Site-directed Mutagenesis Defines the Individual Roles of the Glycosylation Sites on Follicle-stimulating Hormone

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To determine the specific role of each follicle-stimulating hormone (FSH) oligosaccharide, we mutated Asn to Gln at each glycosylation site (aGln52, aGln75, aGln62-75, bGln1, bGln4, and bGln3-24) to selectively inhibit oligosaccharide attachment. For wild-type and mutant FSH, we determined the binding affinity to homogenized rat Sertoli cells and the signal-transducing activity in cultured rat granulosa cells. The binding affinity of FSH lacking any one of the oligosaccharides was increased over wild-type FSH, while the signal-transducing activity of FSH lacking the oligosaccharide at aAsn2 (aGln2 FSH) was markedly reduced, and that of FSH lacking either β oligosaccharide (bGln1 and bGln4 FSH) was slightly reduced. At each FSHβ glycosylation site, we made a second amino acid substitution to inhibit glycosylation (bTyr2 and bTyr21) and an amino acid substitution that preserved glycosylation (bSer2 and bSer21). The amino acid sequence of the second β subunit glycosylation site was important for signal transduction, regardless of the presence or absence of the oligosaccharide. Thus, while each FSH oligosaccharide has a similar impact on binding affinity, the aS2 oligosaccharide has a disproportionate role in signal transduction, and the amino acid sequence at βAsn24 functions in both binding and signal transduction.

FSH, luteinizing hormone, thyroid-stimulating hormone, and CG, make up a family of dimeric glycoprotein hormones with a common α subunit and a β subunit that confers biologic specificity. The α subunit has two N-linked oligosaccharides, and the β subunit has either one (in the case of luteinizing hormone and thyroid-stimulating hormone) or two (in the case of CG and FSH) N-linked oligosaccharides (1-3). Removal of these oligosaccharides by enzymatic or chemical methods decreases adenyl cyclase-stimulating activity, suggesting that in general, the oligosaccharides are required for efficient signal transduction (4-11). Although removal of all four FSH oligosaccharides reduces biologic activity, the role of the individual oligosaccharides remains to be elucidated (12,13). Site-directed mutagenesis can be used to examine the role of individual glycosylation sites by selectively inhibiting oligosaccharide attachment at each site. Using this approach, Matzuk and coworkers (14) identified the oligosaccharide at position 52 on the α subunit as having a greater role in CG activity than the other oligosaccharides. To our knowledge, this type of detailed analysis has not been previously reported for FSH.

Although site-directed mutagenesis has advantages over chemical deglycosylation, it has some limitations that have not been fully appreciated. The first is that deductions made about mutant hormones have been based on immunologic assessments of their concentration. Although this can provide some useful information, it assumes that the epitope(s) involved in immunoreactivity have not been altered by mutagenesis. A more straightforward, non-immunologic approach would be to determine the binding activity in wild-type or mutant media and then measure the steroidogenic activity of equivalent amounts of receptor binding activity (i.e., shifts in potency reflect differences in signal-transducing activity). The second assumption is that differences in mutant hormone activity are due to inhibition of glycosylation rather than to changing the amino acid sequence. Some investigators have used two separate amino acid substitutions to prevent glycosylation at a single site (14), but important additional information could be gained from mutating the amino acid sequence of the region without inhibiting glycosylation.

In the present study, we used site-directed mutagenesis to examine the role of each glycosylation site on FSH. We substituted Gln for Asn at each glycosylation site to inhibit oligosaccharide attachment. We examined the immunologic activity, apparent receptor affinity, and signal-transducing activity of the mutant FSH analogues. At the FSHβ glycosylation sites, we inhibited glycosylation by a second mutation (Thr to Tyr) and included a third amino acid substitution at each site (Thr to Ser) that would not inhibit glycosylation, to further delineate the effects of changing the amino acid sequence from those of inhibiting glycosylation.

EXPERIMENTAL PROCEDURES

Materials—Enzymes for cloning, sequencing, and polymerase chain reaction mutagenesis were obtained from Bethesda Research Laboratories and Perkin-Elmer. The expression vectors, pAV2 and pAV2-α, were gifts of F. Wondisford (NIDDK, NIH). Tissue culture reagents were obtained from Life Technologies, Inc., Biofluids, and Sigma. IRMA kits were obtained from Serono Diagnostics. Human pituitary FSH AFPS417T for radioimmunoassay was obtained from the National Hormone Pituitary Program. Human pituitary FSH F0614 for iodination was obtained from The Scripps Institute.

Mutagenesis and Transfection—Mutagenesis was performed by overlap extension in the polymerase chain reaction using sense and antisense mutant oligonucleotide primers and an α subunit cDNA template or a FSHβ cDNA template constructed by overlap extension from a FSHβ genomic clone (gift of Larry Jameson, MGH, Boston, MA). Polymerase chain reaction products were sequenced to confirm the presence of the intended mutation and the absence of any unintended Tag polymerase mutations. Wild-type and mutant FSHβ cDNAs were cloned into pAV2 and co-transfected with pAV2 containing the wild-type α subunit cDNA using calcium phosphate precipitation into 293 human embryonal kidney cells (gift of Barrie Carter, NIADDK, NIH). Since 293 cells were found to secrete an α subunit that cross-reacted in our α subunit radioimmunoassays, mutant α subunit cDNAs were cloned into
Radioimmunoassay—Employed a rabbit anti-ovine FSH polyclonal antiserum designated H-31, and the second radioimmunoassay used the rat testis FSH radio receptor assay to determine the binding activities of wild-type and mutant media by comparing the slopes of the dose-response curves of wild-type FSH compared with wild-type FSH (Fig. 1). Small changes in the amount of type or type of oligosaccharide present on β-subunit glycosylation sites are not detected by G-75 chromatography. Thus, the progression shift to the right of the G-75 Sephadex elution profiles of βGlu72, βGlu74, and βGlu72-24 FSH (Fig. 1) is consistent with the lack of one or both subunit oligosaccharides secondary to mutations in their respective glycosylation sites. In addition to the Glu mutations, at each site individually, and both sites in tandem, we mutated Asn to Gln (aGln7, αGln24, and aGln7-24) to prevent attachment of the oligosaccharide at one or both glycosylation sites. The oligosaccharides make up approximately 30% of the molecular weight of FSH, contributing substantially to its apparent molecular weight as determined by Sephadex chromatography. Thus, the progressive shift to the right of the G-75 Sephadex elution profiles of βGlu72, βGlu74, and βGlu72-24 FSH (Fig. 1) is consistent with the lack of one or both subunit oligosaccharides secondary to mutations in their respective glycosylation sites. In addition to the Glu mutations, at each β subunit glycosylation site, we mutated Thr to either Tyr (βTyr5 and βTyr26) or Ser (βSer5 and βSer26). The Tyr substitution provides an additional amino acid substitution that prevents oligosaccharide attachment, while the Ser substitution changes the amino acid sequence but maintains a functional glycosylation site. We have previously shown, using radiolabeled glucosamine, that the substitution of Ser for Thr at the analogous site on β thyroid-stimulating hormone does not impair glycosylation (20). Consistent with the presence of a functional glycosylation site, we observed no shift in the G-75 Sephadex elution profile of βSer26 FSH compared with wild-type FSH (Fig. 2). Small changes in the amount of type or type of oligosaccharide present on βSer26 FSH, however, would not be detected by G-75 chromatography.

This information is relevant for understanding the glycosylation sites of FSHβ and their impact on the biological activity of the hormone.
Increased binding affinity of chemically deglycosylated hCG. The dose-response of βTyr\(^{26}\) FSH was indistinguishable from that of pituitary FSH standard in three different immunoassay systems. In contrast, βTyr\(^{26}\) FSH displayed non-parallel immunologic activity in the FSH radioimmunoassay employing polyclonal antiserum DDBB1001 (Fig. 3), indicating a distinct change in the conformation of this FSH analogue. This change can be attributed to the amino acid substitution at this site, rather than the absence of the oligosaccharide per se, since the Gln substitution, which also inhibited glycosylation, did not result in a change in FSH conformation that could be detected by our immunoassay systems (Fig. 3). The finding of a conformational change for βTyr\(^{26}\) FSH, when none of the other mutations produced a detectable change in conformation, suggests that this amino acid is particularly important for the tertiary structure of FSH. The proximity of amino acid 26 to the potential cysteine loop between residues 28 and 52 and to the large Trp at position 27 may make this site vulnerable to conformational changes due to substitution of a bulky Tyr for the smaller Thr. In addition, this region has been identified as a site of interaction between the α and β subunits (21), which may contribute to the conformational changes in the resulting FSH dimer.

Receptor Binding Affinity of the FSH Glycosylation Mutants—The dose-response curves of αGln\(^{24}\), αGln\(^{78}\), and αGln\(^{24-78}\) (Fig. 4A), and βGln\(^{7}, \) βGln\(^{24}, \) and βGln\(^{7-24}\) FSH (Fig. 4B) were shifted to the left of that for wild-type FSH in the radio receptor assay, suggesting that inhibition of oligosaccharide attachment at either or both glycosylation sites on either subunit enhances hormone binding affinity. Indeed, the apparent binding affinities of all the non-glycosylated FSH analogues, except βTyr\(^{26}\) FSH, were greater than that of wild-type FSH (Table 1). Previous chemical deglycosylation studies of the glycoprotein hormones indicate that in general, deglycosylation does not interfere with receptor binding but rather enhances binding affinity (6-13). These studies are limited, however, by the fact that chemical deglycosylation can only remove about 80% of the oligosaccharide. In contrast, site-directed mutagenesis can completely inhibit oligosaccharide attachment at one or more sites on the hormone. A previous site-directed mutagenesis study of CG indicated no change in the binding affinity of non-glycosylated hormone (14), suggesting that the increased binding affinity of chemically deglycosylated hCG may have been an artifact of the deglycosylation procedure. In contrast, our findings for FSH indicate that lack of the oligosaccharides enhances binding affinity. Furthermore, our findings extend this principle to suggest that not only is binding affinity enhanced by removal of all the oligosaccharides, but it

![Diagram](https://example.com/diagram.png)

**Fig. 3.** Dose-response curves of wild-type FSH, βGln\(^{24}\) FSH, and βTyr\(^{26}\) FSH media in a radioimmunoassay using a polyclonal FSH antiserum designated DDBB1001. The dose-response of βTyr\(^{26}\) FSH was not parallel to that of wild-type FSH (p < 0.01). The dose-response of βGln\(^{24}\) FSH, which also lacked the oligosaccharide at Asn\(^{14}\), was indistinguishable from that of wild-type FSH.

**Fig. 4.** Dose-response curves of media containing wild-type FSH or FSH composed of mutant α (A) or β (B) subunit in a radio receptor assay using rat testes homogenate and radiolabeled human pituitary FSH. The dose is the immunologic activity observed in the two-site IRMA in terms of human pituitary FSH standard (AFP8417B).

**Table 1**

| Glycosylation status | Relative binding affinity (mean (95% C.I.)) |
|----------------------|------------------------------------------|
| Wild type            | 1.00                                      |
| Mutations of the α subunit glycosylation sites | |
| αGln\(^{24}\)        | 2.31 (1.29-3.89)                            |
| αGln\(^{78}\)        | 2.07 (1.85-2.63)                            |
| αGln\(^{24-78}\)     | 2.57 (1.95-2.82)                            |
| Mutations of the first β subunit glycosylation site | |
| βGln\(^{7}\)         | 2.49 (1.29-3.89)                            |
| βSer\(^{26}\)        | 1.40 (0.99-2.11)                            |
| βTyr\(^{26}\)        | 2.68 (1.50-4.08)                            |
| Mutations of the second β subunit glycosylation site | |
| βGln\(^{24}\)        | 1.87 (1.11-2.91)                            |
| βSer\(^{26}\)        | 2.98 (1.33-10.51)                           |
| βTyr\(^{26}\)        | 0.16 (0.15-0.64)                            |
| Mutations of both the first and second β subunit glycosylation sites | |
| βGln\(^{24}\)        | 2.76 (1.28-5.00)                            |

\(^2\) Number of assays.
\(^3\) C.I., confidence intervals.
\(^4\) p < 0.01, different from wild type.
occurs with FSH lacking just one oligosaccharide. The reason for the differences in our findings compared with those for hCG may relate to differences in protein conformation or to differences in the terminal oligosaccharides of FSH made in 293 and cos7 cells compared with hCG made in Chinese hamster ovary cells.

At the first FSHβ glycosylation site (Asn21), mutations that inhibit glycosylation (pGln21 and pTyr21) enhanced binding affinity, while a mutation that preserves glycosylation (pSer21) had no effect (Fig. 5A). Thus, increases in binding affinity due to mutations of the first β subunit glycosylation site appear to be related to the absence of the oligosaccharide rather than the amino acid substitution per se.

At the second FSHβ glycosylation site (Asn32), however, both the Gln substitution (pGln32) that inhibits glycosylation and the Ser substitution (pSer32) that preserves glycosylation enhanced binding affinity (Fig. 5B). Thus, increases in binding affinity due to mutations of the second β subunit glycosylation site can be related to changes in the amino acid sequence without inhibition of glycosylation. In contrast to the findings for the other mutants, substitution of Tyr for Thr at position 26 (pTyr26) dramatically reduced binding affinity (Fig. 5B). Apparently, the conformational change induced by this amino acid substitution affects not only its interaction with certain antibodies but also its interaction with the FSH receptor. Amino acid substitutions at position 26 could affect the nearby cysteine loop between residues 32 and 51 that has been identified by previous peptide studies as a receptor binding region (22, 23). It is also possible, however, that the conformational change induced by this amino acid substitution altered more distant receptor binding sites (23, 24).

**Signal-transducing Activity of the FSH Glycosylation Mutants—** Determination of signal-transducing activity does not require accurate immunologic assessments of hormone concentration but involves the straightforward use of a radio receptor assay to determine FSH binding activity in wild-type and mutant media. When equivalent amounts of FSH binding activity were determined in the rat testis radio receptor assay in terms of pituitary FSH standard (AFP817B), the binding activities were dose tracked in the rat granulosa cell bioassay. The ED₅₀, used to determine potency, and the Rₐ, used to determine intrinsic activity, were determined from full dose-response curves in at least two granulosa cell bioassays using the computer program Allfit.

**TABLE II**

| Glycosylation site | Relative signal-transducing activity |
|-------------------|--------------------------------------|
| Potency | Intrinsic activity |
| Mean (95% C.I.) | Mean ± S.D. |
| Wild type | 1.00 | 1.00 |
| Mutations of the α subunit glycosylation sites | | |
| αGln52 | 0.02 (0.02–0.03) | 0.24 ± 0.10 |
| αGln78 | 1.13 (0.86–1.47) | 0.86 ± 0.25 |
| αGln78–79 | <0.05<0.05 |
| Mutations of the first β subunit glycosylation site | | |
| βGln24 | 0.48 (0.30–0.78) | 0.98 ± 0.16 |
| βSer26 | 1.04 (0.81–1.32) | 1.74 ± 0.06 |
| βTyr26 | 0.40 (0.29–0.55) | 0.76 ± 0.08 |
| Mutations of the second β subunit glycosylation site | | |
| βGln32 | 0.41 (0.28–0.60) | 0.97 ± 0.12 |
| βSer32 | 0.20 (0.08–0.35) | 0.73 ± 0.16 |
| βTyr26 | 0.35 (0.16–0.77) | 0.52 ± 0.06 |
| Mutations of both the first and second glycosylation sites | | |
| βGln32 | 0.38 (0.23–0.62) | 0.71 ± 0.22 |

* Number of assays.
* C.I.: confidence intervals.
* p < 0.01, different from wild type.
binding specificity, while the between the glycoprotein hormones and appears to determine these tinct from this similar between glycoprotein hormones and is critical for their recent three-dimensional models of CG and FSH that place oligosaccharide is an additional site of receptor interaction dis-
signal-transducing activity.

Thus, if there are differences in the signal-transducing activity of both hormones. In fact, the reduction in the signal-transducing potency of aGln52 FSH was 10-fold greater than that of CG lacking the α52 oligosaccharide (14). Therefore, the oligosaccharide at position 52 appears to be critical for signal transduction of both hormones. In fact, the reduction in the signal-transducing potency of αGln52 FSH was 10-fold greater than that of CG lacking the α52 oligosaccharide (14). Thus, if there are differences in the α subunit conformation of FSH compared with CG, they appear to accentuate the need for the α52 oligosaccharide.

Recent cassette mutagenesis studies have indicated that a discrete amino acid region in the β subunits of FSH (β87–94) and CG (β93–100) determines the specificity of their interaction with their respective receptors (25). Our findings for FSH, as well as the previous findings for CG, suggest that the α52 oligosaccharide is an additional site of receptor interaction distinct from this β subunit site. This would be consistent with recent three-dimensional models of CG and FSH that place these α and β subunit regions at distinct sites on the same surface of the molecule (26, 27). The β subunit site varies between the glycoprotein hormones and appears to determine binding specificity, while the α subunit site of interaction is similar between glycoprotein hormones and is critical for their signal-transducing activity.

Signal-transducing Activity of FSH Containing Mutant β Subunit—The dose-response curves of the signal-transducing activity of βGln7, βGln24, and βGln7–24 FSHβ were shifted to the right of wild-type FSH in the rat granulosa cell bioassay (Fig. 7, A–C). Although there was no difference in the maximum response of βGln7, βGln24, and βGln7–24 FSH, there was a 2-fold decrease in the their signal-transducing potency (Table II). Thus, the oligosaccharides on the β subunit of FSH appear to influence FSH signal transduction. These data help resolve some of the controversy regarding the relative role of the α and β subunit oligosaccharides in FSH activity. Although some studies have indicated that the α subunit oligosaccharides are essential for FSH activity (13), other studies, using chemically deglycosylated α subunit, have indicated that only the β subunit oligosaccharides are required for signal transduction (12).

It has been difficult to examine the role of the β subunit oligosaccharides directly, due to the limited quantities of purified FSHβ available for chemical deglycosylation. Using recombinant technology, however, we have circumvented this problem and directly examined the properties of FSH lacking one or both β subunit oligosaccharides. Our findings suggest that in contrast to the site-directed mutagenesis findings for CG, indicating no role for the β subunit oligosaccharides, the FSHβ oligosaccharides have a role in signal transduction, albeit a much smaller role than the α52 oligosaccharide. Differences in the conformation of FSH, compared with CG, may require the presence of the β subunit oligosaccharides for the most efficient FSH signal transduction.

The above changes in signal-transducing activity observed for the FSHβ mutants can be attributed either to the lack of the oligosaccharide or to the amino acid substitution used to inhibit glycosylation. At the first FSHβ glycosylation site, amino acid substitutions that inhibit glycosylation (βSerβ) decreased signal-transducing activity, while a mutation that preserves glycosylation (βSerβ) had signal-transducing activity equivalent to wild-type FSH (Fig. 7, A, D, and F) (Table II). This suggests that at the first FSHβ glycosylation site, the
oligosaccharide is the crucial feature for efficient signal transduction.

In contrast, at the second FSHβ glycosylation site, a mutation that preserves glycosylation (βSer<sup>28</sup>) had a marked reduction in signal-transducing activity (Fig. 7E), suggesting that the amino acid sequence influences efficient signal transduction independent of the glycosylation status. These findings have important implications for site-directed mutagenesis studies, because they suggest that in some cases, changes in hormone activity that have been attributed to the lack of the oligosaccharide may actually be due to the amino acid substitutions employed to inhibit glycosylation. We cannot entirely rule out the possibility, however, that the Ser substitution resulted in incomplete or aberrant glycosylation at Asn<sup>24</sup> that may account for the observed changes in signal-transducing activity.

Substitution of Tyr for Thr at position 26 (βTyr<sup>26</sup>) caused a dramatic decrease in signal-transducing activity (Fig. 7G), affecting not only the potency but also the R<sub>max</sub> of signal transduction (Table I). By definition, this reduction occurs at equivalent levels of receptor binding activity; thus, the Tyr<sup>26</sup> substitution not only decreased the binding affinity of FSH for its receptor, it also reduced the efficiency of FSH signal transduction by the hormone-receptor complex. Apparently, the amino acid residue at position 26 is critical for FSH to achieve a conformation compatible with both tight receptor binding and efficient signal transduction. Although it is possible that substitutions at residue 26 could impact on distant FSH regions, it is of interest that nearby residues 34–37 (Thr-Arg-Asp-Leu) have been identified by peptide studies as having a direct role in FSH action (26). Conformational changes brought about by insertion of a Tyr at position 26 might limit the accessibility of this critical region. Thus, our findings indicate that the amino acid sequence near the second FSHβ glycosylation site represents a third region of receptor interaction, in addition to βTyr<sup>7</sup>–94 and α52. This region appears to be critical for both binding and signal transduction.

With the exception of βTyr<sup>26</sup> FSH above, our findings generally agree with previous deglycosylation studies of the glycoprotein hormones, indicating that alterations that enhance binding affinity also decrease signal transduction (5–13, 28, 29). This inverse relationship between binding affinity and signal transduction accounts for the fact that deglycosylated glycoprotein hormones can act as potent antagonists of native hormone (5–11, 28, 29). A model that could explain these findings would be that the oligosaccharides either directly, by lectin-like binding to the receptor (28), or indirectly, by effects on protein conformation (29), promote a hormone-receptor complex with enhanced signal transduction at the expense of some binding energy. This model is supported by epitope-mapping studies indicating a different conformation for deglycosylated hormone-receptor complex than for native hormone-receptor complex (30) and by studies indicating that conformational changes associated with efficient signal transduction reduce binding affinity (28, 29). Our findings suggest that this inverse relationship is not a strict one, however, so that while all the FSH oligosaccharides impact equally on binding affinity, the α52 oligosaccharide has a disproportionate role in signal transduction. This supports a model of FSH action, suggested by previous peptide studies (24), in which there are multiple sites of hormone-receptor interaction; some are involved in binding and others in signal transduction. The site(s) related to binding affinity are equally affected by each of the FSH oligosaccharides, but the site(s) of signal transduction are concentrated at the α52 oligosaccharide. Furthermore, there may be additional sites, such as βThr<sup>26</sup>, that are important for both binding and signal transduction.

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