Enhanced Thermal Stability of Chemically Deglycosylated Human Choriogonadotropin*

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Exposure of aqueous solutions of native human choriogonadotropin (hCG), asialo-hCG (A-hCG), and chemically deglycosylated hCG (DG-hCG) to heat treatment revealed significant differences in their stability. Solutions of hCG and A-hCG were rapidly inactivated above 50 °C. On the other hand, solutions of DG-hCG were comparatively more stable under similar conditions as shown by the retention of significant receptor binding, immunological, and hormonal antagonistic activities. Heated solutions (100 °C) of hCG and A-hCG quickly lost their ability to enhance the fluorescence of the probe 1-anilino-8-naphthalenesulfonate (1,8-ANS) indicating dissociation into subunits. DG-hCG solutions were more stable in this respect suggesting significant preservation of conformational features required for the interaction with 1,8-ANS. Solutions of hCG and A-hCG which had been thermally denatured (100 °C, 10 min) required almost 48 h at 37 °C to regain complete ANS binding ability as well as receptor binding activity. Under the same conditions, heated solutions of DG-hCG completely regained these abilities in less than 2 h. A similar pattern was observed with acid (pH 2.0)-dissociated hCG, A-hCG, and DG-hCG. While heated solutions of hCG had no effect on the action of native hCG in vitro, heated DG-hCG solutions still retained their ability to antagonize the cyclic AMP accumulation in steroidogenesis induced by native hCG in rat interstitial cells. Thus, removal of carbohydrate residues (~75% loss) from hCG renders the hormone more resistant to thermal denaturation.

The bulky carbohydrate chains in human choriogonadotropin form an important part of the structural features of the hormone. Partially deglycosylated preparations of the hormone obtained either by enzymatic methods in successive treatments (1) or by the one-step chemical treatment with anhydrous hydrogen fluoride (2) have been helpful in analyzing the role of carbohydrate moieties in hormone function. It appears that complete integrity of the carbohydrate is required for the expression of full biological response of the hormone including steroidogenesis (2). By virtue of the fact that deglycosylated hCG preparations can efficiently bind to the receptor without activating the cell, they can act as effective inhibitors of the action of the native hormone in vitro (2, 3). Removal of most of the carbohydrate units has no effect on the immunological properties of the hormone as shown by full retention of activity in radioimmunoassays (2).

In continuing the studies on the comparison of the physicochemical and biological properties of hCG and DG-hCG, we have now found remarkable differences in their stability to heat inactivation. The results presented herein will reveal that chemically deglycosylated hCG is more stable to thermal denaturation than the intact hormone.

MATERIALS AND METHODS

hCG from a commercial crude preparation (Ayerst Laboratories and Organon) was isolated and purified as described previously (2). Bovine serum albumin, cyclic AMP binding protein, insoluble neuraminidase, and lactoperoxidase were obtained from Sigma; collagenase and lima bean trypsin inhibitor from Worthington; 3-isobutyl-1-methylxanthine from Aldrich; carrier-free Na^{131}I employed for iodination was purchased from Amersham; Omnifluor and Triton X-100 were from New England Nuclear; 1-anilino-8-naphthalenesulfonate used as the magnesium salt was obtained from Eastman and used without further purification. All other chemicals were of reagent grade from Fisher.

Preparation of DG-hCG—A thoroughly dried powder of the purified hormone was subjected to deglycosylation by exposure to anhydrous HF for 60 min at 0 °C. Details of this methodology including procedures for recovery of the product and characterization have been recently reported (2).

Preparation of A-hCG—Desialylation of the purified hCG was carried out using insoluble neuraminidase (neuraminidase immobilized on Sepharose CL-4B) essentially as described by the manufacturer. To a test tube containing 10 mg of purified hCG in 0.05 M acetate buffer, pH 5.5, and 10 mg CaCl_2 was added 1 unit (1 ml suspension) of insoluble neuraminidase. The test tube was capped and kept on an end over end shaker at room temperature. After 24 h of incubation, the insoluble neuraminidase was separated by passing through a sintered plastic column (Econo column, Bio-Rad). The gel (insoluble neuraminidase) in the column was washed with 10-15 ml of 0.05 M acetic acid buffer. The filtrate and the washings were pooled, dialyzed, lyophilized (designated asialo-hCG). The sialic acid content before and after desialylation was 10.2% and 1.7%, respectively, as determined by microfriozimetric assays (2).

Heat Treatment—Solutions (1 mg of protein/ml) of hCG, DG-hCG, or A-hCG in 0.05 M phosphate buffer, pH 7.4, in Trifton screw cap glass tubes were held in a boiling water bath. Aliquots of 100 μl were withdrawn at designated intervals and mixed with 900 μl of ice-cold solution of 60 μM ANS in 0.02 M phosphate buffer, pH 7.4, containing 0.11 M KCl. Solutions were then scanned for enhancement of ANS fluorescence and kept frozen until assayed for receptor binding, immunoreactivity, biological activity, and antagonistic activities.

In another experiment, solutions (1 mg of protein/ml) of hCG, DG-hCG, or A-hCG in 0.05 M phosphate buffer, pH 7.4, were held in water at various temperatures for 10 min and 200 μl was diluted in 900 μl solutions of 60 μM ANS in 0.02 M phosphate buffer, pH 7.4, containing 0.11 M KCl. The solutions were again scanned for fluorescence and assayed for various activities.

Dissoication of Hormone into Subunits by Heat Treatment and Kinetics of Reassociation—Hormone solutions (1 mg/ml in 0.05 M
potassium phosphate buffer, pH 7.4) were held in boiling water for 30 min. in Teflon-capped glass tubes. The heated solutions were rapidly cooled to 37 °C and incubated at the same temperature. Aliquots of 50 µl were withdrawn at designated time intervals up to 48 h and mixed with 450 µl of ice-cold ANS solution and kept on ice. At low temperatures (0-4 °C), recovery of the quaternary structure of hCG from its a and β subunits is extremely low (4). Then the samples were warmed up to 25 °C and scanned for ANS fluorescence. Their receptor binding activity was also determined. In control experiments, 50 µl of hormone solutions were withdrawn prior to heating and mixed with 450 µl of ANS reagent.

Receptor Binding, Immunological, and Biological Activity Determinations—Receptor binding activity of hCG, DG-hCG, and A-hCG was assayed using adult rat testicular homogenates (2). Radioimmunoassay was performed using the radioimmunoassay kit supplied by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, MD. Bound and free hormones were separated using the double antibody method (2). For the above experiments, NIH-hCG (CR119) labeled with 125I by the lactoperoxidase method (2) and having a specific activity of 50-60 µCi/µg was employed as tracer. Receptor binding and immunological activities were calculated by computing the amount of the sample required to produce a 50% reduction in the binding of labeled hCG to the receptor or the antibody (see figure legends).

Biological activity of hCG, DG-hCG, and A-hCG was determined in vitro using collagenase-dispersed rat testicular interstitial cells by measuring the accumulation of cyclic AMP and stereoidogenesis (3). The cells were incubated with the test samples for 30 min at 37 °C under 95% O2, 5% CO2 in the presence of 0.65 mM 3-isobutyl-1-methylxanthine. When testosterone synthesis was measured, the cells were incubated for 2 h but without 3-isobutyl-1-methylxanthine. The reaction was terminated by immersion in a water bath at 80 °C for 10 min. After removal of the debris by centrifugation, the cyclic AMP accumulated in the medium (supernatant) was estimated by the protein binding assay (3). Testosterone was measured by radioimmunoassay (3). Hormonal antagonistic activity of the DG-hCG and A-hCG was tested in vitro using 100 ng of native hCG (CR119) which usually induced maximal cyclic AMP response (3) and 1 ng of native hCG when testosterone synthesis was assessed (3). In all antagonistic studies, the hormone and the test samples were always added together.

Fluorescence spectra of aqueous solutions of the hormone and derivatives were examined in the presence of the probe 1,8-ANS (2) at room temperature. These were recorded in a Hitachi-Perkin-Elmer MFP-3 spectrofluorimeter.

RESULTS

Solubility Properties—hCG is highly soluble in aqueous solutions and this property was not altered following the loss of about 75% of its carbohydrate moiety by the deglycosylation which conferred a net increase in hydrophobicity to the molecule (2). Despite this, neither DG-hCG nor the other preparations (hCG and A-hCG) showed any tendency to precipitate following exposure to the various heat treatments. All solutions remained completely clear.

Heat Inactivation—Preliminary studies indicated significant differences (Fig. 1) in the relative stability of the aqueous solution of hCG and DG-hCG in a boiling water bath. The unheated A-hCG and DG-hCG showed 150 ± 10% and 280 ± 20% (n = 4) receptor binding activity as compared to native hCG. DG-hCG preparations retained their ability to bind to the receptor even after being at this temperature for 1 h. This was abolished in the case of native hCG and A-hCG.

In other experiments, the kinetics of the heat inactivation was studied. Fig. 2 shows the relative stability of hCG, A-hCG, and DG-hCG at different temperatures. hCG, A-hCG, and DG-hCG all retained full receptor binding and immunoreactivity when heated for 10 min up to 50 °C. At 75 °C, hCG and A-hCG underwent a transition that resulted in over 95% inactivation while DG-hCG retained about 35-50% of its original activity. Since the DG-hCG samples were more potent than hCG in the receptor binding assay (see Fig. 1), the receptor binding activity in this instance becomes equivalent to about 100% relative to hCG.

Fig. 1. Differential effects of heat treatment on receptor binding activity of hCG, A-hCG, and DG-hCG. Equal concentrations of the hormones (1 mg/ml in 0.05 M phosphate buffer, pH 7.4) were kept in a boiling water bath for 30 min, cooled, diluted, and assayed for receptor binding activity. The receptor binding activity of the native unheated hCG sample is set as 100% for reference. (The unheated A-hCG and DG-hCG show 150 ± 10% and 280 ± 20% activity, respectively, as compared to the native hormone.) Note that the activity scale is logarithmic.

Fig. 2. Stability of hCG, A-hCG, and DG-hCG at various temperatures. Solutions (1 mg of protein/ml) were held in water at various temperatures for 10 min, cooled immediately, and assayed for activity in radioreceptor assay (A) and radioimmunoassay (B). The activity of untreated hormone was set as 100% in each instance.

Fig. 3. Kinetics of heat inactivation of hCG, A-hCG, and DG-hCG. The solutions (1 mg of protein/ml) were immersed in a boiling water and aliquots were withdrawn at designated time intervals and assayed in specific radioreceptor assay (A) and specific radioimmunoassay (B). The activity of untreated hormone was set as 100% in each instance.
When the inactivation of the hormone preparations was studied as a function of time (Fig. 3), again DG-hCG was quite different as compared to hCG and A-hCG. For example, in the boiling water bath both hCG and A-hCG quickly and consistently lost 95% of their receptor binding activity within 5 min. Under the same conditions, DG-hCG solutions retained over 50% of the original receptor binding activity. Even after being in a boiling water bath for 1 h, DG-hCG was not completely inactivated and retained approximately 20% of its original binding activity.

Fluorescence Spectra—The enhancement of fluorescence of the probe 1-anilino-8-naphthalenesulfonic acid upon interaction with the hormone was evaluated as a biophysical parameter. Similar to the data obtained in Fig. 2, all three preparations (viz. hCG, A-hCG, and DG-hCG) showed no alteration in their ability to bind and enhance the fluorescence of the probe, after exposure for 10 min up to 50 °C. At higher temperatures of 75 and 100 °C, this characteristic was considerably reduced (Fig. 4). The loss was again more pronounced in hCG and A-hCG than in DG-hCG. When the experiment was repeated with solutions kept in a boiling water bath for 1 h, the loss in ability to enhance the fluorescence of ANS by hCG, A-hCG, and DG-hCG was 86%, 95%, and 70%, respectiv-
tively, indicating the greater stability of DG-hCG preparations.

Thermally Induced Reversible Change in Conformation—
The complete or partial loss in the ability of heated hormone solutions to enhance ANS fluorescence implied a change in native conformation that most likely resulted in a breakdown of the quaternary structure. It was therefore of interest to know if this change was reversible and if so to what extent. Thus, heated (10 min at 100 °C) solutions were cooled and reincubated at 37 °C. The enhancement of ANS fluorescence and receptor binding activity were monitored at various intervals of time. As seen in Fig. 5, A and B, there was a complete regain of both ANS fluorescence and receptor binding activity in all three instances (hCG, A-hCG, and DG-hCG) indicating the reversible nature of the thermal denaturation process. There was a significant difference in the rate of recovery of the original structure. Heat-denatured DG-hCG solutions regained their original conformation in less than 2 h at 37 °C while the extent of recovery in the case of similarly treated hCG and A-hCG solutions was only about 20% in the same period. Approximately 48 h of incubation was required to effect a complete reversal.

The differences between DG-hCG, A-hCG, and hCG were assessed in another manner by inducing dissociation under acidic conditions (4). The samples were first dissociated by incubation at pH 2.0 overnight. Complete dissociation was evident as shown by the loss of ANS fluorescence and receptor binding activity (Fig. 5, C and D). The solutions were then neutralized to pH 7.4 and aliquots were withdrawn at specified time intervals, mixed with ANS, and kept on ice. When the final aliquot was withdrawn, ANS fluorescence and receptor binding activity were determined. The pattern of recovery of these two parameters, which reflect a regain of quaternary structures was identical with the heat dissociation phenomena observed in the previous experiment (Fig. 5, A and B). Again, acid-dissociated DG-hCG subunits recovered the original

![Graph A](image1)

**Fig. 6. Hormonal agonistic and antagonistic activity of DG-hCG and heated DG-hCG solutions.** In each of these separate experiments (A-D), about 200,000 collagenase-dispersed rat testicular interstitial cells were incubated in the presence of 0.06 mM 3-isobutyl-1-methylxanthine for 30 min or 3 h at 37 °C in a total volume of 0.6 ml. In A and C, cyclic AMP was measured after 30 min of incubation, by the protein binding assay. In B and D, testosterone in the medium was measured by radioimmunoassay following a 3-h incubation. When tested for agonistic activity (C and D), DG-hCG (unheated or heated) samples were added to the cells at the same time as the challenging dose of hCG. A, cyclic AMP accumulation in the presence of hCG, DG-hCG, and heated samples. B, steroidogenic activity of hCG, DG-hCG, and hCG after heating (hCG H30'). C, inhibition of hCG-induced cyclic AMP accumulation. The net cyclic AMP accumulation of 510 ± 30 pm (n = 5) in cells treated with 100 ng of hCG was considered as 100% response. Note that a 30-min heated hCG sample has no inhibitory activity against native hCG. D, inhibition of hCG-induced testosterone production (n = 3 or 5).
structure fully within 2 h, whereas hCG and A-hCG required a much longer time. The extent of recovery noted with hCG and A-hCG at the concentrations employed is very similar to that reported by other investigators using acid-deglicosidase hCG (5).

**Hormonal Antagonistic Activity**—One of the striking changes in the hormone induced by deglycosylation is the induction of hormonal antagonistic activity which is highly specific (2, 3). Thus, it was of interest to see if this property was affected by heat treatment. Hence, the antagonistic activity of DG-hCG before and after heat treatment was evaluated using a selected dose of hCG in collagenase-dispersed rat interstitial cells. It was not surprising to find that heat treatment of DG-hCG had no effect as regards its failure to induce cyclic AMP accumulation. On the other hand, the native hormone lost this activity as well as its ability to enhance testosterone synthesis (<0.2%, Fig. 6, A and B). Heat-treated DG-hCG was still a potent antagonist of the action of hCG in vitro. The inhibitory potency of DG-hCG calculated from the amount (nanograms) of sample required to inhibit the action of hCG by 50% was approximately one-third of the unheated sample (Fig. 6C). A 30-min heat-treated sample of DG-hCG also antagonized the steroidogenic action of hCG (25% loss of 50% stimulation). This clearly indicates that the antagonistic property of DG-hCG is also stable to heat treatment. Heated preparations of native hCG had no inhibitory effect on the hCG response, suggesting the lack of nonspecific effects induced by thermal denaturation.

**DISCUSSION**

hCG appears to be more stable than human lutropin in its ability to withstand the effects of elevated temperature. Mori (6) has reported that following treatment at 80 °C for 15 min, hCG still retained about 77% of its biological activity and 90% of the immunological activity. In contrast to this, Ingham et al. (4) who used enhancement of ANS fluorescence to monitor structural changes in human lutropin found that conformational breakdown was rapid at temperatures above 40 °C. The pronounced loss of ANS binding to the extent of 95% in 10 min at 65 °C suggested rapid destruction of the native structure. The denaturation process even at temperatures such as 55 °C was not completely reversible in human lutropin.

In the present work, we have found that hCG and A-hCG underwent drastic changes in structure between 50 and 75 °C (Fig. 2), resulting in considerable loss of hormonal activity. In light of the data of Mori (6), it can be assumed that temperatures greater than 60 °C are detrimental to the maintenance of the native structure. Under identical conditions of the experiment, DG-hCG was definitely more stable than either hCG or A-hCG (Figs. 2-5). From the differences in stability patterns of A-hCG and DG-hCG, one may conclude that the removal of carbohydrate units in the interior of the sugar chains must be required for conferring this enhanced stability to thermal inactivation. It is possible that the net increase in hydrophobicity of the molecule resulting from the removal of more than 75% of the hydrophilic sugar residues (2) might have been responsible for the increased stability. Whatever may be the cause, it is clear that changes that lead to loss of receptor binding activity and ANS fluorescence occur more slowly in DG-hCG than in hCG or A-hCG as shown by data in Fig. 5. These experiments show that the reversal of the conformational changes induced by thermal treatment occur slowly in the native hormone and asialo-hCG while similar changes are more readily reversible and complete in DG-hCG. The rapid recovery of ANS fluorescence and receptor binding activity from the acid-deglicosidated subunits of DG-hCG again supports these conclusions (Fig. 5, A-D). The present study imply that the loss in activity of hCG and A-hCG following heat exposure was due to a breakdown of the quaternary structure resulting in dissociation.

The heat stability characteristics of DG-hCG extend our recent findings of the resistance of deglycosylated ovine lutropin (7) and ovine folliculin (8) to thermal inactivation. We have speculated elsewhere (7, 8) that in view of the heat stability characteristics and specific hormonal antagonistic properties, the deglycosylated hormones may provide an important clue in the identification of the so-called gonadotropin-inhibiting materials found in human urine of both sexes of all ages. The presence of such an activity in human urine can only be demonstrated after destruction of the accompanying biologically active gonadotropins by heat treatment of the aqueous solutions for 1 h in a boiling water bath (9-14). The relative stability of the gonadotropin-inhibiting material is similar to the results obtained with DG-hCG in the present studies. It is, however, not possible to conclude from the present work whether the gonadotropin-inhibiting materials reported by previous workers (9-14) represent some form of deglycosylated gonadotropins. Such an activity has also been reported to be present in crude hCG (15) and again is detectable only after keeping the preparations in a boiling water bath. This activity is lost from purified hCG preparations, suggesting its elimination during the purification procedure. Gonadotropin inhibitory activity has also been detected in some glycoprotein fractions (16) obtained during hCG purifications. The lack of true biological effects typical of lutropin in such fractions suggests that some degree of deglycosylation occurring in vitro may have been responsible for the antagonistic properties. A reinvestigation of the nature of such gonadotropin-inhibiting materials may be helpful in resolving their mystery and offer clues for possible involvement of deglycosylation phenomena in the regulation of gonadotropic action.

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