CHEMICAL MODIFICATION OF HUMAN CHORIONIC GONADOTROPIN AND ITS BIOLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION[a]

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1. Introduction

In the preceding paper [1] we describe a simple chromatographic method which can be easily reproduced for preparation of a highly purified human chorionic gonadotropin (HCG), which nevertheless separated in starch gel electrophoresis without urea and in polyacrylamide electrophoresis with urea into 5–7 bands, respectively. This report deals with chemical modifications of the highly purified glycoprotein hormone, giving some information about the prerequisites for its biological and immunological active site.

2. Methods

Acetylation of HCG was done according to Riordan et al. [2]. Acetanhydride was added in 200 fold excess[e] in half saturated ammonium acetate solution. The incubation lasted for 70 min at 0°.

Succinylation of amino groups was carried out after Klotz [3] adding succinanhydride in phosphate buffer, pH 7.5 in 25, 200 and 770 fold excess[e]. During the reaction with 200 and 770 fold excess[e] the pH decreased to 7.2 and 6.8, respectively. The incubation time was 60 min at 22°.

For carbamylation of amino groups, HCG was incubated with a 150 and 1500 fold excess[e] of potassium cyanate in triethanolamine–EDTA–HCl buffer, pH 7.4 after Stark [4] for 120 min at 22°.

Deamination was performed with sodium nitrite, 500 and 1800 fold excess[e] in 0.1 M ammonium acetate buffer pH 4.0. Incubation time was 30 min at 4°.

Nitration was accomplished after the method of Sokolovsky et al. [5] with a 0.05 M tris buffer, pH 8.0. Tetranitromethane (TNM) was dissolved in ethanol and added to the HCG solution. The final concentration of ethanol was 20%. Incubation time was 45 min at 22°, excess of reagent 100 and 300 fold[f].

Polymerization of HCG with glutaraldehyde was carried out according to Markus [6] in 0.1 M phosphate buffer, pH 7.0 with a 12.5 and 125 fold excess [3] of glutaraldehyde. It was done for 30 min either in the cold at 4° with a low concentration or at 22° with a higher concentration of glutaraldehyde.

Polymerization with bisdiazobenzidine was done as described by Silman et al. [7]. HCG was incubated with this solution at pH 7.4 for 14 hr at 4°.

Polymerization with Woodward reagent K was performed according to Patel et al. [8]. The reaction lasted for 30 min at 22° in 0.1 M phosphate buffer, pH 7.5 using different quantities of the reagent. For identification of the modified products, the following methods were employed: (1) the amino groups were determined by the ninhydrin reaction after Moore et al. [9]; (2) the number of tyrosine groups were

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[e] Based on 8 moles Lys/mole HCG (MW 40,000) determined after [9].
[f] Based on 6 moles Tyr/mole HCG (MW 40,000) determined after [10].

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analysed photometrically after Holeyšovský et al. [10]; (3) active esters were analysed after Lipman et al. [11]. Unicam spectrophotometers models SP 800 and SP 8000 were used. The methods for biological and immunological characterization are given in the preceding paper [1].

3. Chemical and biological results

Acetylation: The purified hormone (see preceeding paper [1]) contained 8 amino groups (determined on the basis of a MW 40,000) using the procedure described above; 5 amino groups could not be acetylated. The remaining activity of this product was 65% (limit of confidence l.o.c. +34 −38%). After reacetylation with the same amount of acetanhydride a rest-activity of 68% (l.o.c. +32 −23%) was obtained. Even after using a larger quantity of reagent, the biological activity did not further decrease. In starch gel electrophoresis the acetylated product moved faster to the anode with 5 bands as compared to the movement of the original hormone.

Succinylation: The biological activity decreased with increasing succinylation. Thus with a 25 fold excess of succinanhydride about 3 amino groups were substituted and with a 200 and 770 fold excess of the reagent about 5 amino groups were modified[e] The corresponding figures for the biological activity were 87, 31 and 7%, respectively. No ester formed with OH groups could be detected. Due to the new negative charges the succinylated hormone moved in starch gel electrophoresis much faster to the anode as one band.

Carbamylation: Under all conditions tested cyanate reacted with 3 amino groups (maximum). There was no concomitant loss of biological activity.

Deamination: 3 amino groups per mole HCG were removed by addition of nitrite. No significant loss of biological activity was observed.

Nitration: In the absence of urea, a 100 and a 300 fold excess of TNM caused a modification of 2 out of 6 tyrosine groups. The latter product showed a fall in biological activity to 66% (l.o.c. +25 −18%). However with a 100 fold excess of TNM no activity was lost.

Polymerization with glutaraldehyde: Although 3 amino groups were substituted with the lowest amount of glutaraldehyde, the molecular weight did not increase remarkably. The sedimentation constant, $s_{20,w} = 3.1$. Ultracentrifuge analysis of the product obtained with a 125 fold excess of glutaraldehyde revealed 2 peaks with an $s_{20,w} = 2.9$ and 6.2, respectively. About 3.5 amino groups per mol HCG were substituted. While the biological activity of the latter hormone derivative was completely destroyed, the first product retained 42% activity.

Polymerization with bisdiazobenzidine: Substitution of 3 tyrosine groups[f] yielded a rest-activity of 41% (l.o.c. +20 −10%). The single peak sedimentated with $s_{20,w} = 2.7$.

Polymerization with Woodward reagent K: Increasing amounts of Woodward reagent K caused a linear increase of sedimentation constant $s_{20,w}$ (table 1). Each time a single peak was observed in the ultracentrifuge. Parallel to the increasing MW, the biological activity of the polymerized hormone decreased rapidly to 2% in the presence of the highest amount of reagent.

Table 1
Biological activity and molecular weight of HCG treated with increasing amounts of Woodward reagent K.

	Execess of reagent (X mole/mole Lys)	Biol. activity (%)	l.o.c. (%)	$s_{20,w}$	MW
Highly purified HCG + Woodward reagent K	2.5	98	(+23 −24%)	3.5	45,000
	8.0	123	(+15 −48%)	4.2	60,500
	25.0	42	(+7 −12%)	7.1	106,000
	80.0	2	(+ 1.5 − 0.7%)	7.1	131,000
Control		100	(+36 −25%)	3.3	40,000

4. Immunological results

The antiserum to purified HCG reacted in quantitative precipitation reactions with the above modified antigen. However, the amount of antigens needed to precipitate a given amount of antibody varied, so did the amount of antibody precipitated. On the basis of their precipitation curves three different groups could be distinguished:

Group I: a) the succinylated and b) acetylated HCG precipitated the least amount of antibody with about 4 μg antigen at equivalence point.

Group II: from the a) carbamylated, b) deaminated and c) nitrated HCG about 8 μg antigen each was needed to precipitate the maximal amount of antibody.

Group III: this group is represented by the antigens polymerized with a) bisdiazobenzidine, b) glutaraldehyde and c) Woodward reagent K. In this case 12–14 μg antigen was needed to precipitate at equivalence point.

5. Discussion

Comparing the chemical with the biological results, the experiments show that substitution or cleavage of up to 3 amino groups does not change the activity of the hormone. There is no difference in the kind of reaction occurring at the amino group, except if some polymerization is taking place at the same time. This result demonstrates that in the absence of urea 3 out of 8 amino groups present in HCG are accessible to chemical reagents. Furthermore their presence is not an essential prerequisite for full biological activity.

Nevertheless during acetylation HCG lost some of its activity, though no more than 3 amino groups were modified. However, one should remember that because of inherent difficulties, the biological test for HCG represents only approximate figures. Nitration of one third of the tyrosines present in the hormone caused only a limited loss of activity. Further experiments to clarify the specific role of the aromatic amino acids are needed.

In the present investigation an attempt was made to polymerize a gonadotropic hormone. For this purpose Woodward reagent K seems to be especially suitable because of its mild and specific reaction. A larger hormone molecule would be expected to remain longer in blood stream and thus have a favourable effect. However, the loss of biological activity parallel to the increase in MW shows that the intact three dimensional structure of the gonadotropic hormone is essential for hormonal activity. Nevertheless the polymerization reaction of HCG has some interesting features and a better understanding should facilitate the current work on the relationship between chemical structure and biological and immunological activity of the gonadotropic hormone. The deamination, nitration and carbamylation causes no substantial change in antigenicity compared to the highly purified hormone. Thus, cleavage or carbamylation of the amino groups as well as the nitration of the tyrosine groups did not markedly influence the antigenicity of the molecule. Substitution of the lysine residues with acetyl and charged succinyl groups causes a decrease in the total antibody precipitated. Polymerized HCG (precipitated always with a constant amount of antibody) causes a marked shift of the equivalence point up to an average of 12 μg antigen. This is comparable with the 8 μg range of the equivalence point for the purified hormone, the deaminated, nitrated and carbamylated products and with the 4 μg range for the acetylated and succinylated derivatives.

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