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The Common α Subunit of Bovine Glycoprotein Hormones: Limited Formation of Native Structure by the Totally Nonglycosylated Polypeptide Chain

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The folding of the bovine glycoprotein hormone α subunit, synthesized in bacteria following insertion of the nucleotide sequence coding for this polypeptide, has been studied to determine the effect that a complete lack of carbohydrate has on this process. The bacterially derived α polypeptide (bac-α), extracted from *E. coli* in the presence of reductant and denaturant, had an estimated 0.2% native structure as determined by a conformationally sensitive radioimmunoassay. Upon reduction of disulfide bonds and reoxidation in air, the amount of native structure increased about 18-fold. Approximately 2% of the refolded bac-α preparation combines with the β subunit of human chorionic gonadotropin (hCGβ) to form a complex that binds to the gonadotropin receptor and elicits a biological response. Since the correct folding (by immunological criteria) of bac-α (ca 3%) is significantly greater than expected from a random formation of disulfide bonds (0.1%), it appears that correct folding of α subunit can occur in the complete absence of carbohydrate, though in very low yield. Native bovine lutropin α subunit (LHα) and chemically deglycosylated LHα (which retains two asparagine-linked N-acetyl glucosamine residues per α oligosaccharide) were subjected to the same reduction/reoxidation regimen as the bacterially produced α subunit. As has been reported previously [Giudice LC, Pierce, JG, J Biol Chem 251: 6392, 1976] intact LHα fully regained its native structure. The partially deglycosylated LHα also refolds to a native-like structure in high yield as assessed by immunological assays and by its ability to combine with HGCβ to form a biologically active complex. The

Abbreviations used: TSH, thyrotropin; LH, lutropin; FSH, follitropin; hCG, human choriogonadotropin; bac-α, bacterially derived bovine glycoprotein hormone α subunit; GdHCL, guanidine hydrochloride; SDS, sodium dodecyl sulfate; RIA, radioimmunoassay; RRA, radioreceptor assay; RCM, reduced carboxymethylated; DTE, dithioerythritol; HPLC, high pressure liquid chromatography; ME, mercaptoethanol; ref, refolded; dg, deglycosylated.

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data show that carbohydrate, while not obligatory for correct folding, greatly facilitates the formation of functional α subunit.

Key words: protein folding, carbohydrate deglycosylation

Several aspects of the oligosaccharide structures of the glycoprotein hormones LH, TSH, FSH, and CG are of considerable interest with respect to their biosynthesis and function. This family of hormones each consists of two glycosylated peptide chains, α and β, held together by noncovalent interactions (for review see Pierce and Parsons [1]). Within a species, the amino acid sequence of the α subunit is the same in all glycoprotein hormones. The β subunit is unique to each hormone and confers biological specificity to the α-β dimer. The α subunit contains two asparagine-linked oligosaccharides at postions 56 and 82. Depending on the β subunit, one or two asparagine-linked oligosaccharides are present. The oligosaccharides of the pituitary hormones are unusual in that they contain N-acetylgalactosamine as well as N-acetyl glucosamine residues. In addition, negligible amounts of galactose and sialic acid, common peripheral constituents of N-linked oligosaccharides, are found in bovine LH and TSH. Rather, the terminal amino sugars of these hormones are O-sulfated [2,3].

In terms of function, the carbohydrate is not necessary for interaction with the receptor but its removal by chemical or enzymatic procedures causes a marked attenuation in hormone-stimulated biochemical events occurring subsequent to binding [eg, 4–10]. Folding and assembly of the subunits into functional hormones may also depend on their carbohydrate moieties. For example, Weintraub *et al* [11] observed that nonglycosylated TSHα synthesized by mouse thyrotropic tumor cells, treated with tunicamycin to inhibit glycosylation, failed to combine with TSHβ. Furthermore, the nonglycosylated TSHα also would not combine when challenged with additional TSHβ [12]. Bovine α subunit synthesized in a cell-free translation system folds poorly without addition of carbohydrate while glycosylated subunits fold to native-like structures [13]. In earlier studies of the *in vitro* folding of the bovine and human α subunits, reoxidation after full reduction resulted in a fully functional tertiary structure that does combine with β subunit [14,15]. Subsequently, enzymatically deglycosylated HCGα was found to refold after reduction to yield a native structure, as determined by immunological criteria and ability to combine with β subunit [8,13]. However, because enzymatic deglycosylation does not result in complete removal of carbohydrate (at least, the asparagine-linked N-acetyl glucosamines remain [8]), it is difficult to assess whether the remaining carbohydrate groups contributed to the folding process.

The above results, *in toto*, strongly suggest that some attachment of sugar, even if only the glucosamine vicinal to asparagine, is necessary for the formation of the correct tertiary structure of α subunits during the folding process. To address this question directly, we investigated the *in vitro* folding and refolding properties of carbohydrate-free bovine α subunit expressed from its nucleotide sequence [16,17] inserted into *E coli*. Described are the characterization of the biosynthetic product together with data leading to the conclusion that, while not obligatory, carbohydrate greatly facilitates correct folding of the glycoprotein hormone α subunit.

MATERIALS AND METHODS

Materials

The bovine glycoprotein hormone α polypeptide was expressed in *E coli* and purified as described elsewhere (Thomason et al, manuscript in preparation). Briefly,

the protein was solubilized in a GdHCL/mercaptoethanol solution and subjected to DEAE- and CM-cellulose chromatography in the presence of urea. Bovine LH was isolated as previously described [18] and bovine LHα was prepared by reverse phase high performance liquid chromatography using a trifluoroacetic acid/acetonitrile system [19]. hCG and hCGβ (batch CR123) were provided by the Center for Population Research (National Institute of Child Health and Human Development, Bethesda, MD). Before use, the hCGβ was incubated in 6 M GdHCL and gel filtered via HPLC [19] to remove any remaining intact hCG. Antisera against bovine LHα and RCM bovine LHα were produced in rabbits as before [20].

Deglycosylation and Carbohydrate Analysis

LHα was deglycosylated using anhydrous trifluoromethanesulfonic acid according to the procedure of Kalyan and Bahl [9]. The deglycosylated product was isolated by chromatography on a column of Sephadex G-100 developed with 1% ammonium bicarbonate. Analyses for neutral and amino sugars were performed as previously described [21].

Refolding of α Subunits

All three α subunit forms, LHα, deglycosylated LHα, and bac-α were refolded using the same procedure. A 1-mg/ml solution of subunit was prepared in 0.25 M Tris-CL (pH 8.5) and 6 M GdHCL. Dithioerythritol was added to a final concentration of 2.5 mM, the solution was incubated for 2 hr at room temperature, and then diluted into 200 volumes of freshly prepared 1% ammonium bicarbonate (final protein concentration, 5 μg/ml). This solution was then stood for 48 hr at room temperature exposed to air to allow reoxidation of the cysteine residues. After lyophilization, the protein was dissolved in water to a concentration of 0.5 mg/ml and desalted on a column of Sephadex G-25 (fine) developed with 1% ammonium bicarbonate. The oxidation state of the cysteine residues was monitored during the reduction/reoxidation procedure by treating an aliquot of the solution with iodoacetic acid before and after addition of DTE and after reoxidation and lyophilization and assaying for carboxymethyl-cysteine by amino acid analysis. In both the initial and reoxidized α solutions, the half-cystine residues were all in the form of disulfide bonds. In the reduced α solutions, all were present as cysteine.

Radioimmunoassays

Radioimmunoassays were performed as previously described [22] with the following modifications: 1) the initial incubation was for 18 hr at room temperature, 2) the normal rabbit serum was omitted, and 3) a 30-min incubation with 10 μl of a 10% suspension of formalin-fixed *Staphlococcus aureus* cells (Enzyme Center, Boston, MA) was used in place of the second antibody to precipitate the immune complex. The protein concentrations of solutions tested in the RIAs were determined by amino acid analysis.

Recombination With hCGβ

Recombination of the various forms of α subunit was performed by dissolving the subunit in a 1-mg/ml solution of hCGβ (0.1 M potassium phosphate, 10 mM sodium azide, pH 7.5). The mixture was incubated at 37°C for 48 hr. In the case of LHα, refolded LHα, deglycosylated LHα, and refolded deglycosylated LHα the

molar ratio of hCGβ to α was slightly greater than one. With refolded bac-α, where less of the total α was capable of recombination, an hCGβ to α ratio of approximately 0.2 was employed. The extent of recombination of each α subunit form was monitored by HPLC gel filtration. To test for the combination with β subunit of LHα, refolded LHα, deglycosylated LHα, and refolded deglycosylated LHα, equal aliquots of combination mixtures were applied to a series of three Bio-Sil HPLC gel filtration columns (Bio-Rad, Richmond, CA) with and without prior incubation of 6 M GdHCL. The amount of α subunit observed in the sample preincubated with GdHCL represents the total subunit while the α seen in the absence of GdHCL is that proportion of the subunit that did not combine with hCGβ. From these two values, the percentage of the α subunit that had combined was calculated. The amount of α subunit was measured by cutting out and weighing the chart paper representing the peaks recorded at 280 nm on the chromatogram. The above method could not be used with the refolded bac-α since too small a percentage combines to measure the change $\pm$ GdHCl accurately. Rather, the combination of refolded bac-α was measured by radioimmunoassay of fractions collected from HPLC gel filtration. Of the applied α immunoreactivity 92% was recovered from the column. hCGβ did not cross react in the α RIA.

Radioreceptor and Steroidogenesis Assays

The radioligand receptor assay was performed using a crude rat testis homogenate [23] except that ^{125}I-hCG was used as the labeled ligand, $MgCl_2$ and $CaCl_2$ were omitted, and the total incubation volume was increased to 1 ml. Leydig tumor cells, prepared as described from the M5480P murine Leydig cell tumor [24], were used in the steroidogenesis assay as described [25] except that the volume of cells used was decreased to 1 ml.

RESULTS

The reduced and carboxymethylated bac-α preparation is indistinguishable from RCM-LHα in a radioimmunoassay using anti-RCM-LHα, as shown in Figure 1. As this antiserum is specific for RCM-LHα [13], this coincidence confirms the identity of the bac-α.

The mobility of bac-α in polyacrylamide gel electrophoresis in the presence of SDS is shown in Figure 2. In the absence of mercaptoethanol (Fig. 2A, lane 3), the preparation contains a major band that migrates slightly faster than deglycosylated LHα, as well as several higher molecular weight components. After reduction (Fig.2B), the higher molecular weight species migrate with monomeric bac-α, suggesting they are disulfide-linked oligomers. The lower molecular weight species may be degradation products. Table I shows the amino acid composition of bac-α to be in generally good agreement with the composition of native LHα.

The immunological activity of the bac-α preparation both before and after reduction and reoxidation is shown in comparison with several other α preparations in Figure 3. The antiserum is directed against the native α subunit; linear α, ie, RCM-LHα, shows no cross-reactivity at 10 μg per assay tube. Bac-α that had not been subjected to the refolding procedure gave a competition curve that is not parallel to that of LHα, indicating related but nonidentical determinants [26]; its approximate

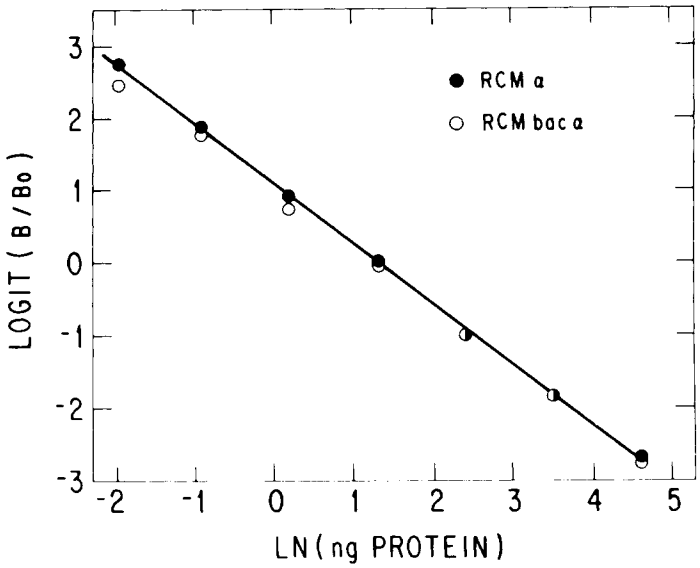

Fig. 1. RCM-α radioimmunoassay. RCM-LHα and RCM bac-α were examined in an RIA that employed antiserum directed against RCM-LHα and ^{125}I-RCM LHα as tracer as described in Materials and Methods.

relative reactivity was 0.2% of bLHα itself.* In a protein containing ten half-cystines, 945 combinations to form five disulfides are potentially possible [27]. If, during formation of the gene product, disulfide formation had been random, only one of the 945 (approximately 0.1%) would have correct disulfide bonds and hence the correct ("native") conformation. Thus, the 0.2% native structure observed for the bac-α preparation is little better than random; the low yield may have resulted in part from the presence of urea during the bac-α purification. The yield of immunoreactive bac-α was increased approximately 18 times by reduction and reoxidation. Furthermore, the preparation was largely monomeric as shown by gel electrophoresis (Fig. 2A, lane 4) and gel filtration (HPLC, data not shown). The solubility properties of the refolded bac-α also differed from those of the original preparation (both soluble at pH 5.0, only refolded bac-α readily soluble at pH 7.0 [1–2 mg/ml]). The displacements of radioiodinated LHα by "native" α and chemically deglycosylated α, both before and after refolding, are also given in Figure 3. The deglycosylated LHα described herein contained essentially only the chitobiose core (analysis gave 4.3 residues of glucosamine per mole of α and only traces of other sugars) and its approximate relative immunopotency is 19% that of native LHα. Upon reduction and reoxidation, about 50% of the initial immunoreactivity of deglycosylated LHα was retained. The preparation appears thus to have suffered some damage during degly-

*Because the displacement curves are not fully parallel, the amount of native-like structure in bac-α (relative potency in the RIA) was estimated by comparison of the amount of bac-α and LHα required to decrease the ^{125}I-LHα bound to 50% (logit = 0).

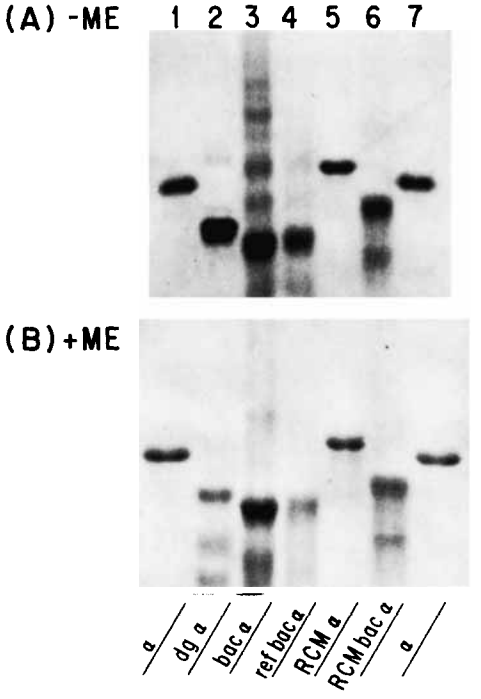

Fig. 2. Polyacrylamide gel electrophoresis in the presence of SDS. The different α forms were subjected to electrophoresis in 15% gels either in the absence (A) or presence (B) of reductant. Approximately 20 μg of each protein was applied, and staining is with Coomassie blue R. Lanes 1 and 7) LHα; lane 2) chemically deglycosylated LHα; lane 3) bac-α; lane 4) refolded bac-α; lane 5) reduced carboxymethylated LHα; lane 6) reduced carboxymethylated bac-α.

cosylation; SDS gel electrophoresis indicated 50% of the material to have at least one peptide bond cleaved (probably owing to traces of moisture during the deglycosylation reaction.)† The correct refolding of the α subunit containing only a chitobiose core in reasonable yield (when the damage to the preparation is considered) is in agreement with earlier studies [8,13] that showed that enzymatically deglycosylated α subunit of hCG refolds with a nearly 100% yield. The control "native" LHα fully regained its structure upon reduction and reoxidation as before [14] (Fig. 3).

Combination With β Subunit

hCGβ was used in these studies because its larger size relative to other glycoprotein hormone β subunits permits easier monitoring of combination by gel filtration. A profile showing combination of LHα with hCGβ is shown in Figure 4A, left; after dissociation by preincubation in GdHCL only the subunits are seen (Fig. 4A, right). With either deglycosylated or bac-α, the α-β complex coelutes with uncombined β subunit and, as shown in Figure 4B, the difference in the amount of uncombined

†Studies from other laboratories in which moisture was more rigorously excluded during deglycosylation have resulted in higher yields of undamaged material [eg, 7,9,10].

TABLE I. Amino Acid Composition of Bac-α

Amino Acid	Residues found[a]	Residues from bovine α sequence
Lys	8.5 (0.7)	10
His	2.8 (0.3)	3
Arg	3.0 (0.5)	3
Asx	5.9 (0.5)	6
Thr	8.6 (0.6)	9
Ser	5.5 (0.3)	6
Glx	8.1 (0.5)	8
Pro	6.4 (0.7)	7
Gly	4.0 (0.2)	4
Ala	6.8 (0.4)	7
Cys[b]	1.5 (0.5)	10
Val	5.1 (0.2)	5
Met[c]	3.6 (0.3)	4
Ile	1.8 (0.2)	2
Leu	2.4 (0.5)	2
Tyr	4.2 (0.7)	5
Phe	4.1 (0.4)	5

[a]Average values (standard deviation in parentheses) from nine analyses performed over a 6-month period.
[b]Destroyed during hydrolysis. Analysis of both reduced S-carboxymethylated bac-α and a control α for carboxymethyl cysteine gave values of 16 half-cystines per molecule.
[c]The bac-α is expected to contain an amino terminal methionine not found in bovine α. Thus, a total of five methionine residues are expected for bac-α.

α subunit versus uncombined plus dissociated material (Fig. 4B, right) is a measure of the extent of α-β combination. For bac-α as described in Materials and Methods, radioimmunoassy was necessary to determine how much of the correctly folded material combined. The gel filtration patterns are given in Figure 4C. Our results showed that 71, 51, 35, and 18%, respectively, of the LHα, refolded LHα, deglycosylated LHα, and refolded deglycosylated LHα combined with hCGβ.[‡] For bac-α a large excess of refolded bac-α preparation versus hCGβ was needed (see Fig. 4C, left) because only about 3.4% was active in the α RIA. Approximately 2%, or 55% of the available (on the basis of its RIA) refolded bac-α, combined with hCGβ.

Figures 5 and 6 show the ability of the various α-β complexes, respectively, to bind to LH receptors in crude testes preparations and to stimulate steroid production. The relative potencies in both assays were as expected from the content of combined material found in each preparation. The biological activities are summarized in Table II, together with the immunological data and the percentage of subunit that had combined. Since the isolated subunits of glycoprotein hormones have negligible receptor binding or biological activity [23], correcting the potencies of the different recombinants in the radioligand receptor and steroidogenesis assays for the amount

[‡]About twice as much of the two deglycosylated LHα samples combined than the amount of "native" structure determined by the α radioimmunoassay. The discrepancy most probably results from the uncertainty of estimating 50% displacement from nonparallel inhibition curves in the radioimmunoassay.

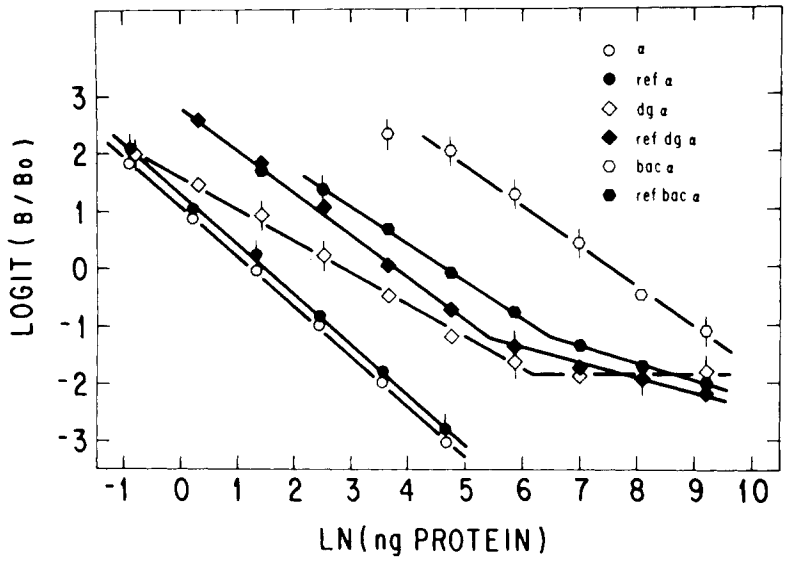

Fig. 3. LHα radioimmunoassay. LHα, deglycosylated LHα, bac-α, and their refolded counterparts were examined in an RIA employing antiserum directed against native LHα and ^{125}I-LHα as tracer as described in Materials and Methods.

of recombinant present allows comparison of the intrinsic activity of each α-β dimer. All α-β complexes both bound to the receptor preparations and stimulated steroid production while the hCGβ was completely inactive (Fig. 6). As expected, α-β complexes containing LHα or refolded LHα stimulated steroidogenesis to the same level as native hCG. Only submaximal stimulation could be achieved by the complexes containing either chemically deglycosylated or bac-α subunit in agreement with earlier observations with partially deglycosylated hCG preparations [8,9]. Although the potencies are only approximate, it is clear that the majority of any complex formed is biologically active.

DISCUSSION

The data presented demonstrate that the product expressed after insertion of cDNA for the α subunit of bovine glycoprotein hormones into *E coli* has the expected amino acid composition of the α polypeptide and is indistinguishable from α subunit isolated from bovine lutropin in an RIA utilizing antiserum directed solely against linear determinants. The availability of this completely nonglycosylated α subunit has permitted a more full definition of the role glycosylation plays in correct folding of α subunits. The major conclusion derived is that while covalently attached carbohydrate is not obligatory for correct formation of three-dimensional structure, it greatly facilitates this process and increases the yield of correctly folded subunit. Thus, while correctly folded bac-α, which is totally nonglycosylated, constituted only 2% of the reduction-reoxidation mixture, that amount is significantly greater than the 0.1% that would result from random formation of disulfide bonds. Although it is possible that under other *in vitro* conditions the yield of correctly folded bac-α could be increased,

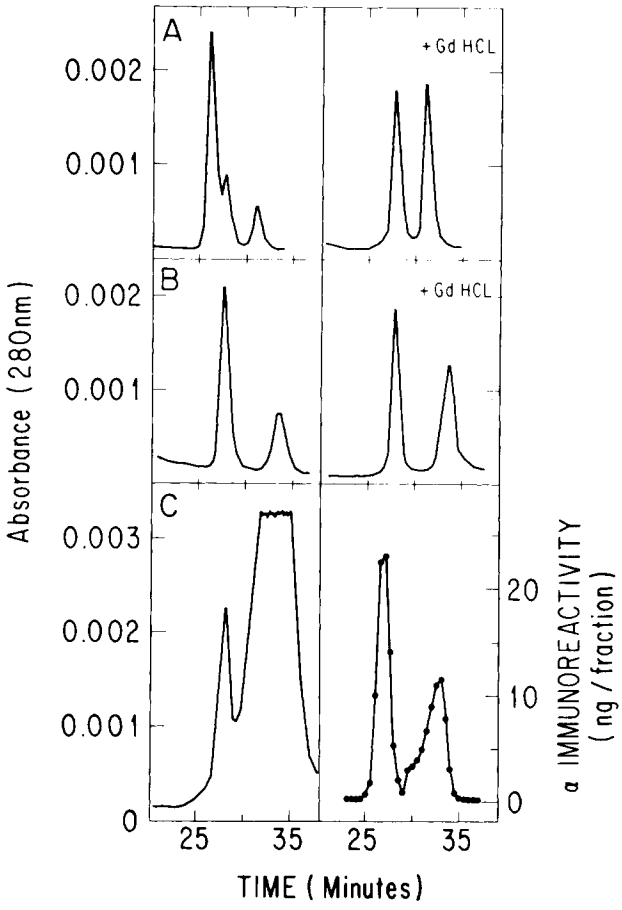

Fig. 4. Recombination of α with hCGβ. The different forms of α subunit were incubated with hCGβ under conditions favorable to recombination as described in Materials and Methods. Aliquots of each recombination mixture were subjected to HPLC gel filtration on a series of one Bio-Sil TSK-125 and two Bio-Sil TSK-250 columns (7.5 × 300 mm, Bio-Rad). The columns were developed with 0.2 M ammonium acetate (pH 6.0) containing 30% acetonitrile. As described in Materials and Methods, the percentage of recombination of LHα (A) and deglycosylated LHα (B) were determined by chromatography with (right side) and without (left side) a preincubation with GdHCL. The percentage of recombination of refolded LHα and refolded deglycosylated LHα were determined in the same manner (chromatographs not shown). Approximately 10 μg of total protein (α+β) were applied in A and B and the effluent was monitored at 280 nm. In A, LHα, hCGβ, and the dimeric LHα-hCGβ elute at approximately 31, 28, and 26 min, respectively. Deglycosylated LHα, hCGβ, and the dimer of deglycosylated LHα-hCG elute at approximately 33.5, 28, and 28 min, respectively, as shown in B. In the left side of C the optical density recording of the mixture of refolded bac-α and hCGβ is shown. The right side shows the α immunoreactivity of the fractions collected from this column.

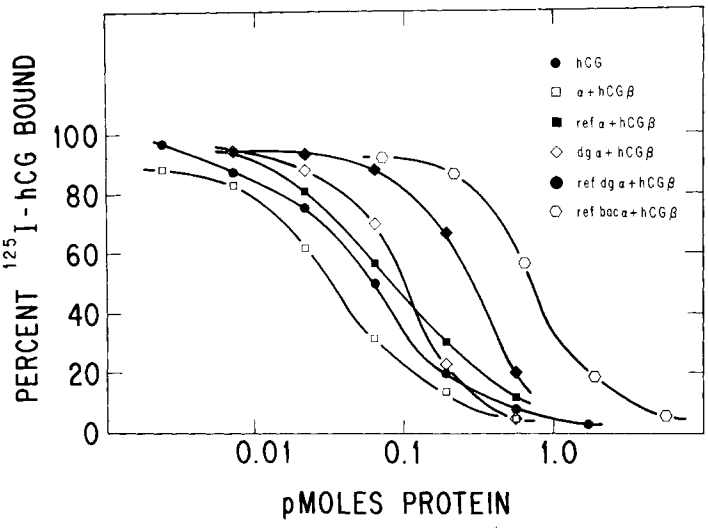

Fig. 5. Radioligand receptor assay of α recombinants. The total recombination mixture resulting from incubation of the various α forms with hCGβ, with no separation of α-β dimer from free subunits, was tested for its ability to inhibit ^{125}I-hCG binding to a rat testicular homogenate as described in Materials and Methods.

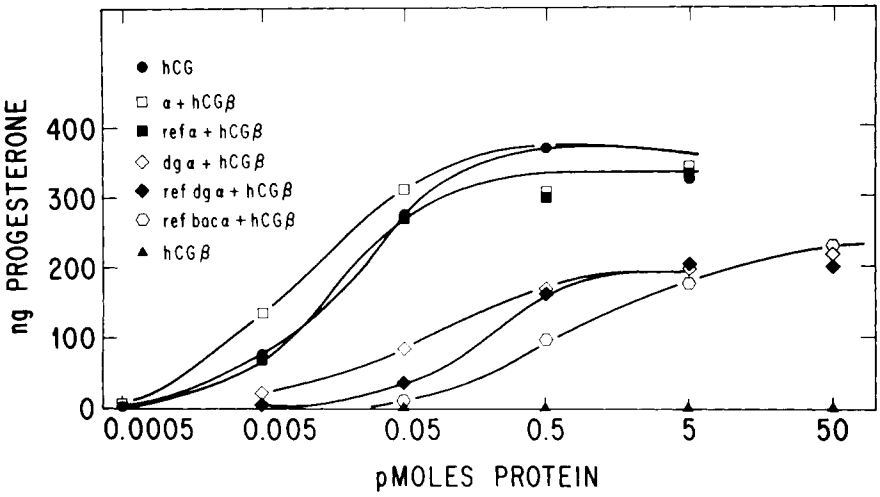

Fig. 6. Steroidogenesis assay of α recombinants. The total recombination mixture resulting from incubation of the various α forms with hCGβ was tested for its ability to stimulate steroid production in mouse Leydig tumor cells as described in Materials and Methods.

the present work as well as several earlier studies on the folding or refolding of α subunits suggests that even incomplete oligosaccharides allow high yields of functional α subunit to be obtained. The present *in vitro* results also are in full

TABLE II. Summary of Assay Data

	Radio immunoassay potency[a]	Percentage of recombination[b]	Corrected radioreceptor assay potency[c]	Corrected steroidogenesis potency[d]
α	100	71	100	100
Refolded α	76	51	60	74
Deglycosylated α	19	35	73	24
Refolded deglycosylated α	7.6	18	48	18
Bac-α	0.19	—	—	—
Refolded bac-α	3.4	1.9	175	37

[a]Percentage of cross-reactivity relative to native LHα in the α subunit RIA. Calculated from data shown in Figure 3 by comparing the amount of each subunit required to reduce ^{125}I-LHα binding by 50% (logit = 0).

[b]Determined as described in Materials and Methods.

[c]Corrected RRA potencies relative to LHα-hCGβ. Determined from data shown in Figure 5. Gross potencies were determined by comparing the amount of protein required to decrease ^{125}I-hCG binding by 50%. To correct for the amount of recombinant present, the gross potencies were divided by the percentage of recombination.

[d]Corrected steroidogenesis assay potencies relative to LHα-hCGβ. From the data in Figure 6, the gross potency was taken to be the amount of protein required to increase steroid production to 50% of the maximal level for that recombinant. The potencies were corrected by dividing these values by the percentage of recombination.

agreement with observations, both by Weintraub et al colleagues [11,12] and by this laboratory [13], which show that, both in mouse thyrotropic tumor cells and in cell-free translation of mRNA for the α subunit, glycosylation is a prerequisite for correct folding of α subunit and assembly into hormone. Based in the sequence determined for the cDNA of bovine α subunit and the characterization of the subunit expressed by *E coli*, the only likely difference in the protein portion vis à vis that of α subunit obtained from bovine glycoprotein hormones is the probable presence of a methionine at the amino terminus. While it is possible that this residue, which is not found in α subunit from the mature hormones, might influence folding, it is unlikely because this region of the molecule can differ without affecting folding, eg, human α subunit is four amino acids shorter than bovine and heterogeneity of the amino terminus is common in α subunits [28,29]. Most important in this context is that the α peptide formed during cell-free translation also does not form a functional structure before glycosylation [13]. This material does not have a methionine at or near its amino terminus.

It is also of interest that with another glycoprotein, vesicular stomatitis virus G protein, the size of the oligosaccharide affects folding since some variants only fold if they contain the full-size, high mannose oligosaccharide [30]. In the case of both hCGα [8] and bovine α, the presence of either one or two sugars per carbohydrate unit suffices to allow correct folding in good yield.

The low yield of correctly folded bac-α is presumably a consequence of formation of nonnative disulfide bonds during the reoxidation, which results in stabilization of structures that then do not rearrange to give the correct structure. Thus, in

competition between native and nonnative folding pathways, the native pathway is completed only rarely by the nonglycosylated α. The role of carbohydrate in the mechanism of folding of α subunit can be envisioned in several ways. The oligosaccharides may, from steric considerations, prevent the formation of certain conformers and thereby inhibit the formation of nonnative disulfide bonds. Alternatively, the carbohydrate moieties may, by their hydrophilic character, insure that certain portions of the peptide chain remain on the surface of the molecule during the folding reaction and thereby lessen the number of incorrect interactions. In this regard, it is of interest to note that oligosaccharide moieties are often located at positions in the protein that are predicated to be β turns [31,32] and β turns are crucial elements in protein folding [33–35].

For many proteins, the amino acid sequence alone contains sufficient information to direct folding to the native structure [36–38]. This is not true, however, of some glycoproteins that, when devoid of carbohydrate, do not fold properly [39]. In other glycoproteins, the lack of carbohydrate appears to have no adverse affect on protein folding [39]. It is interesting to consider how and at what point in the evolution of certain glycoproteins correct folding became dependent on the presence of carbohydrate. In glycoproteins where oligosaccharides are necessary for folding, this is probably not their sole purpose. Rather, they probably serve another purpose and have become important for folding only through amino acid changes subsequent to acquisition of carbohydrate. One can envision that after a protein possesses a carbohydrate unit for a period of time, certain amino acid changes may occur that no longer allow the protein to fold in the absence of the carbohydrate. Thus, all bacterial proteins fold correctly in the absence of carbohydrate as they have not had the opportunity to become "dependent" as some eukaryotic proteins have. In this scenario, the carbohydrate of the glycoprotein hormone α subunit would be of primary importance to the biological activity of the hormone with participation in the folding pathway of α a secondary occurrence.

Finally, it is clear that unless better conditions can be obtained for correct *in vitro* folding of nonglycosylated α subunits, efficient expression of α and β subunits will necessitate the use of eukaryotic cells where glycosylation and subunit combination will occur.**

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