IMMUNOLOGICAL PROPERTIES OF THE β-SUBUNIT OF HUMAN CHORIONIC GONADOTROPIN*

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SUMMARY

The β-subunit of human chorionic gonadotropin (hCG-β) has been modified to a varying degree by the cleavage of the disulfide bonds by reduction with dithioerythritol followed by S-carboxamidomethylation. The resulting derivatives of hCG-β show a preferential loss in their immunological cross-reactivity with human luteinizing hormone (hLH). The immunological specificity of the partially reduced and S-alkylated derivatives can be further enhanced by conjugation with tetanous toxoid using glutaraldehyde. Neither conjugation of hCG-β with the toxoid nor its treatment with anti-hLH immunoadsorbent affects its cross-reactivity with hLH.

Possible potential application of the β subunit of human chorionic gonadotropin (hCG-β)[1] to contraception by immunological means was recognized during the course of our investigations on its structure (1-4). However, one serious problem which hampered its immediate use, was the lack of immunological specificity which is highly desirable for such an application. Because of considerable amino acid sequence homology in the NH_2-terminal three quarters of the molecule, hCG-β shows significant immunological cross-reactivity with human luteinizing hormone (hLH). To overcome this problem two choices were considered - one to remove NH_2-terminal sequences which are homologous to hLH and secondly, to modify it specifically to

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1. Abbreviations used are: NaCl-P buffer, 0.9% sodium chloride in 0.01M sodium phosphate buffer, pH 7.4; hCG, hCG-α and hCG-β, human chorionic gonadotropin and its α and β subunits; hLH, human luteinizing hormone; PRA-hCG-β, partially reduced and S-carboxamidomethylated hCG-β; hCG-β-TT, hCG-β-tetanous toxoid conjugate. IU, International Unit, defined as the activity contained in 0.001279 mg of the international standard for chorionic gonadotropin.

minimize the cross-reactivity. This communication describes some of our initial studies based on the latter approach and includes the effect of the alteration of the tertiary structure on the immunological specificity of hCG-β.

MATERIALS AND METHODS

hCG, 12,000 IU/mg was prepared from a commercial preparation with a potency of 4,100 IU/mg essentially according to Bahl (5) (Organon, West Orange, N.J.) except that a pH of 7.4 was maintained throughout the purification. The β subunit was prepared by dissociation of hCG with 8 M urea followed by chromatography on DEAE-Sephadex using stepwise elution rather than continuous gradient followed by chromatography on Sephadex G-100 (6,7). hCG-β thus prepared had hCG activity of 0.4% as determined by the radioligand receptor assay (8). hLH and tetanous toxoid were generous gifts of Dr. Saxena of Cornell Medical School and Dr. Hosley of Lilly Research Labs., respectively. Dithioerythritol and iodoacetamide were products of Pierce Chemical Co. and Sigma Chemical Co., respectively.

Preparation of Antisera. Antisera against hCG-β and hLH were raised in New Zealand male rabbits. A three-injection schedule was followed by administering 200 μg of the antigen every other week. The antibody titer was measured by using ^{125}I-labeled hormones.

Radioimmunoassays of hCG-β and hLH. hCG-β and hLH were iodinated with Na^{125}I using chloramine-T procedure (9). The radioimmunoassays were carried out by the procedures described previously (10). The free and the bound hormone were separated by alcoholic ammonium acetate (10) or by the double antibody technique using sheep anti-rabbit γ-globulin (11). The relative activities of hCG-β and its derivatives were determined in hCG-β-anti hCG-β and hLH-anti hLH systems by measuring their ability to displace 50% of the labeled ^{125}I-hCG-β and ^{125}I-hLH.

Treatment of ^{125}I-hCG, ^{125}I-hLH and ^{125}I-hCG-β-tetanous Toxoid Conjugate (^{125}I-hCG-β-TT) with Anti-hLH immunoadsorbent. The immunoadsorbent was prepared as described (12). Prior to use, the immunoadsorbent was tested for nonspecific binding by using bovine serum albumin. The immunoadsorbent was considered unsatisfactory if the nonspecific binding was more than 10%.

A sample of each of 5 ng of ^{125}I-hLH, ^{125}I-hCG and ^{125}I-hCG-β-TT ($\sim 10^5$ cpm) in NaCl-P buffer was treated, with continuous stirring, with 0.5 g of the immunoadsorbent for 1 hr at 25°. The suspension was centrifuged and the supernatant, after measuring the radioactivity, was again treated with the fresh immunoadsorbent as described above. This operation was repeated twice.

Treatment of hCG-β with Anti-hLH Immunoadsorbent. 2.0 mg of the highly purified hCG-β in 5 ml of NaCl-P buffer was treated with 10 g (wet weight) of the immunoadsorbent for 2 hr at 25°. The adsorbent was centrifuged and the sediment was washed with 10 ml of the buffer. The supernatant was lyophilized and desalted on Sephadex G-25. hCG-β adsorbed on the immunoadsorbent was eluted by shaking with 10 ml of 0.2 M glycine HCl buffer, pH 2.8 twice for 15 min at 0° and centrifuged. The supernatant was immediately adjusted to pH 7.4 with 1 M K_2HPO_4. The pooled supernatant was lyophilized and desalted on coarse Sephadex G-25. Aliquots of the unadsorbed hCG-β and that eluted from the immunoadsorbent was subjected to amino acid analyses and the radioimmunoassays in both of the above systems.

Graded Reduction and S-alkylation of hCG-β. To 0.2 μmoles of hCG-β in 5 ml of 0.5 N Tris-HCl buffer, pH 8.5 containing 8 M urea and 2% EDTA was added 2.5 to 25 μmoles of dithioerythritol and the reaction mixture was incubated for 30 min at 37° under nitrogen. Subsequently, 6 to 60 μmoles of iodoacetamide were added and the incubation was continued for another 30 min. The reduced and alkylated derivatives of hCG-β (PRA-hCG-β) were desalted on coarse Sephadex G-25 and lyophilized.

Conjugation of hCG-β and PRA-hCG-β with Tetanous Toxoid. hCG-β or PRA-hCG-β was conjugated to tetanous toxoid with a bifunctional reagent, glutaraldehyde, as described by Avrameas (13). The conditions for the purification of the tetanous toxoid conjugate (hCG-β-TT) were established using ^{125}I-hCG-β. After centrifugation to remove the polymerized material, the supernatant was subjected to chromatography on Sephadex G-150 (1.5 x 52 cm) to separate the free hCG-β from hCG-β-TT conjugate. The toxoid-hCG-β conjugates containing varying amounts of the toxoid were prepared by altering the relative proportion of the toxoid and hCG-β.

Amino Acid Analyses. Hydrolysis was performed in evacuated sealed bulbs with 6 N HCl at 110° for 24 hours. 10 μl of thioglycolic acid and 50 μl of phenol were added to avoid any loss of S-carboxymethyl cysteine and tyrosine residues during the acid hydrolysis. The hydrolysates were analysed on a Spinco Automatic Amino Acid Analyzer.

RESULTS AND DISCUSSION

hCG-β has 10% of the immunological activity of hCG in hCG-anti hCG radioimmunoassay system (4,14) and 6% of that of hLH in hLH-anti hLH system (Fig. 1, Table I). Obviously, hCG-β has antigenic determinants some of which are common to hLH and hCG and some that are specific to hCG in the same molecule. The hLH/hCG (common) determinants in hCG-β are likely to be located in the regions of the polypeptide chain which are homologous to hLH-β and the hCG specific determinants reside in the variable region including, in particular, the 32-residues COOH-terminal peptide.

These determinants are predominantly conformational in nature since complete reduction and S-carboxamidomethylation causes the loss of both activities (Table I, Fig. 1). Further evidence in support of the conformational rather than sequential determinants comes from the failure of tryptic, chymotryptic and cyanogen bromide peptides of the reduced and S-carboxamidomethyl hCG-β and hCG-α (1-3) to compete for binding at 10^4 molar excess with ^{125}I-hCG, ^{125}I-hCG-β, ^{125}I-hCG-α for anti hCG, anti hCG-β and anti hCG-α (4). Recognizing the possibility of weak affinity of the peptides for the various antibodies, the double antibody precipitation

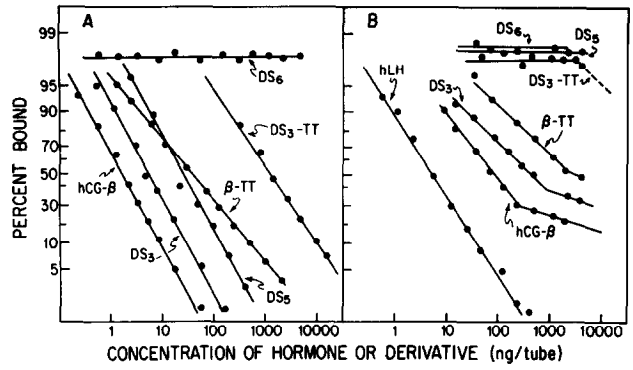

Fig. 1 Radioimmunoassays of hCG-β and its derivatives: A. in hCG-β-anti hCG-β system. B. in hLH-anti hLH system. DS3, DS5 and DS6 represent partially reduced and S-alkylated derivatives of hCG-β with 3, 5 and 6 disulfide bonds cleaved respectively. β-TT and DS3-TT are tetanous toxoid conjugates of hCG-β and DS3 respectively.

Table I. Immunological Activity of hCG-β and its Derivatives

Subunit or derivative[1]	Number of disulfide bonds	Percent activity hCG-β system	Percent activity hLH system	Ratio hCG-β:hLH
hCG-β	6.0	100[2]	100[3] (6.2)	1:1[4]
DS3 hCG-β	2.9	37.2	16 (0.97)	2:1
DS5 hCG-β	1.1	6.6	0	∞
DS6 hCG-β	0	0	0	-
hCG-β-TT	6.0	4	3.92(0.24)	1:1
DS3 hCG-β-TT	2.9	0.16	0.0098 (0.0006)	16:1

[1]Derivatives of hCG-β prepared by the cleavage of 3 to 6 disulfide bonds (DS3 hCG-β, DS5 hCG-β and DS6 hCG-β). hCG-β-TT, DS3 hCG-β-TT are conjugates of hCG-β and DS3 hCG-β with tetanous toxoid.
[2]Values calculated from 50% inhibition of binding ^{125}I-hCG-β or ^{125}I-hLH to anti hCG or anti LH by hCG-β or its derivatives.
[3]Values in the parenthesis represent the actual LH activity of hCG-β or its derivatives.
[4]Ratio of hCG-β and hLH activities.

technique for the separation of free and bound hormone was used. Since all of the disulfide bonds are present in the NH_2-terminal 115-residue segment, which has considerable homology with hLH-β, one would have expected the retention of some of the hCG-β activity, particularly that which is associated with the carboxy-terminal peptide. It appears that the effect of the conformational change in the NH_2-terminal portion brought about by the cleavage of the disulfide bonds is transmitted also to the COOH-terminal peptide

Table II. Treatment of ^{125}I-hLH, ^{125}I-hCG-β and ^{125}I-hCG-β-TT with Anti-hLH Immunoadsorbent[1]

Experiment	cpm in the supernatant		
	^{125}I-hLH	^{125}I-hCG-β	^{125}I-hCG-β-TT
Initial cpm	100,130	118,414	118,919
I cycle	9,421 (9.4)[2]	34,819 (29.4)	43,844 (36.8)
II cycle	2,818 (2.8)	7,598 (6.4)	11,467 (9.6)
III cycle	915 (0.9)	1,186 (1.0)	943 (0.8)
IV cycle	518 (0.5)	498 (0.4)	673 (0.6)

[1] See text for details.
[2] Values in parentheses represent percent unadsorbed from the preceding cycle.

resulting in the total loss of the immunological activity. It was thought that partial reduction and S-alkylation might leave sufficient constraint in the molecule so as to localize this effect and thus preserve the conformational integrity of the COOH-terminal peptide. The resulting derivatives in that case should have less or no cross-activity with hLH but still retain some hCG-β or hCG specific activity. Consequently, hCG-β was partially reduced and S-carboxamidomethylated. Two derivatives were obtained in which 3.1 (DS3-hCG-β) and 4.9 (DS5-hCG-β) disulfide bonds were split as determined from their amino acid analyses. As it is clear from Table I that there is a preferential loss of hLH activity of hCG-β with the increase in the number of disulfide bonds cleaved. DS5-HCG-β has about 5% of the hCG-β and none of the hLH activity at a level of even 5 μg (Fig. 1, Table I).

Tetanous toxoid hCG-β conjugates containing the toxoid in equimolar or higher concentrations were found to have the same relative proportion of hCG-β and hLH activities although overall both activities were drastically reduced. The reduction in the activities was greater in the conjugates in

Table III. Amino Acid Compositions[1] of hCG-β and Anti-hLH Immunoadsorbent Treated hCG-β[2].

Amino Acid	hCG-β	Unadsorbed hCG-β	Adsorbed & eluted hCG-β
Lysine	2.5	2.6	2.2
Histidine	1.7	1.4	1.6
Arginine	7.4	8.5	6.95
Aspartic acid	8.5	8.96	8.36
Threonine	6.8	7.0	6.95
Serine	8.5	9.6	8.3
Glutamic acid	7.2	7.2	6.3
Proline	15.9	17.8	16.7
Glycine	5.9	6.2	5.97
Alanine	5.8	6.4	5.97
Half cystine	6.2	n.d.[3]	6.7
Valine	7.8	8.3	7.97
Methionine	1.3	0.46	0.43
Isoleucine	3.2	3.3	3.2
Leucine	8.2	9.2	8.8
Tyrosine	2.1	1.8	2.0
Phenylalanine	1.5	1.4	1.3

[1]Values represent moles per 100 moles of amino acids
[2]See text for details
[3]n.d. not determined

which higher proportion of toxoid was present. However, when DS3 hCG-β was conjugated with an equimolar proportion of tetanous toxoid, there was a preferential loss of LH activity (Table I). Therefore, unlike hCG-β-TT conjugate, DS3-hCG-β-TT conjugate does improve the specificity of hCG-β.

Talwar et al (15) and Segal (16) have recently claimed the specificity of hCG-β after treatment with anti-ovine LH immunoadsorbent. It seems inconceivable that one can remove hLH/hCG determinants from hCG-β without

the removal of hCG specific determinants since both of them are present in the same molecule. As a matter of fact hCG-β should completely adsorb on such a column. When we treated repeatedly ^{125}I-hLH, ^{125}I-hCG-β and ^{125}I-hCG-β-TT with anti hLH immunoadsorbent, in three cycles almost all the hormone was absorbed (Table II). However, when the treatment of hCG-β with the immunoadsorbent was carried out once, only 38% of it was adsorbed. The adsorbed material was eluted and the amino acid analysis of the eluate was identical to that of the unadsorbed or hCG-β (Table III) indicating that there was no specific effect of adsorption. The radioimmunoassays of the adsorbed and eluted material and that of the unadsorbed hCG-β showed that the ratio of hCG-β and hLH activities were unaltered.

The studies reported here demonstrate that hCG-β has hLH/hCG and hCG specific determinants and these are predominantly conformational in nature. Furthermore, it is possible to preferentially destroy the common determinants in hCG-β by specific modification. The mere conjugation of hCG-β with tetanous toxoid does not improve its specificity. Finally, these results should facilitate the development of a contraceptive vaccine for human use.

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