V) for wavelengths inferior to 210 nm, the exact position of this band could not be determined.

4. Discussion

This work has shown that modification of carboxyl-groups of the hormone to carboxamido-groups leads

to its dissociation into subunits. As the modification involves the replacement of a negatively charged group by a neutral one, this suggests that the dissociation is due to the breakage of electrostatic bond(s) between carboxyl-group(s) and positively charged group(s). However, this result does not imply that these groups are located on different subunits.

If carboxyl-amino pairs are indeed involved in the maintenance of the quaternary structure, in the absence of added nucleophile the amino-group is expected to attack the nearby active *O*-acyl isourea derivative formed by the treatment with EDC. Indeed, from the gel filtration patterns, it appears that, in contrast to the native hormone, EDC-treated hormones do not dissociate into subunits upon treatment with acidic 8 M urea. This is clearly confirmed by SDS-gel electrophoresis indicating that covalent linkage has occurred between the two subunits.

The biological activity of undissociable luteinizing hormones prepared with 0.1 M EDC and 0.01 M EDC respectively clearly differ (table 1). While the latter is still fully active the former is completely inactivated. This drastic difference in potency can be related to a difference in the number of amide bridges in the two derivatives. The β subunit of pLH contains no free amino-groups [12], thus it is obvious that intersubunit amide bridge(s) involve(s) carboxyl-group(s) from this subunit and amino-group(s) from the α subunit [13]. The fact that an LH derivative with covalently-linked subunits is biologically active shows that after binding with its receptor the hormone does not dissociate to ellicit its biological function.

As with its biological activity, the immunological reactivity of LH is markedly decreased after treatment with 0.1 M EDC and remains intact with 0.01 M EDC. CD spectra of native pLH and of the two undissociable derivatives in the 320–240 nm region show no difference. It is thought that the broad negative aromatic Cotton effect observed at 280 nm in the spectrum of native pLH is due to shielding of tyrosyl residues as a consequence of the association of the subunits [10,14,15]. Thus, the establishment of at least one covalent linkage in the binding area of the subunits apparently does not disturb the status of these tyrosyl residues. In the 200–250 nm region, the CD spectra of native LH and of the active undissociable hormone are not significantly different. In contrast, that of the inactive derivative is different as its negative maximum is enhanced and shifted from 212–214 nm to a wavelength under 210 nm. It is impossible to draw precise conclusions from these observations but, together with biological and immunological data, they suggest an important effect of 0.1 M EDC on the 3-dimensional structure of pLH. The loss of biological and immunological activities of 0.1 M EDC-treated hormone might be due to a loss of 'flexibility' of the molecule arising from the establishment of additional covalent bridges. This rigidity of the hormone could prevent the binding of the molecule to its receptor and its antibodies. Nevertheless, disappearence of amino- or carboxyl-groups essential for biological function cannot be excluded.

When the reaction with 0.01 M EDC is stopped with sodium acetate, the biological activity of the derivative is completely destroyed and cannot be restored by treatment with 0.1 M hydroxylamine (table 1). In contrast immunological reactivity of this derivative is comparable to those of native and active undissociable hormones (fig. 2). This loss of biological activity is attributed to the acetylation of an essential lysine residue of the α subunit. This hypothesis is in agreement with the recent data of De La Llosa et al. [16].

The location of the amide bridge(s) of the active undissociable hormone is under study and should provide information concerning the binding area between subnits. It has been postulated [15,17,18] that binding area between subunits of both luteinizing hormone and thyrotropin are similar. The fact that we have recently succeeded in the preparation of undissociable thyroid-stimulating hormone using EDC supports this view. Lastly, as active undissociable hormone exhibits immunological activity similar to that of native hormone it may be of interest to use this derivative in the purification of specific antibody to dimeric hormone by affinity chromatography.

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