Equine gonadotropins as models for glycosylation studies

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Gonadotropins require carbohydrate for their normal physiological function; however, determining the mechanisms involved is complicated by the existence of several glycosylation sites decorated with a variety of oligosaccharide structures that contribute to various aspects of gonadotropin structure and function. Partially overlapping functions of these oligosaccharides provide additional complications. N-linked oligosaccharides contribute to protein folding, heterodimer stability, circulatory survival, receptor-binding affinity, and cellular activation. O-linked oligosaccharides influence N-linked oligosaccharide processing and extend circulatory survival. Equine LH and eCG provide useful subjects for studying the influence of carbohydrate on gonadotropin action because they differ only in their carbohydrate moieties, which produce substantial effects on their activities. Moreover, because these hormones bind FSH receptors, as well as LH receptors, they can be used to study the role of carbohydrate in hormone activation of both receptors.

The gonadotropins are the most abundant members of the classical glycoprotein hormone family. Two of the three pituitary glycoprotein hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are gonadotropins. The third, thyroid-stimulating hormone (TSH), is the single non-gonadotropin member. Two groups of mammals, primates and equids, elaborate a placental glycoprotein hormone, chorionic gonadotropin (CG). These heterodimeric molecules are structurally related because they possess a common β subunit combined with a hormone-specific α subunit. The crystal structures of hCG and hFSH revealed that despite less than 10% sequence homology between them, both α and β subunits possess a cystine-knot motif in common. This makes the glycoprotein hormone members of the cystine knot growth factor superfamily and identifies the basis for the structural relationship between the α and β subunits first suggested by Dayhoff. Other cystine knot growth factor superfamily members include the nerve growth factor family, the platelet-derived growth factor family, and the transforming growth factor β superfamily. Glycoprotein hormone subunits derived from LH, FSH, and TSH are N-glycosylated at one or two conserved sites. Chorionic gonadotropin β subunits and eLHβ are O-glycosylated in the C-terminal extension unique to these hormones. A review of the vast variety of roles carbohydrates play in glycoprotein hormone structure and function was appropriately entitled: "Biological roles of oligosaccharides: all of the theories are correct," could be applied to the glycoprotein hormones alone. Glycosylation influences gonadotropin subunit folding, subunit association, carbohydrate processing, heterodimer stability, secretion, circulatory survival, receptor binding affinity, signalling, internalization, and sensitivity to proteolytic digestion.

Glycoprotein hormones require carbohydrate to be functional and their activities are modulated by the structures of the attached oligosaccharides. The literature is replete with studies showing retention of substantial receptor-binding activity, but almost complete loss of biological activity resulting from elimination of all or virtually all of the carbohydrate from gonadotropins. Classical chemical and enzymatic deglycosylation experiments indicated α subunit carbohydrate was more important than β subunit carbohydrate. Site directed mutagenesis provided valuable information regarding the functional roles of individual glycosylation sites. For example, Asn was identified as the site of the α subunit oligosaccharide that was critical for hCG and hFSH biological activity. However, the impact of altering the structures of the oligosaccharides, other than removing terminating sialic acid residues, is virtually unexplored. This is a curious deficiency because glycoprotein hormone preparations consist of a collection of isoforms that possess the same primary structure, but differ in the structures of the oligosaccharides attached to three or more glycosylation sites. The carbohydrate structures are believed to change under different physiological conditions, implying a
N-Glycosylation patterns in the glycoprotein hormones

The common α subunit possesses two N-glycosylation sites at Asn⁶⁰ and Asn⁸² (Fig. 1. Note the corresponding sites in human glycoprotein hormones will be designated as hαAsn⁶³ and hαAsn⁶⁰ because its numbering differs from all other mammalian α subunits due to an amino terminal deletion of four amino acid residues that results from alternative splicing). A variable number of glycosylation sites located at conserved positions exist on the hormonespecific β subunits (Fig. 2). LHβ and CGβ subunits are N-glycosylated at Asn¹³ or Asn¹⁰, or both. FSHβ subunits are potentially N-glycosylated at Asn⁶³ and Asn⁸⁴ (homologous to LH/CGβ Asn¹³ and Asn¹⁰, respectively), while TSHβ subunits are glycosylated at Asn⁶³ (homologous to LH/CGβ Asn¹³). Partially glycosylated hCGβ and FSHβ isoforms have been identified in recent years.

O-glycosylation patterns in equid and primate glycoprotein hormones

O-glycosylation has been less well characterized even though it is often invoked to explain the functional differences between hCG and eLH. In primates, the four O-glycosylation sites observed in hCGβ are reduced to three in baboon CGβ and one in macaque CGβ, although a potential N-glycosylation site is substituted in the macaque at one of the hCGβ O-glycosylation sites (Fig. 4). Greater conservation of O-glycosylation sites is observed among the equids, as the bulk of the twelve O-glycosylation sites identified in both eLHβ and eCGβ are present in donkeys and zebras as well. Two possible exceptions include a predicted Cys residue in place of Ser¹⁸ in the donkey and a potential N-glycosylation site at position 138 in the zebra sequence. The greater conservation of O-glycosylation in the equid species could be related to the use of carbohydrate to distinguish between...
Luteinizing Hormone $\beta$

- ovine, bovine, porcine, rabbit, mouse, rat, whale, salmon.
- human
- equine

Follicle-Stimulating Hormone $\beta$

- $6$ or $7$
- $23$ or $24$
- \(\text{eFSH}\beta\)
- \(\text{hFSH}\beta\)

Thyroid-Stimulating Hormone $\beta$

- $23$ (in Xenopus)

Chorionic Gonadotropin $\beta$

- human
- baboon
- macaque
- horse
- donkey
- zebra

Fig. 2—Beta subunit glycosylation sites. N- and O-glycosylation sites are indicated with the same symbols as in Fig. 1. Incompletely glycosylated FSH$\beta$ isoforms patterns are indicated for the naturally occurring hormone. The glycosylation sequences exist, but no carbohydrate is attached. An insect cell-expressed hFSH$\beta$ isoform bearing only Asn' oligosaccharide has been reported (vide infra).
Fig. 3—Sequences of CGβ subunits. Primary structures determined for eLH/CGβ and hCGβ along with the predicted amino acid sequences for donkey, zebra, baboon, and macaque CGβ subunits. Glycosylation sites are indicated by open boxes enclosing the N-glycosylation sequon or O-glycosylated Thr or Ser residue. Potential glycosylation sites are indicated by shading.
pituitary LH and placental CG in these species. Free α subunit isolated from the anterior pituitary is O-glycosylated at Thr\(^{31,54}\). In the horse, the relative abundance of O-glycosylated pituitary α subunit is 50\%\(^{55}\), while less than 10\% free hCGα is modified in this manner.\(^{56}\)

**Gonadotropin glycoforms**

Largely as a result of oligosaccharide structural heterogeneity, it is difficult to isolate enough of a homogeneously glycosylated, glycoprotein hormone isoform preparation for extensive characterization. For example, human pituitary FSH was separated into 20 fractions by a combination of isoelectric focusing and anion exchange chromatography\(^{35}\), yet provided only enough material for static acid measurements, leaving the remainder of the oligosaccharide uncharacterized.\(^{58}\) Moreover, individual isoforms often exhibit small differences in biological activity that make it difficult to define the contribution of each glycosylation site to the overall activity of the hormone. In this regard, equine LH and CG offer a unique opportunity to study substantial differences in biological activity that result from variations in carbohydrate structure. Both hormones display both LH and FSH activities\(^{59-62}\), possess identical amino acid sequences\(^{63-65}\), yet differ significantly in their carbohydrate moieties\(^{73,66-71}\). Therefore, all functional differences that are observed between eLH and eCG in vivo and in vitro are due only to glycosylation. In the rat, the apparent affinity of eLH for either the LH or FSH receptor is 5 to 10 times that of eCG\(^{74,52,72}\), while in the horse, the affinity of eLH for the LH receptor is 50- to 75-fold greater than that of eCG\(^{73}\). In vivo, the plasma half-life of eCG is 6 days, while that of eLH is 5 hours, almost a 30-fold difference. A 5.7-fold difference in clearance rates for eLH and eCG was noted in the rat.\(^{70}\)

### Carbohydrate and biosynthesis

Carbohydrate plays a facilitating role in glycoprotein hormone biosynthesis as evidenced by reduced

![Fig. 4](image-url) — Oligosaccharide heterogeneity in gonadotropins. A. Examples of the variety of oligosaccharide structures found in gonadotropin preparations. All three major classes are represented. Only a few high mannose structures have been reported, but others are probably uncharacterized due to low yield. Hybrid complex/high mannose are commonly attached to αAsn\(^6\) (heAsn\(^7\)). Complex oligosaccharides represent the most diverse class. Bi-antennary oligosaccharides are the most abundant, although FSH and TSH possess more extensively branched oligosaccharides. Many pituitary gonadotropin oligosaccharides are terminated with sulfated GalNAc residues. Facosylation of the proximal GlcNAc residue is subunit-specific. Less than 25\% of α subunit oligosaccharides are fucosylated while more than 90\% of β subunit oligosaccharides are fucosylated. B. Carbohydrate heterogeneity in human glycoprotein hormones. Site-specific studies from Renwick’s laboratory\(^{46,71,72}\) determined the distribution of oligosaccharide structures at each N-glycosylation site in three human glycoprotein hormones, hLH, hTSH, and hCG. The structure of the most abundant oligosaccharide at each site is shown. The percentage indicates the relative abundance of that structure at that site and the number above the oligosaccharide indicates the number of different oligosaccharide structures characterized by NMR. Positions of the O-linked glycosylation sites in hCGβ are indicated along with an oligosaccharide structure proposed by Bahl’s laboratory\(^{73}\). Structures of hFSH oligosaccharides are shown along with their abundance in one study\(^{12}\), however, these were obtained from all four N-glycosylation sites\(^{12,14}\).
yields of recombinant glycosylation mutants. Mutation of any of the three TSH glycosylation sites resulted in a reduced yield of recombinant hormone. Reduced yields of recombinant hCG were noted when either α or β subunit glycosylation sites were eliminated with the effects of α subunit glycosylation greater than β subunit glycosylation. Reduced secretion of hFSH glycosylation mutants, particularly when α subunit glycosylation sites were eliminated, was reported when all four glycosylation sites were mutated, no secreted hormone and little intracellular hormone were detected. The greater impact of α subunit glycosylation was probably related to the contribution of its carbohydrate, particularly hαAsn, to subunit folding. Bovine and human α subunits have been denatured under reducing conditions, yet efficiently refolded when the reducing and chaotrophic agents were removed. Bovine α subunit expressed in bacterial cells, which lacked the N-glycosylation machinery, folded to such a low extent that only a sensitive, conformation-dependent RIA could detect the presence of folded molecules. In vivo, the folding of the hCGα proceeded so rapidly that after a two-minute pulse labeling experiment, the disulfide bonds were completely oxidized. The carbohydrate attached to hαAsn appears to participate in α subunit folding. Located between hairpin loops L1 and L3 its proximal GlcNAc residue forms hydrogen bonds with several amino acid residues in both loops. In contrast, the β subunit N-linked oligosaccharides are located in two antiparallel β strands, Pro–Val and Ile–Tyr, and do not appear to interact with the polypeptide moiety. Indeed, folding of hCGβ lacking both N-linked oligosaccharides has been obtained. Rotation of the hαAsn Man(B1-4)GlcNAc(B1-4)GlcNAc (B1-N) trisaccharide is restricted due to interaction with α loops L1 and L3, while the presence of oligosaccharide distal to GlcNAc1 contributes to hCGα stability.

Oligosaccharide attached to hαAsn is located in loop L2 and does not appear to interact with the α subunit polypeptide, although it seems to play a role in stabilizing the heterodimer. It is located in the part of αL2 that is embraced by the seabeat loop and it is differentially glycosylated due to the different β subunit context. Since hCG heterodimer formation appears to occur prior to latching of the seabeat in vitro and because agents that promote partial disulfide bond reduction facilitate hCG subunit association in vitro, hαAsn oligosaccharide may limit the time when dimer formation can occur. In hCG biosynthesis, this appears to be the interval between formation of the 93-100 disulfide bond, when dimerization competence is obtained, until formation of the 26-110 seabeat latch disulfide, when steric hindrance on the part of hαAsn GlcNAcManGlc3 high mannose oligosaccharide blocks dimer formation. For in vitro studies, the fact that the oligosaccharide must be threaded through the seabeat loop selects for smaller oligosaccharides such as the hybrid oligosaccharides attached to hCG and eLH. Indeed, we have noted that the increased size of equine α subunit Asn oligosaccharides is accompanied by a reduction in dimer formation efficiency that is largely relieved by eliminating the oligosaccharide from eLHα and eFSHα with peptide-N-glycanase, but only partially relieved in the case of eCGα. Free hCGα preparations exhibited impaired ability to associate with hCGβ because of increased oligosaccharide branching and this appears to reflect the relative abundance of triantennary oligosaccharides attached to free hCGα. The corollary that triantennary oligosaccharides stabilize the heterodimer by preventing denatured subunits from completely dissociating, thus permitting the native conformation to be recovered has not been demonstrated. Although dissociation-resistant forms of oLH, hFSH, eLH, and eFSH are known to exist, the structures of the oligosaccharides in these preparations have not been characterized. Whether or not the oligosaccharide contributes to the stability of the heterodimer depends on the particular gonadotropin under investigation. A recent report claimed that the hCGα Asn oligosaccharide stabilized the heterodimer and the loss of biological activity attendant upon elimination of this glycosylation site by mutagenesis was due to heterodimer dissociation into inactive subunits rather than the carbohydrate participating in hCG action. This report is in contrast to the increased thermal stability of chemically deglycosylated oLH, eFSH, and hCG preparations. Moreover, in our hands, an N59dg-eLHαβ preparation experienced no loss of receptor-binding activity following 72 hr incubation at 37°C. Despite the greater stability of this preparation than N59α-hCG mutants, the N59dg-eLHαβ preparation exhibited an 89% reduction in LH biological activity, while retaining 100% LH receptor binding activity. Moreover, recombinant eLH/hCG expressed in human embryonic kidney 293 cells
remained stable even when the seatbelt latch disulfide 26-110 was eliminated by mutation\(^3\). Biological activity of stabilized N\(^5\)DG-rechCG was measured in a cell line overexpressing the LH receptor. If oligosaccharide is involved in ligand-occupied self-association\(^9\), then the greater than 10-fold higher receptor concentration may obviate the need for oligosaccharide in this process. In the case of eFSH, the αAsn\(^{56}\) oligosaccharide definitely contributed to heterodimer stability as 85% receptor-binding activity of the latter was lost after 24 hr incubation at 37°C, while the intact eFSH preparation required was only 50% dissociated after 72 hr\(^9\). However, heterodimer stability is not a good predictor of biological activity. Equine FSH is less stable than pFSH and hFSH\(^{96}\), yet is significantly more active than both hormones in the rat granulosa cell bioassay\(^97\).

O-glycosylation of hCG and eCG, as well as eLH\(_{\beta}\), is characterized by only a fraction of the potential glycosylation sites bearing oligosaccharide chains\(^3\). Automated Edman degradation indicated partial glycosylation at 10 of the 12 sites in eLH\(_{\beta}\) and eCG\(_{\beta}\) preparations ranging from a low of 10-20%, respectively, to 100%\(^3\). Mass spectrometry of hCG peptides indicated mutually exclusive glycosylation of Ser residues 127 and 132\(^98\). O-glycosylation of hCG\(_{\beta}\) was reported to occur during the last phase of post-translational modification prior to secretion\(^3\), however, recent studies have indicated that at least one of these sites influences processing of the N-linked oligosaccharides\(^100,101\).

While both α subunit N-glycosylation sites as well as LH, CG, and TSH β subunit N-glycosylation sites are always decorated with carbohydrate in naturally occurring hormone preparations, N-glycosylation of FSH\(_{\beta}\) is not quantitative. Partial glycosylation of recombinant bFSH\(_{\beta}\) was reported in abstract form, with no indication of the particular glycosylation site\(^3\). We reported that eFSH\(_{\beta}\) consisted of two isoforms, one glycosylated at both Asn\(^7\) and Asn\(^{24}\), while the other was partially glycosylated\(^37\). N-terminal sequence analysis indicated that Asn\(^7\) was partially glycosylated, while no conclusion could be made regarding the glycosylation status of Asn\(^{24}\) due to N-terminal heterogeneity. Automated Edman degradation revealed the presence of phenylthiohydantoim (PTH)-Asn at position 7, which indicated that carbohydrate had never been attached to this position. The relatively low yield of PTH-Asn initially suggested that partial glycosylation also occurred at Asn\(^{24}\), however, analysis of tryptic peptides released from eFSH\(_{\beta}\) by mass spectrometry revealed only a non-glycosylated peptide containing Asn\(^7\) (unpublished data). Moreover, automated Edman degradation of the partially glycosylated eFSH\(_{\beta}\) band separated from deglycosylated eFSH\(_{\alpha}\) and eFSH\(_{\alpha}\) by SDS-PAGE and blotted on PVDF also indicated a low yield of PTH-Asn at cycles 5 and 7. Dias and colleagues reported that Asn\(^{24}\) was only partially glycosylated in insect cell-expressed recombinant hFSH\(_{\beta}\). We observed a glycosylation isoform of hFSH\(_{\beta}\) derived from human pituitaries that was present in all hFSH charge isoforms derived from chromatofocusing experiments\(^3^9\). Mass spectrometry suggested that this hFSH\(_{\beta}\) isoform was not glycosylated and automated Edman degradation corroborated the absence of carbohydrate and indicated that it had not been attached to the protein because PTH-Asn was observed for both glycosylation sites. Because N-glycosylation followed by carbohydrate removal by peptide-N-glycanase produces PTH-Asn\(^{102}\), the relatively greater abundance of this PTH-amino acid derivative associated with Asn\(^7\) and Asn\(^{24}\) than that associated with Asn\(^1\) raised the possibility that some of the non-glycosylated hFSH\(_{\beta}\) resulted from this process. As in the case of eFSH\(_{\beta}\) above, the yields of PTH-Asn\(^1\) and PTH-Asn\(^{24}\) were both low for the non-glycosylated hFSH\(_{\beta}\) isoform derived from human pituitary extracts\(^103\). Electrophoretic mobility of pFSH bands provided preliminary evidence that similar β subunit isoforms exist for this hormone\(^104\). Because Coomassie blue and silver-stained gels did not reveal the presence of hFSH\(_{\beta}\) isoforms, western blotting with FSH\(_{\beta}\)-specific monoclonal antibodies appears to be a more reliable method to detect FSH\(_{\beta}\) isoforms\(^105\). The initial reports indicating changing patterns of FSH glycosylation were based on changes in the elution volumes during gel filtration\(^105,106\). Deletion of hFSH\(_{\beta}\) N-glycosylation sites produced altered gel filtration patterns for recombinant hFSH\(_{\beta}\)\(^107\), suggesting that elimination of one or more FSH\(_{\beta}\) oligosaccharides occurred under physiological conditions. Taken together, these results suggest that the activity of the enzyme that initiates N-glycosylation, oligosaccharyltransferase, is regulated in gonadotropes. A recent report indicated that protein kinase C inhibited the glycosylation activity of yeast oligosaccharyltransferase\(^108\). As the gonadotropin releasing factor, GnRH, regulates protein kinase C activity in gonadotropes\(^109\).
this suggests a possible mechanism for producing partially glycosylated or non-glycosylated FSHβ isoforms. It also raises an interesting question, why don’t we observe partial glycosylation of LHβ and the common α subunit?

**Carbohydrate regulation of metabolic clearance rates**

Studies with TSH\(^1\)\(^{10}\) and FSH\(^1\)\(^{11}\) have indicated that the β subunit N-linked oligosaccharides modulated metabolic clearance rates. Oligosaccharides terminated with SO\(_4\)-4-GalNAc have been the focus of attention for regulating LH clearance by glycosylation because these are the most abundant in the more readily studied oLH, bLH, and pLH preparations\(^1\)\(^{12}\). A liver lectin that binds oligosaccharides terminated with sulfated GalNAc was discovered\(^1\)\(^{13}\). This appears to be a Cys-rich carbohydrate recognition domain of the macrophage mannose receptor\(^1\)\(^{14}\)\(^{11}\)\(^{17}\). The existence of this lectin provides a mechanism to explain the relatively short half-life of pituitary LH, in which sulfated oligosaccharides are the most abundant type, as compared with placental gonadotropins, which are exclusively sialylated\(^2\). Studies on hLH isoforms suggested that the location of the sulfate moiety was critical for determining metabolic clearance\(^1\)\(^{18}\). However, studies on LH have been conducted on a hormone-wide scale rather than at the level of individual glycosylation sites\(^1\)\(^{112}\). TSH hybrids incorporating pituitary-derived subunits possessing sulfated oligosaccharides and the complementary recombinant subunits possessing only sialylated oligosaccharides indicated that β subunit glycosylation rather than α subunit glycosylation dictated metabolic clearance rates\(^1\)\(^{10}\). Studies with hCG have focused on the role of the C-terminal extension, ignoring the potential contribution of the N-linked oligosaccharides. When the hCGβ C-terminus was coupled to recombinant bLHβ, a long-acting LH derivative was produced that had a 2- to 3-fold longer half-life than recombinant bLH lacking the C-terminal extension\(^1\)\(^{119}\). A 3- to 5-fold increase in FSH potency in vivo was noted when the hCGβ C-terminal extension was coupled to recombinant hFSHβ\(^1\)\(^{20}\). However, the role of the βAsn\(^1\)\(^{13}\) oligosaccharide in modulating metabolic clearance rates should not be ignored. Almost 30% of the \(^{125}\)I-eLH injected into rats was found in the liver 30 min later, while only 10% of \(^{125}\)I-eCG was present in this tissue and 75% remained in the circulation\(^5\). Binding of \(^{125}\)I-eLH to isolated hepatic endothelial cells was demonstrated and, while cold eLH could compete with \(^{125}\)I-eLH for these binding sites, cold eCG could not. For exclusively sialylated glycoprotein hormones, such as hCG and hFSH, the liver does not appear to be the site for metabolic clearance. Despite the textbook model for sialylated glycoprotein clearance involving desialylation by peripheral neuraminidase to expose galactosyl residues that are cleared by the liver asialoglycoprotein receptor\(^2\)\(^{121}\), this mechanism has been shown to be invalid for hCG\(^2\)\(^{122}\). The same may be true for hFSH, which possesses only 7% sulfated oligosaccharides, unlike the 30-40% sulfated oligosaccharide content of bovine, porcine, ovine, and equine FSH preparations\(^8\)\(^9\)\(^{23}\). Studies with recombinant mutant hFSH indicated that elimination of either β subunit N-glycosylation site produced a 2- to 3-fold increase in metabolic clearance rate (MCR), while elimination of both sites resulted in a 10-fold increase\(^1\)\(^{11}\). No statistically significant effect of α subunit N-linked oligosaccharides was observed, although elimination of αAsn\(^7\) glycosylation appeared to increase MCR. The crystal structure of hFSH has revealed a molecule similar in shape to hCG\(^8\). The β subunit oligosaccharides protruded outward, effectively doubling the narrow diameter (the molecular dimensions of deglycosylated hCG have been reported to be 75 x 35 x 30 Å\(^6\), while an N-linked oligosaccharide can extend as much as 30 Å\(^{24}\)). This change in shape could affect ultrafiltration rates in the kidney, thereby altering clearance rates for the hormone. Baenziger\(^4\) proposed that sulfated oligosaccharides were responsible for the rapid clearance of LH, which resulted in an episodic pattern of LH secretion. The longer half-life and lower levels of circulating FSH have made it more difficult to demonstrate episodic release of this hormone\(^2\)\(^{25}\). While episodic release of GnRH is required for proper pituitary function\(^1\)\(^{26}\), demonstration that the accompanying episodic gonadotropin release is necessary for proper ovarian function has not yet been obtained. FSH is responsible for several different activities in the ovary, including pre-antral stage granulosa cell proliferation, antrum formation, induction of aromatase, induction of LH receptor, and resumption of meiosis by the oocyte\(^2\)\(^{17}\). The pre-ovulatory LH rise provides the ovulatory signal, although the two-cell model for estradiol synthesis assumes LH stimulation of theca cells to provide the necessary androgen precursors to the granulosa cells\(^2\)\(^{28}\). The hFSHβ double glycosylation mutants were completely inactive in the classical weight gain
Carbohydrate inhibits receptor-binding

Once gonadotropins reach their target cells, both receptor-binding and cellular activation are influenced by glycosylation. Chemical and enzymatic deglycosylation revealed that cellular activation was affected to a greater extent than receptor-binding\(^{15-25}\). The latter was either unchanged or modestly increased, while the former was significantly reduced. The absence of carbohydrate increased the association rate for deglycosylated oLH, but had no effect on its dissociation rate\(^{23}\). In contrast, a recombinant mutant hCG lacking all four N-linked oligosaccharides exhibited essentially irreversible binding to both human and rat LH receptors\(^{31}\). Deglycosylation of individual subunits demonstrated that \(\alpha\) subunit carbohydrate was more important for signal transduction than the \(\beta\) subunit carbohydrate, but carbohydrate from both subunits had to be eliminated to obtain maximum loss of biological activity\(^{20,22,23,26}\). Site-directed mutagenesis identified hCG\(\Delta\text{Asn}^{13}\) as the critical oligosaccharide for signalling in the hCG\(\alpha\) subunit and \(\beta\text{Asn}^{12}\) as a secondary critical site in the hCG\(\beta\) subunit\(^{22}\). Interestingly, the effect of mutating the Asn\(^{13}\) glycosylation site was not detectable until after the hCG\(\alpha\text{Asn}^{12}\) site had been eliminated\(^{27}\). This combination of \(\alpha\) and \(\beta\) subunit mutants has not been replicated with the other glycoprotein hormones. However, the requirement for chemical deglycosylation of both oLH\(\alpha\) and oLH\(\beta\) subunits for maximum loss of biological activity suggested \(\beta\text{Asn}^{13}\) oligosaccharide is involved in LH biological activity, as oLH\(\beta\) possesses only the Asn\(^{13}\) glycosylation site\(^{23,26}\). The partial loss of FSH activity following removal of \(\alpha\text{Asn}^{56}\) carbohydrate with PNGase in N\(^{56}\)dg-eLH (cefF) hybrid preparations suggests FSH\(\beta\) oligosaccharides also participate in FSH action. While carbohydrate deletion studies were valuable for providing evidence that carbohydrate was involved in the biological activity of the gonadotropins, they were limited by the fact that the \(\alpha\) subunit is always decorated with carbohydrate. (Although the presence of hFSH inhibitory activity in serum samples derived from women treated with GnRH antagonists suggested that FSH lacking one or more \(\alpha\) subunit oligosaccharides might exist\(^{32}\), these have not yet been isolated.) Composition analysis demonstrated hormone-specific differences in glycosylation of porcine glycoprotein hormones\(^{133}\). This suggested that the effects of altered glycosylation could be studied by isolating \(\alpha\) subunits from gonadotropin preparations, combining them with the same \(\beta\) subunit preparation, and comparing the biological activities of the resulting hybrid preparations.

We employed gonadotropins derived from the horse because it was possible to obtain large amounts of each gonadotropin from this species\(^{134,135}\). The plan was to prepare all possible combinations of equine gonadotropin subunits, perform receptor-binding assays to confirm formation of functional heterodimers, and proceed to functional bioassays. Because it was somewhat controversial as to whether or not deglycosylated LH and hCG were somewhat more active, somewhat less active, or as active as the intact hormone in receptor-binding assays\(^{15-25}\), we anticipated that the receptor-binding phase of the investigation would be brief. Instead, we found a wide range of receptor-binding potencies with a 600-fold difference in potency between the most active and least active preparations. These resulted from both \(\alpha\) subunit and \(\beta\) subunit glycosylation affecting subunit association as well as receptor-binding activity\(^{72}\). Oligosaccharide mapping of \(\alpha\text{Asn}^{56}\) carbohydrate selectively removed by PNGase digestion of intact \(\alpha\) subunit preparations had revealed a hormone-specific pattern of glycosylation at this site\(^{71}\). The major structural variation between eLH\(\alpha\), eFSH\(\alpha\), and eCG\(\alpha\) Asn\(^{56}\) oligosaccharides consisted of differences in the size of the Man(\(\alpha1\)-6)Man branch (Fig. 5). In eLH\(\alpha\) this consisted
Fig. 5—Structures of equine α subunit Asn\(^{56}\) oligosaccharides. Oligosaccharides released from Asn\(^{56}\) by PNGase digestion of intact α subunit preparations are illustrated. These are largely based on composition and average mass data. Two oligosaccharide structures were characterized by NMR analysis of the indicated peaks in panels B and D. A cautionary note was served when the hybrid complex sulfated/high mannos glossy saccharide believed to populate eLHα Asn\(^{56}\) was revealed to be high mannos glossy saccharide when the purified oligosaccharide fractions were characterized.
of two Man residues derived from exoglycosidase processing of the Glc₃Man₉GlcNAc₂ precursor. In eFSHα, either sulfate-4-GalNAc(β1-4)GlcNAc (β1-2) Man(α1-6) or NeuNAc(α2-3)Gal(β1-4) GlcNAcMan (α1-6) were present, while in eCGα, the NeuNAc (α2-3) Gal(β1-4)GlcNAcMan(α1-6) structure was extended by lactosamine repeats. Since it was not possible to determine the masses of individual oligosaccharides due to the negative charges provided by terminal sulfate and sialic acid moieties, the difference in mass between each α subunit preparation and its Asn⁵⁶-deglycosylated derivative was determined using mass spectrometry. This analysis revealed a progressive increase in the average Asn⁵⁶ oligosaccharide mass ranging from 1482 mass units for eLHβ to 2327 for eCGα. Increased size of the Man(α1-6) branch was responsible for the increased mass of eFSHα and eCGα oligosaccharides and produced significant (P<0.05) correlation between carbohydrate size and reduced dimerization efficiency leading to an eventual 23% reduction (Fig. 6B). Removing the αAsn⁵⁶ oligosaccharide with PNGase eliminated the correlation between increased oligosaccharide size and reduced dimer formation (P>0.05). Both eFSHβ and eCGβ combined less efficiently with the α subunit preparations than did eLH(β) by revealing an inhibitory effect of β subunit carbohydrate on dimer formation. The inhibitory effects of α and β subunit carbohydrate on dimer formation complicated interpretation of results obtained with eLHβ and eCGβ hybrids because the heterodimer and β subunits co-purified in every available chromatographic method. We then removed the C-terminal 29 residues from eLHβ and eCGβ, making it possible to purify the heterodimer fraction. Experiments with des(121-149)-eLHβ and -eCGβ hybrids confirmed the inhibitory effects of increasing αAsn⁵⁶ oligosaccharide size on both LH and FSH receptor-binding affinity (Fig. 7). However, they also revealed that eCGα Asn⁵⁶ oligosaccharide inhibited receptor-binding activity of hybrids possessing eCGα. Comparison of the inhibitory effects of α subunit carbohydrate on eLHβ and eFSHβ hybrids revealed an attenuated influence of oligosaccharide size on FSH receptor binding. Thus, eLHβ:eFSHβ was more active than eCGα:eFSHβ, however, the magnitude of the difference between these hybrids was only 2-fold, while the difference in FSH receptor-binding activity between eLHα:eLHβ and eCGα:eLHβ was 5-fold.

We proposed two explanations for the greater magnitude of the inhibitory response to increased αAsn⁵⁶ oligosaccharide size in eLHβ hybrids as compared with eFSHβ hybrids. The first was steric hindrance on the part of the carbohydrate accompanied by a difference in the orientation of eLHβ hybrids and eFSHβ hybrids in the FSH receptor. The increased size of the more flexible Man(α1-6) branch inhibits receptor binding by steric hindrance because it is located closest to the putative receptor-binding site and simply gets in the way. The problem with this rationale is the differential effect observed in LHand
and FSHβ hybrids. It is not simply a matter of steric hindrance on part of the carbohydrate, as the same oligosaccharides produce a bigger effect in the former than in the latter. A model for the interaction of deglycosylated hCG with a model of the LH/CG receptor extracellular domain placed the GlcNAc residues of the hAsn52 oligosaccharide core close to the receptor, illustrating how large oligosaccharides attached to this position could get in the way. The other N-linked oligosaccharides were located on the opposite side of the hormone, facing away from the putative hormone-receptor interface. The C-terminal portion of the cystine knot, which is important for FSH binding to its receptor, was also directed away from the receptor. Rotation of the hormone to orient this region toward the putative hormone-binding site of the receptor, moved the hAsn52 oligosaccharide away from the receptor, potentially reducing its steric influence. We suggested that eLH and eCG interact with both LH and FSH receptors in the same orientation that LH and hCG engage the LH receptor. This has the consequence that αAsn56 oligosaccharides

![Graph](image-url)

Fig. 7—Receptor-binding activities of purified equine gonadotropin hybrid hormone preparations. A. LH radioligand assay using 125I-hCG tracer and rat testis homogenate. B. FSH radioligand assay employing 125I-eFSH tracer and rat testis homogenate.
inhibit eLHβ hybrid binding to both receptors to the same degree. Moreover, the partial activation of FSH target cells by eLH, eCG, and hybrids possessing their β subunits, is consistent with a partially inappropriate interaction with the FSH receptor. The relative potency of eLH and eLHβ hybrids in a rat testis receptor-binding assay ranged from 5% to 19% that of eFSH. In the granulosa cell assay the relative potencies were all lower, ranging from 1% to 7%, averaging 3.8-fold lower biological potency than FSH receptor-binding potency.

The second explanation was that increases in oligosaccharide size altered the conformation of the protein, thereby reducing receptor-binding affinity. In this model, the conformation of eLHβ hybrids changed more than that of FSHβ hybrids producing a more pronounced reduction in receptor binding affinity for the former set of hybrid preparations than for the latter. Preliminary data supporting a conformational change in eLHβ hybrids have been obtained using circular dichroism and more extensive studies involving both eLHβ and eFSHβ hybrid preparations are in progress. Since the two explanations are not mutually exclusive, the inhibitory effects of carbohydrate structure on hormone activity might reflect both steric hindrance by the carbohydrate moieties as well as conformational changes in the peptide moieties. The crystal structure of hFSH suggested the nature of the conformational change was likely to consist of variations in the positions of the α and β subunits relative to each other. Two conformational isomers of hFSH were observed in crystals of insect cell-expressed recombinant hFSH. While each α subunit could be overlaid without significant differences in the positions of the backbone atoms, the complementary β subunits did not align well and vice versa. A comparison of the 3D structures for chemically deglycosylated hCG and desialylated hCG complexed with antibody Fv regions revealed changes in backbone conformation in both subunits, however, it was not clear whether the presence of virtually complete oligosaccharide chains or antibody binding were responsible for the changes in conformation. Based on changes in affinity of monoclonal antibodies for various hCG and LH derivatives, Moyle and colleagues have argued that a change in hormone conformation occurs with receptor binding. Since the epitopes recognized by these antibodies remain largely undefined, it is not yet known what sort of conformational changes are involved. If changes in the relative positions of the α and β subunits are important elements in hormone-receptor activation, then the role of the α Asn56 oligosaccharide may indeed consist primarily of a stabilizing effect, permitting subunits to shift in their orientation relative to one another without falling apart and terminating cellular activation. This would support the model proposed by Moyle and colleagues that a minimal oligosaccharide structure is necessary for biological activity. Too small an oligosaccharide, such as the GlcNAc3 remnant following chemical deglycosylation, could only partially activate LH receptor-mediated responses. However, in this model, the oligosaccharide interacts with the receptor to alter its conformation, thereby activating it. The stability model, suggests that below a minimum size, oligosaccharide

Fig. 8—In vitro bioassays of equine gonadotropin hybrids. A, Diethylstilbestrol-primed rat granulosa cell FSH bioassay. The endpoint was progesterone synthesis after 72 hr incubation. B and C: Rat testis Leydig cell steroidogenesis LH bioassay. Because of the short-term incubation involved in this assay, fewer samples could be accommodated at one time.
cannot prevent subunit dissociation during hormone-receptor interaction.

**Surprisingly small effect of altered receptor-binding affinity on in vitro biological activity of equine gonadotropin hybrids**

While the effects of carbohydrate on receptor-binding affinity revealed by comparison of equine gonadotropin hybrid preparations were larger than expected, the differences in steroidogenic potency between the preparations were reduced to the extent that they were no longer significant. This may have resulted from amplification of the ligand-dependent receptor activation as well as the variability introduced by the two-assay nature of the steroidogenesis assay; target cell incubation to induce steroidogenesis followed by steroid RIA to measure the product. One possible conclusion was that oligosaccharide structure at αAsn\(^{56}\) beyond a minimal basic structure was irrelevant for biological activity. However, when the ratios of receptor-binding IC\(_{50}\) to steroidogenic EC\(_{50}\) were calculated for each preparation, a different pattern emerged. Mendeleison et al. reported that G protein activation was compromised by elimination of hCG carbohydrate, suggesting partial receptor activation. The nature of gonadotropin receptor activation is unknown. For the prototype of G protein coupled receptors, the β2-adrenergic receptors, ligand-binding in the transmembrane helical region alters the relative positions of helices 3 and 6. This appears to alter the conformations of cytoplasmic loops 2 and 3, permitting G protein activation by exposing buried residues in these loops. The decapeptide releasing factor, GnRH, appears to bind the extracellular loops of its receptor to achieve the same rearrangement of transmembrane helices. The glycoprotein hormone receptors differ in that high affinity binding occurs in an enlarged N-terminal domain that comprises roughly half the receptor. Activation of the receptor has been suggested to involve a low affinity site in the extracellular loops. Alternatively, co-expression of cDNAs encoding the extracellular domain and transmembrane domain of the LH receptor produced a functional receptor. Changes in extracellular domain resulting from hormone binding could alter the conformation of the transmembrane domain via interactions between the extracellular domain and the extracellular loops of the transmembrane domain. In contrast to other G protein coupled receptors, no glycoprotein hormone antagonists have been developed. Chemically and genetically deglycosylated hCG retain residual biological activity. We prepared an interesting FSH inhibitor consisting of Asn\(^{56}\)-deglycosylated eLHβ combined with des(121-149)eLHβ (N\(^{56}\)-eLHβ:eLHβ) that exhibited no FSH activity in the rat granulosa cell bioassay, although it did exhibit 5% LH activity in the rat Leydig cell bioassay. N\(^{56}\)-eLHβ:eLHβ completely inhibited the steroidogenic response to 300 pg eFSH or 2000 pg hFSH. However, when other variants of this derivative were tested for FSH inhibitory activity, it was either reduced 10-fold or else completely eliminated. These results illustrate a complicating factor in studies involving gonadotropin carbohydrate, although αAsn\(^{52}\) plays a primary role in coupling receptor-
binding to cellular activation, other oligosaccharides, such as βAsn play secondary roles. These provide partial compensation for the absence of αAsn oligosaccharide and imply that carbohydrate-mediated receptor aggregation is involved in cellular activation.

A model for gonadotropin receptor activation proposed over 20 years ago, but inadequately tested was that carbohydrate was involved in receptor aggregation, as labeled hCG appeared to be clustered at the surface of target cells. Recently, Roes and colleagues presented the results of fluorescence resonance energy transfer studies involving individual cells expressing LH receptor-green fluorescent protein or -yellow fluorescent protein chimeras that indicated that dLH and hCG binding induced ligand-receptor self-association. Earlier studies showing antisera directed against hCG restored biological activity to chemically deglycosylated hCG were interpreted as restoring a native conformation to the deglycosylated molecule. Indeed, the determinant loop of hCG complexed with recombinant Fv fragments of α subunit- and β subunit-directed monoclonal antibodies displayed an altered conformation, which might represent the functional conformation of the hormone. On the other hand, wheat germ agglutinin (WGA) could also restore the activity of deglycosylated hCG. As WGA was used in the isolation of the LH receptor, it could probably aggregate cell surface receptor on its own. However, WGA by itself had no effect on cellular activity, therefore, association of ligand-activated receptors appears to be necessary for target cell activation. In support of this, constitutively activated receptors only partially activate target cells. While inactive LH receptor-GFP chimeras failed to self-associate, constitutively activated receptors were not tested. Ligand-mediated receptor dimerization has been known to be a mechanism for receptor activation ever since the crystal structure of growth hormone bound to two extracellular domains was reported. The standard textbook model for G protein coupled receptor action predicted isolated receptors should be fully functional. However, GnRH antagonists were reported to become agonists when crosslinked by antibodies, and this has subsequently been confirmed by reports that GnRH antagonists do not initiate fluorescence energy transfer by GnRHR-GFP, GnRHR-YFP chimeras expressed in the same cell. The premier system for GPCR studies, the β2-adrenergic receptor provides a progressive model for hormone-receptor induced receptor phosphorylation leading to G, to G, switching followed by GRK phosphorylation which is followed by β-arrestin-mediated desensitization and internalization, which by clustering with growth factor receptors leads to activation of MAP kinase pathways. Although the dimerization determinants of the gonadotropin receptors do not appear to reside in the transmembrane domains, β-arrestin-mediated desensitization and internalization occur, albeit largely in the absence of receptor phosphorylation. Studies involving cells expressing LHR-GFP chimeras indicated that concentration of the LH receptor was distributed over a large portion of the cell, rather than the punctate pattern observed for the β2-adrenergic receptor. Studies with hCG glycopeptides indicated that carbohydrate, especially that obtained from hCGα, could inhibit hCG-stimulated adenyl cyclase activation at doses that had no effect on 125I-hCG binding. Indeed, the amount of G-protein associated with the LH receptor was greater when cells were treated with intact hCG than when treated with chemically deglycosylated hCG. If ligand-receptor self association represents the mechanism for gonadotropin receptor activation, then the secondary role for βAsn oligosaccharide in hCG biological activity might stem from its ability to participate in self aggregation. The partial compensation may result from hormone-receptor complexes oriented improperly that may need reorientation for functional activation.

O-glycosylation effects on gonadotropin activity
Deletion of the C-terminal extension from eLHβ and hCGβ by mild acid hydrolysis and by mutation of recombinant hCGβ had little effect on the in vitro biological activities of the eLH and recombinant hCG derivatives. This led to the conclusion that the C-terminal extension only affected these hormones in vivo. Enhanced in vivo potency following introduction of the CTP into both LH and FSH has been experimentally confirmed. However, truncation of the hCGβ C-terminus by site-directed mutagenesis revealed its influence on N-linked oligosaccharide processing, thereby extending the list of functional activities associated with this structural element. Recent studies involving eCG isoforms revealed that the high degree of O-glycosylation associated with eCG-H inhibited LH and FSH receptor-binding activity. Because reduced receptor-binding activity was not compensated by enhanced ability to stimulate cellular activity, reduced LH steroidogenic
potency was also observed (Butnev and Bousfield, unpublished). Removal of residues 121-149 by mild acid hydrolysis of Asp$^{120}$–Pro$^{121}$ virtually eliminated the difference in receptor-binding activity between eLHβ and eCGβ hybrids sharing a common α subunit, despite the fact that the mass of eLHβ was 1053 mass units less than that of eCGβ. This result was obtained with all three equine α subunit preparations. Recombinant eLH/CG mutated to eliminate the αAsn$^{120}$ glycosylation site was reported to lose LH biological activity, but not FSH biological activity suggesting that O-glycosylation can selectively affect the latter.$^{10}$

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