PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 181, 432–437 (1986)

Deglycosylation of Gonadotropins with an Endoglycosidase (42277)

JASON R. SWEDLOW, ROBERT L. MATTERI, AND HAROLD PAPKOFF

Hormone Research Laboratory, University of California at San Francisco, San Francisco, California 94143

Abstract. A commercially available endoglycosidase (*N*-glycanase, Genzyme, Boston, Mass.) purified from *Flavobacterium meningosepticum* with a specificity for cleaving asparagine-linked carbohydrate moieties in glycoproteins was tested on several pituitary and chorionic gonadotropins as substrates. All intact hormones tested were resistant to the action of the enzyme as were all β subunits from the respective gonadotropins. All α subunits, however, were susceptible to the enzyme as evidenced by a decrease in molecular size when examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Preparative experiments with ovine luteinizing hormone subunit (oLHα) indicated that only 35–40% of the carbohydrate was removed after *N*-glycanase treatment, suggesting that perhaps only one of the two carbohydrate moieties was cleavable under the conditions employed. The enzyme-modified subunit (DG-oLHα) was able to recombine with untreated oLH β. An *in vitro* steroidogenic bioassay (rat Leydig cell) showed that the recombinant (DG-oLHα-oLHβ) was about 22% as potent as the native oLH, but in a testicular membrane binding assay for LH, it was equal in potency to the native hormone in competing with the radioligand.

There is general agreement that the carbohydrate of the glycoprotein hormones plays an important role in the manifestation of the hormonal activity. In the past few years there have been many studies directed toward elucidating the structure–function relationships in which the carbohydrate of the gonadotropins participate. These studies for the most part have focused on the effects of removal of sialic acid (1–4) or the removal of variable amounts of the other carbohydrate constituents either chemically (4–7) or enzymatically (4, 8, 9). The pituitary gonadotropins all possess two asparagine-linked carbohydrate moieties in their α subunit and 1–2 in their β subunits (10). Human and equine chorionic gonadotropin (hCG, eCG) are similarly composed and possess, in addition, a number of threonine- (or serine-) linked small oligosaccharide units (4). Thus far it has not been possible to remove the major asparagine-linked carbohydrate moieties intact and without damage to the polypeptide chains. In this report we describe the results of initial experiments with a commercially obtained glycosidase (*N*-glycanase), purified from *Flavobacterium meningosepticum*, which appears to be able to effect cleavage at the asparagine-*N*-acetyl-glucosamine bond liberating the intact carbohydrate moiety.

Materials and Methods. The following highly purified hormones were used in these experiments and were prepared in this laboratory: ovine luteinizing hormone (oLH) (11) and follicle stimulating hormone (oFSH) (12), equine luteinizing hormone (eLH) and follicle stimulating hormone (eFSH) (13), equine chorionic gonadotropin (eCG) (14), and human chorionic gonadotropin (hCG). Subunits were prepared as previously described (15, 16).

The endoglycosidase *N*-glycanase (Genzyme) was used to deglycosylate glycoprotein hormones and subunits. The enzyme is purified from cultures of *F. meningosepticum* and is distinct from the endo-β-*N*-acetylglucosaminidase F (Endo F) activity described by Elder and Alexander (17, 18). *N*-Glycanase hydrolyzes N-linked oligosaccharides from glycoproteins to give free oligosaccharide and a protein-containing aspartic acid at the glycosylation site. Protease activity in the *N*-glycanase was reported to be absent by the manufacturer.

The hexose, hexosamine, and sialic acid content of the preparations were determined by colorimetric methods which were scaled down to conserve material (19). A rat Leydig cell bioassay for LH (20) and a competitive binding assay using equine testicular membranes and [^{125}I]-eLH as a radioligand (21)

0037-9727/86 $1.50

were used to biologically characterize the preparations. All dose–response curves in assays were linearized by a weighted log–logit method. Tests for parallelism were performed using Student's t test. Relative potencies were calculated by comparing the resulting ED_{50}s.

Analytical deglycosylation of glycoprotein hormones and subunits was performed as follows: 30 μg of hormone was dissolved in 10 μl 0.2 M $NaPO_4$/10 mM EDTA (pH 8.6). One microliter of N-glycanase (26 U/ml) was added to the solution and the reaction mixture was incubated at 37°C for 18 hr. Samples were then centrifuged for 3 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (22). The gels (15% polyacrylamide, 0.1% SDS) were run under reducing conditions (2-mercaptoethanol) and stained with Coomasie brilliant blue R-250. Electrophoretic mobility of the enzyme-treated hormone was compared to that of an equal amount of untreated protein.

Preparative deglycosylation experiments employed larger quantities of material: five milligrams of α subunit was dissolved in 1.0 ml 0.2 M $NaPO_4$/10 mM EDTA (pH 8.6). One microliter of N-glycanase (260 U/ml) was added to the solution and the reaction mixture was incubated at 37°C for 18 hr. A 5-μl aliquot of the reaction mixture was subjected to SDS–PAGE to confirm the completion of the reaction. The reacton mixture was then applied to a 1.6 × 25.0-cm Sephadex G-50 Fine (Pharmacia) column equilibrated with 0.05 M NH_4HCO_3 and 40 fractions (1.5 ml/tube) were collected. Elution profiles were determined by monitoring optical density of the fractions for protein at 230 nm and for hexose (orcinol reaction) at 505 nm. Peak fractions were pooled and lyophilized. The oligosaccharide fraction was dissolved in 250 μl 0.05 M NH_4HCO_3 and desalted on a 1.6 × 22.0-cm Sephadex G-25 (Pharmacia) column equilibrated with 0.05 M NH_4HCO_3. Peak profiles were determined as above.

Recombination of subunits was achieved as follows: 2 mg each of either intact or deglycosylated oLHα and oLHβ was incubated in 250 μl 0.01 M KPO_4, pH 7.0 for 48 hr at room temperature. One hundred microliters 0.05 M NH_4HCO_3 was then added and the solution was applied to a 1.6 × 85.0-cm Sephadex G-100 SuperFine (Pharmacia) column. The elution profile was determined by monitoring optical density at 230 nm. Sixty fractions were collected (2.0 ml/tube). Peak fractions were pooled and lyophilized.

Results. When native oLH, OFSH, eFSH, eCG, and hCG were treated with N-glycanase as described and examined by SDS–PAGE, no increase was seen in electrophoretic mobility for any of the preparations. Since an increase in mobility would indicate a decrease in molecular weight (i.e., cleavage and removal of carbohydrate moieties), it was concluded that all the above glycoprotein hormones were resistant to the action of the enzyme. The results obtained with the individual subunits of these hormones were somewhat different, however, and are shown in Fig. 1 (oLH subunits), Fig. 2 (eLH and eFSH subunits), and Fig. 3 (eCG and hCG subunits). The results are similar to those with the intact hormones in that none of the β subunits were affected by the N-glycanase treatment. All of the α subunits, however, when treated with N-gly-

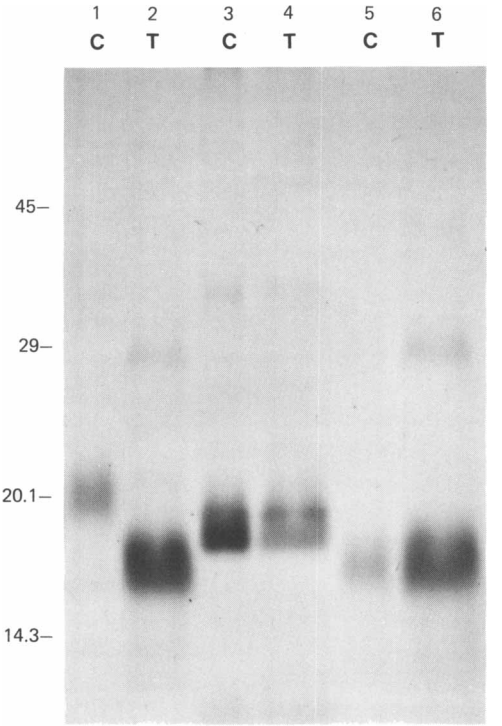

FIG. 1. SDS–PAGE of N-glycanase-treated ovine LH subunits. C: control; T: enzyme-treated. Lanes 1 and 2: oLHα; 3 and 4: oLHβ; 5 and 6: DG-oLHα.

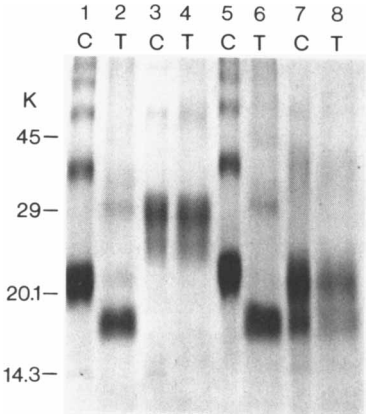

FIG. 2. SDS–PAGE of *N*-glycanase-treated equine gonadotropin subunits. C: control; T: enzyme-treated. Lanes 1 and 2: eLHα; 3 and 4: eLHβ; 5 and 6: eFSHα; 7 and 8: eFSHβ.

canase, migrated with a faster mobility in the gels than the untreated materials. The increase in mobility in most cases corresponded to a decrease in apparent molecular weight of about 3000.

To study the effects of *N*-glycanase on an α subunit in greater detail, a preparative experiment was performed with 5 mg of ovine LHα. Following the treatment with *N*-glycanase, the digestion mixture was applied to a column of Sephadex G-50. The elution profile is seen in Fig. 4. It is evident that the protein peak

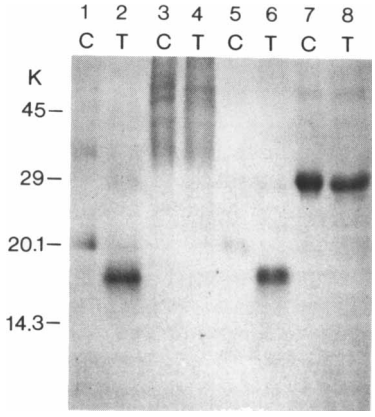

FIG. 3. SDS–PAGE of *N*-Glycanase-treated equine and human chorionic gonadotropin subunits. C: control; T: enzyme-treated. Lanes 1 and 2: eCGα; 3 and 4: eCGβ; 5 and 6: hCGα; 7 and 8: hCGβ.

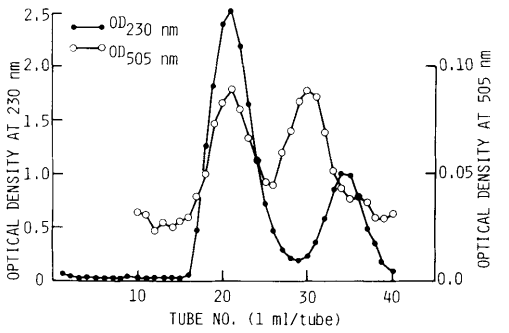

FIG. 4. Gel filtration of 5 mg of ovine LHα treated with *N*-Glycanase on a column (1.6 × 25 cm) of Sephadex G-50 Fine in 0.05 *M* NH_4HCO_3. Void volume is at tube 16; peak of salt (phosphate) elution is at tubes 34–35.

($OD_{230\ nm}$) which emerges with the void volume of the column still contains appreciable carbohydrate ($OD_{505\ nm}$). This material was designated DG-oLHα and was followed by a retarded peak which appears to contain only $OD_{505\ nm}$ absorbing material (i.e., carbohydrate only; designated oLHα-OS).

Analysis of DG-oLHα by SDS–PAGE (Fig. 1) shows that the preparation (lane 5) behaves identically to the analytical sample of oLHα treated with *N*-glycanase (lane 3). A second incubation of DG-oLHα with the enzyme was without effect (Fig. 1, lane 6), but denaturation and reduction of DG-oLHα before treatment with the enzyme resulted in a further decrease in apparent molecular weight of 3000 as determined by SDS–PAGE (data not shown). Carbohydrate analysis of DG-oLHα showed that the hexose and hexosamine contents were reduced by 34 and 39%, respectively, compared to the untreated oLHα. The same two classes of sugars were found in oLHα-OS but quantitation was poor due to the small amount of material recovered.

DG-oLHα was incubated with untreated oLHβ to determine if it was feasible to prepare a recombinant molecule for study. The incubation mixture was purified by gel filtration (Sephadex G-100) and resulted in the elution profile seen in Fig. 5. The major peak seen (tubes 32–36) represents the recombinant and was pooled and lyophilized for further study. The V_e/V_0 of this peak was about 1.7. The small peaks before (tubes 26–30) and after (tubes 39–44) the main peak are probably

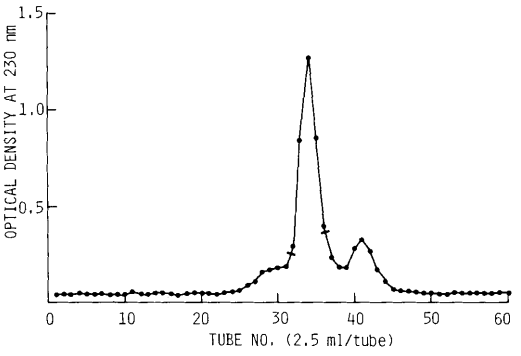

FIG. 5. Gel filtration of a recombinant mixture of DG-oLHα and oLHβ (2.0 mg each) on a column of Sephadex G-100 SuperFine (1.6 × 85 cm) in 0.05 *M* NH_4HCO_3. Void volume is at tube 20; salt elution is at tube 66.

subunit aggregates and unrecombined subunits, respectively. In a control experiment with untreated subunits, the recombinant was slightly less retarded (i.e., larger molecular size) and eluted with a V_e/V_0 of 1.6.

Preliminary assays (Table I) show that DG-oLHα, as expected, has minimal activity in both the Leydig cell bioassay for LH and the equine LH testicular binding assay. The recombinant DG-oLHα-oLHβ, however, was 22% as active as oLH in the bioassay and equal in potency (1.22×) to oLH in the binding assay. Its activity in the latter assay was significantly better (1.22 vs 0.67×) than the recombinant prepared from the untreated subunits. The oligosaccharide fraction (oLHα-OS) was without activity in the rat Leydig cell assay at a dose of 1000 ng.

Discussion. Previous studies on the role of the carbohydrate of gonadotropins to the biological activity have involved either sequential removal of sugar residues with enzymes (4, 8, 9) or chemical modifications (4–7) which result in varying degrees of deglycosylation. In the present study, an endoglycosidase (*N*-glycanase) with specificity for cleavage of the asparaginyl–sugar bond linking carbohydrate moieties to the peptide chain was tested on a number of pituitary and chorionic gonadotropins and their subunits. None of the intact gonadotropins nor their desialylated derivatives or their β subunits proved to be susceptible to *N*-glycanase action as no evidence was seen that carbohydrate moieties had been released. On the other hand, evidence was obtained for the release of carbohydrate from the α subunits tested (from oLH, oFSH, eLH, eFSH, eCG, and hCG). The above experiments were qualitative in that SDS–PAGE was employed to gain evidence of a change in molecular weight (Figs. 1–3). In the case of the α subunits, the decrease in apparent molecular weight was about 3000.

Verification that *N*-glycanase could effect removal of carbohydrate moieties came from a preparative experiment (Fig. 4) in which 5 mg of enzyme-treated oLHα was gel-filtered. Although carbohydrate was released and separated from the protein, it was also evident that the reaction was incomplete in that carbohydrate was still associated with the subunit. Since retreatment of this material (DG-oLHα) did not result in the release of additional carbohydrate (Fig. 1, lane 6), it is tempting to believe, pending further experiments, that only one of the two carbohydrate moieties was cleaved and removed. The carbohydrate removed apparently does not affect the ability of the subunit derivative (DG-oLHα) to recombine with the untreated oLHβ as seen in Fig. 5. The recombinant eluted from the gel filtration column was slightly retarded relative to oLH which reflects the loss of carbohydrate from the α subunit. Similar results have been obtained by others (4–7) who effected partial deglycosylation of subunits with HF. Preliminary assays on the recombinant (Table I) show that its activity in a competitive binding assay employing testicular membrane and radioiodinated LH as ligand is equal to native oLH while its steroidogenic activity (*in vitro* rat Leydig cell assay) is reduced 78% compared to oLH. These results, too, are similar to those

TABLE I. RELATIVE POTENCIES OF SUBUNITS AND RECOMBINANTS IN COMPETITIVE BINDING AND LEYDIG CELL ASSAYS

Preparation	Binding assay	Bioassay
oLH[a]	1.00	1.00
oLHα	0.025 ± 0.014	0.03
oLHβ	<0.01	<0.01
DG-oLHα	<0.01	0.02
oLHα-OS	—	<0.01
oLHα + oLHβ	0.67 ± 0.23	0.52 ± 0.22
DG-oLHα + oLHβ	1.22 ± 0.18	0.22 ± 0.04

[a] Purified ovine LH is taken as 1.0 in each assay.

obtained with HF-treated gonadotropin preparations.

The resistance of the intact hormones and β subunits to *N*-glycanase is probably related to the conformational properties of the molecules (23). It is known, for instance, that the conformational properties of the subunits change upon dissociation. Thus, in the intact hormone, the asparagine–carbohydrate linkage may not be accessible to the enzyme. It is also known that dissociated oLHβ tends to form aggregates in neutral solutions (24) which could also result in specific loci becoming inaccessible. Supporting the thought that conformation is important with regard to susceptibility to *N*-glycanase action is the observation that heat denaturation and reduction of the disulfide bonds yield a denatured product which is completely available to the action of the enzyme.

Calvo and Ryan (25) have recently shown that glycopeptides obtained enzymatically from hCG have the ability to inhibit hCG-induced adenylate cyclase in rat luteal tissue and a receptor lectin is postulated as part of the mechanism involved. Oligosaccharides devoid of peptide components would clearly be of value in such studies. In addition, there is the potential for isotopic labeling of oligosaccharide units which could be employed to study direct interaction with receptors and also to study the metabolism of the carbohydrate units of gonadotropins separate from the peptide moieties. The experiments described here point to a potential method for obtaining both oligosaccharide moieties and deglycosylated peptide chains for studying the contribution of each on hormone–receptor interactions.

We thank Mr. Dan Ng for his expert technical assistance. This work was supported in part by a grant from the National Institutes of Health; HD-05722.

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Received September 16, 1985. P.S.E.B.M. 1986, Vol. 181.
Accepted November 20, 1985.