Comparative Glycomics of the Glycoprotein Follicle Stimulating Hormone: Glycopeptide Analysis of Isolates from Two Mammalian Species†

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ABSTRACT: Follicle stimulating hormone (FSH) is one of the important hormones that regulate gonadal functions. This hormone is glycosylated, and the glycans greatly influence the biological properties. In the present study the negatively charged glycopeptides of equine and human pituitary follicle stimulating hormone (eFSH and hFSH) have been characterized in a glycosylation site-specific manner using FT-ICR-MS and Edman sequencing. The characteristic pattern of glycan distribution at each glycosylation site has been deduced and compared between horse and human FSH preparations. The data suggest that site-specific differences exist between glycoforms of human and equine FSH. For instance, except for one site in the β subunit (Asn^{7}) of hFSH all other sites in both species have sulfated glycoforms. Also, glycoforms at Asn^{52} of hFSH are all complex type, whereas in eFSH, both complex and hybrid structures exist at this site. There is also a higher percentage of sulfated glycans in the latter site compared to the former. This is the first study that characterizes the glycans from this hormone in a glycosylation site-specific manner, and these data can be used to begin correlative studies between glycosylation structure and hormone function.

Follicle stimulating hormone (FSH) is a heterodimeric glycoprotein consisting of two noncovalently linked α and β subunits found in the pituitary of all mammals (*1*, *2*). It is one of the hormones that regulate reproductive functions of the body. Specifically, it is responsible for stimulating follicle maturation in females and for supporting spermatogenesis in males (*3*, *4*). While the amino acid sequence of this glycoprotein is highly homologous between species (*5*), the glycan structures differ significantly not only between species (*5*) but also between the glycosylation sites for any given species (*6*). It is known that the glycans greatly influence the biological properties of this hormone (*6*). For example, signal transduction (*7*), receptor binding (*8*), and half-life in the circulation (*9*) are all controlled by glycans, and the final biological response of the target cell [granulosa and Sertoli cells (*10*)] in vivo is determined by these factors (*11*). Previous studies focused on a single critical glycosylation site in the common α subunit have been unable to account for differences in biological activity (*1*, *2*) due to compensatory effects of β subunit glycans (*3*). Hence, to relate their biological functions to their structures, it is essential to determine the variability of the glycans in a glycosylation site-specific manner. The knowledge gained from this approach can be applied in the development and production of analogues that can be used to regulate gonadal functions.

FSH initiates its action by binding to G protein-coupled receptors, which are on the surface of the target cells (*3*, *12*). This binding causes adenylate cyclase activation, resulting in hormonal responses. Previous studies have reported that this process is greatly affected by glycans at particular sites. For instance, when site-directed mutagenesis was performed on Asn^{52} of hFSH[1] to selectively eliminate the glycan at this position, it resulted in either increased (*13*, *14*) or unaltered (*15*) receptor binding activity with a reduction of receptor activation and signal transduction. Similar results have also been observed for eFSH when deglycosylation was performed at the α Asn^{56} site using either PNGase digestion (*11*) or site-directed mutagenesis (*16*). It is still unclear about which glycans are responsible for this function even though there is a clear indication that Asn^{52}/Asn^{56} glycans play an essential role.

Studies done on recombinant human variants of hFSH reveal that glycans from the β subunit play a more important role than those from the α subunit in metabolic clearance/half-life circulation (*9*). It is evident that the glycans mediate different functions at different glycosylation sites. Therefore,

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[1] Abbreviations: eFSH, equine follicle stimulating hormone; hFSH, human follicle stimulating hormone; LH, luteinizing hormone; PNGase, peptide:*N*-glycosidase; Asn, aspargine; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; LTQ-FTICR-MS, linear ion trap-Fourier transform ion cyclotron resonance mass spectrometry; MS, mass spectrometry; ESI, electrospray ionization; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; MeOH, methanol; VBA, visual basic algorithm; PhNCS, phenyl isothiocyanate; PLC, phospholipase C; HexNAc, *N*-acetylhexosamine.

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to accurately study glycan structure and relate it back to its functional significance, one needs to characterize glycans in a glycosylation site-specific manner.

The glycans from the pituitary and recombinant FSH have been previously characterized by various methods. Conventional approaches employed for this type of analysis involve cleaving the glycans enzymatically from the peptide backbone (*2*, *17*, *18*−*21*) followed by a combination of techniques such as gel filtration, high-performance liquid chromatography (HPLC), and lectin affinity chromatography with endo- and exoglycosidase digestion (*2*, *17*). While these techniques are clearly useful, purifying carbohydrates by chromatographic methods is quite a daunting task. ^{1}H nuclear magnetic resonance (NMR) has also been used in this type of analysis; however, this analysis also requires purified samples (and large sample quantities) (*19*). Despite these limitations, several abundant glycoprotein hormones have been characterized in a site-specific manner. Alternatively, mass spectrometry has the advantage of small sample requirements and the ability to profile complex mixtures of glycans. But unlike the other methods, MS does not provide structural information for isomeric structures (*20*, *21*). In addition to all pros and cons already discussed, most conventional methods of glycosylation analysis suffer from the fact that no glycosylation site-specific information is obtained, because the carbohydrates are cleaved from the protein prior to analysis.

To obtain glycosylation information in a site-specific fashion, glycoproteins can be subjected to proteolysis, followed by MS analysis of glycopeptides (*22*−*25*). We implement this method herein to characterize FSH glycopeptides in two species (human and equine). A nonspecific protease is used for proteolysis to produce small glycopeptide fragments, and a combination of Edman sequencing and mass spectrometry is used to analyze the digestion products. The information obtained from this method is used to compare the glycans at different glycosylation sites of human and equine FSH preparations.

EXPERIMENTAL PROCEDURES

The horse pituitary glycoprotein hormone preparation, eFSH, was prepared as previously described (*26*). Human FSH (AFP-4161B) was obtained from the NIDDK National Hormone and Pituitary Program and Dr. A. F. Parlow (*27*, *28*).

Protease Digestion. Glycoprotein hormone preparations, which typically contain approximately 2−5 mg of protein, were reduced with dithiothreitol and alkylated with iodoacetic acid as described previously (*26*). The reaction mixtures were desalted using 4 mL Amicon (Millipore Corp., Bedford, MA) Ultra-4 centrifugal ultrafiltration cartridges (MW cutoff 10000) in a Sorvall (Kendro Laboratory Products, Newtown, CT) RC-3B Plus centrifuge (5000 rpm, LA/S-400 rotor). After being washed three times with 0.2 M ammonium bicarbonate, pH 8.5, the protein solutions were transferred to 2 mL polypropylene screw cap tubes, and 10% (w/w) proteinase K (Boerhinger Mannheim, Indianapolis, IN) was added. The tubes were incubated overnight at 37 °C with continuous shaking. The next day, samples were dried in a Thermo Savant (Marietta, OH) Speed Vac centrifugal evaporator.

Superdex Gel Filtration. A 10/30 Amersham Biosciences (Piscataway, NJ) Superdex peptide column was connected to a Waters (Milford, MA) model 600 HPLC system equipped with a model 484 variable wavelength detector controlled by Empower Pro (build 1154) control/acquisition software. The column was equilibrated with 0.2 M ammonium bicarbonate buffer at a flow rate of 0.4 mL/min. Glycopeptide samples (typically 1−2.5 mg) were dissolved in 200 μL of 30% acetonitrile in 0.2 M ammonium bicarbonate and injected into the HPLC. The chromatogram was developed at a flow rate of 0.4 mL/min. Fractions were collected by hand in 12 × 75 mm polypropylene tubes and dried in the Speed Vac. The dried residues were dissolved in 500−1000 μL of water; 25 μL aliquots were removed and dried in 2 mL screw cap polypropylene tubes. Dried samples were hydrolyzed in 200 μL of 4 N trifluoroacetic acid at 100 °C for 4 h. TFA was removed by evaporation, and the hydrolysates were dissolved in 100 μL of water containing deoxyglucose. Monosaccharide composition was determined using a Dionex (Sunnyvale, CA) carbohydrate analyzer consisting of a model GP50 gradient pump and LC20 chromatography enclosure equipped with a 4 × 50 mm BorateTrap column, a 4 × 50 mm AminoTrap column, which functioned as a guard column, a 4 × 250 mm CarboPak PA-10 column, and a model ED50 electrochemical detector, controlled by Chromeleon control/acquisition software. A Waters model 717 refrigerated autosampler maintained the hydrolysates at 4 °C and injected 20 μL samples from each vial. Carbohydrate-positive samples were subsequently analyzed in duplicate. The fraction containing the largest amount of carbohydrates (90% of the glycopeptides in eFSH and 75% of the glycopeptides in hFSH) was subjected to mass spectral analysis.

Peptide Sequencing. Automated Edman degradation was carried out using an Applied Biosystems model 492 Procise sequencer (*24*). Samples were applied to glass fiber membranes coated with Biobrene that had been precycled in the sequencer. Typical sequencer experiments consisted of seven Edman degradation cycles, sufficient to sequence the entire length of three to five residue peptides that were the most common products of proteinase K digestion.

Mass Spectrometry. MS data were acquired on a high-resolution Thermo Finnigan linear ion trap−Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FTICR-MS) (San Jose, CA) equipped with a 7 T actively shielded magnet. The dried glycopeptides were first dissolved in water and diluted with MeOH−H_2O (4:1) containing 0.3% acetic acid, to a final concentration of 0.03 μg/μL. Samples were directly infused into the mass spectrometer using a syringe pump at a flow rate of 5 μL/min. High-resolution data were acquired by maintaining resolution at 50000, for *m*/*z* 400. The instrument was externally calibrated over the mass range of interest (*m*/*z* 800−2000) prior to the analysis. ESI spray voltage was maintained between −3.0 and −4.0 kV for data acquisition in the negative mode. Capillary temperature was held between 200 and 230 °C, and N_2 was used as a nebulizing gas at 20 psi. Data were acquired and processed using Xcalibur 1.4 SR1 software (Thermo Finnigan, San Jose, CA).

Glycopeptide Composition Assignment. The glycopeptide compositions were assigned for the peaks in the mass spectrum using the aid of two computer algorithms written

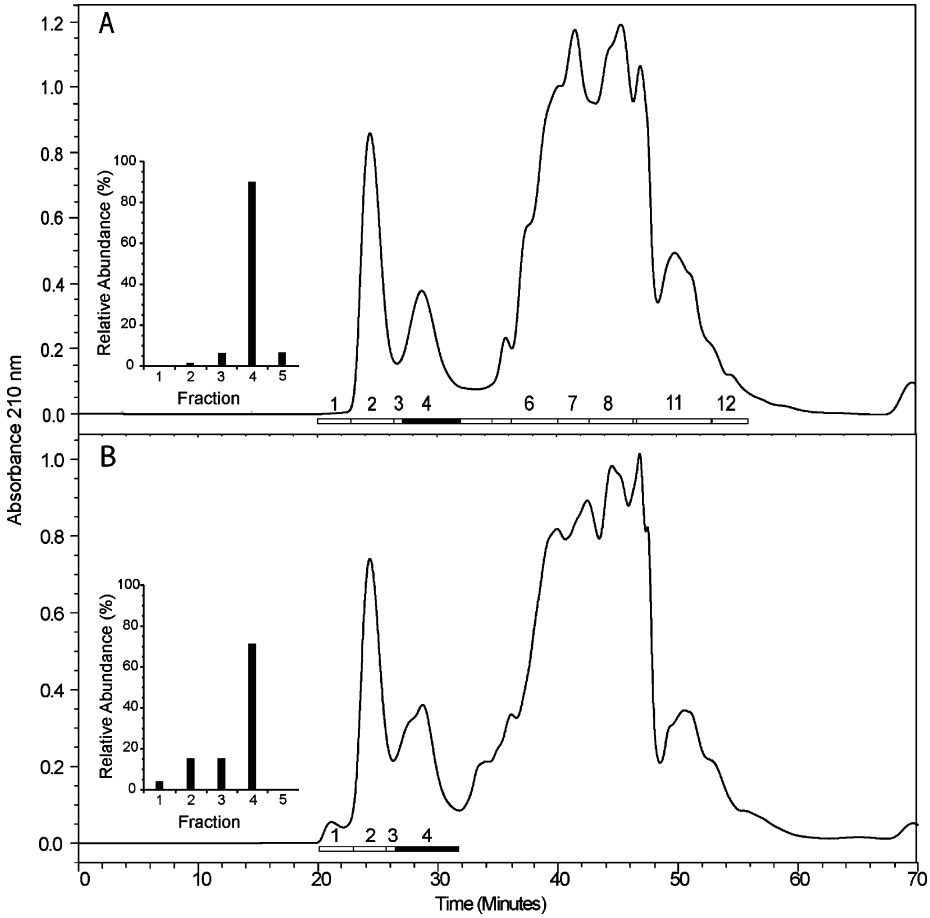


FIGURE 1: Fractionation of 200 μL samples containing 2500 μg of (A) eFSH or (B) hFSH proteinase K digest on a Superdex peptide column (absorbance at 210 nm) equilibrated with 0.2 M NH_4HCO_3 at a flow rate of 0.4 mL/min. The protease peak elutes first, followed by the glycopeptide peak and then the peptide peaks. Inset: The solid bars represent monosaccharides quantified from each of the different fractions, collected manually in 12 × 75 mm tubes. For each fraction, carbohydrate content was determined by hydrolyzing any glycopeptides present with TFA to release monosaccharides. These were quantified against an internal standard, deoxyglucose, using a Dionex HPAEC system, as described in Experimental Procedures. Quantitative results from five fractions of the dozen collected are shown here, because carbohydrate composition analysis indicated that glycans were present only in the first four to five fractions.

in VBA and Perl. The VBA algorithm was described previously (*24*). The Perl program follows a mass fingerprinting algorithm that matches theoretical and experimental masses at a given tolerance. Whenever possible, MS/MS analysis was performed on the glycopeptides to verify the peak assignments. This provided supporting information but could not be used to unequivacably assign branching.

RESULTS

The approach utilized for characterizing the glycosylation in a site-specific fashion is as follows. The hormones were digested with a nonspecific serine protease, proteinase K. Typically, smaller peptides are generated from the proteinase K digestion than from a tryptic digest. (Pronase, another nonspecific enzyme, was also tested in preliminary experiments, but it produced less optimal signal.) The small peptides produced by proteinase K facilitate separation of nonglycosylated peptides from glycopeptides by gel filtration. Other strategies, including reverse-phase HPLC and separations using porous graphite, were also investigated but showed less optimal results. Reverse-phase HPLC irreversibly adsorbed eFSH glycopeptides. Adsorption to Carbograph graphitized charcoal cartridges removed proteinase K but not the small peptides. Some glycopeptides were lost during the Carbograph adsorption process, and resulting mass spectrometry signals were weak, perhaps due to residual TFA. Gel filtration of the total digest removed both protease and small peptides.

An example of the gel filtration fractionation data appears in Figure 1. The eFSH and hFSH proteinase K digest chromatograms included a large protease peak followed by a small peak that was eluted before the peptide peaks. As low-UV absorbance is characteristic of glycopeptides, we surveyed hydrolyzed samples of all the column fractions for carbohydrate and found it was largely restricted to the putative glycopeptide peak (Figure 1, solid bar). Most of the eFSH carbohydrate was restricted to a single fraction (Figure 1A). However, 13% of the hFSH carbohydrate coeluted with the protease fraction, 13% emerged in the next fraction, and 67% was recovered from the 27−33 min fraction (hFSH glycopeptide yield is increased to 90% by altering collection times to 25−31 min) (Figure 1B). This was consistent with the larger size of hFSH oligosaccharides as compared with those derived from eFSH. The size fractionation strategy enhances the mass spectral signal of the glycopeptides by removing the nonglycosylated peptide interference.

Subsequently, the glycopeptide fractions were subjected to both Edman sequencing and FT-ICR-MS for analysis. Edman data are useful for identifying the amino terminus and predicting the peptide sequence for each of the glyco-

a) **Cycle 1 -** K (185pmole) ; I (182pmole)
Cycle 2 - V (46pmole) ; H (127pmole) ; I (65pmole)
Cycle 3 - T (318pmole) ; V (182pmole)
Cycle 4 - T (234pmole)

Possible sequences in hFSH

KNVT, NVT, NHT, NIT, INTT

b) **Cycle 1 -** L (361pmole)
Cycle 2 - E (310pmole) ; H (70pmole) ; I (477pmole)
Cycle 3 - T (388pmole)
Cycle 4 - H (176pmole) ; Q (63pmole)
Cycle 5 - T (207pmole)

Possible sequences from eFSH alpha subunit

LENHTQ, NHTQ, NIT

c) **Cycle 1 -** S (112pmole) ; I (171pmole) ; T (41pmole)
Cycle 2 - I (299pmole)
Cycle 3 - I (40pmole) ; T (266pmole)
Cycle 4 - T (281pmole)
Cycle 5 - T (152pmole)
Cycle 6 - T (66pmole)

Possible sequences from eFSH beta subunit

SINTT, INTT, TNIT, NIT

FIGURE 2: Quantitative output of Edman data from different cycles of hormone isolates: (a) hFSH; (b) eFSH α subunit; (c) eFSH β subunit. The absolute amount of aspargine (N) is absent in these cycles because glycosylated amino acid derivatives are not extracted from the Edman chemistry cartridge.

a) eFSH

Amino acid sequence of eFSH α subunit

FPDGEFTTQDCPECKLRENKYFFKLGVPIYQCKGCCFSRAYPTPARSRKTMLVPK
N ^{56}ITSESTCCVAKAFIRVTVMGNIK**LEN^{82}HTQ**CYCSTCYHHKI

Amino acid sequence of eFSH β subunit

NSCEL**TN^{7}IT**IAVEKEECGFCI**SIN^{24}TT**WCAGYCYTRDLVYKDPARPNIQKTCTFKELVY
ETVK VPGCAHHADSLYTYPVATACHCGKCNSDST DCTVRGLGPSYCSFGDMKE

b) hFSH

Amino acid sequence of hFSH α subunit

APDVQDCPECTLQENP FFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQK**N^{52}VT**SE
STCCVAKSYNRVTVMGGFKVE**N^{78}HT**ACHCSTCYYHKS

Amino acid sequence of hFSH β subunit

NSCELT**N^{7}IT**IAIEKEECRFCIS**IN^{24}TT**WCAGYCYTRDLVYKDPARPKIQKTCTFKELVY
ETVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE

FIGURE 3: Amino acid sequences for both (a) eFSH and (b) hFSH α and β subunits showing the glycosylation sites.

peptides released from the digest. Quantitative output from the Edman degradations is shown in Figure 2, and interpretation of Edman data is illustrated below. This study focuses on characterizing glycopeptides in a glycosylation site-specific manner, and special emphasis is placed on determining the unique properties of glycans that are present and absent from each site in human and equine FSH, respectively.

Peptide Compositions from Edman Sequencing. To interpret the Edman data for a mixture of glycopeptides, the protein sequence and the position of glycosylation sites must be known. The amino acid sequences of eFSH and hFSH in Figure 3 show that α subunit glycosylation takes place at Asn^{52} and Asn^{78} in human FSH (*29*) while the corresponding glycosylation sites in eFSH are Asn^{56} and Asn^{82}. Both hormones are glycosylated on the β subunit, at Asn^{7} and Asn^{24} (*29*). From this information, the peptide sequences from Edman data can be inferred.

The Edman data are interpreted by matching the sequences of amino acids near the known glycosylation sites of the protein (Figure 3) with the amino acid derivatives observed in the Edman sequencing experiments (Figure 2). For example, the glycopeptide KNVT is presumed to be present in the hFSH digestion, since >PhNCS-Lys is abundant in cycle 1, >PhNCS-Val is in cycle 3, and >PhNCS-Thr is present in cycle 4. No >PhNCS-Asn (N) was detected in cycle 2, because glycosylated amino acid derivatives are not extracted from the Edman chemistry cartridge by the adsorptive protocol employed in these studies. In addition to KNVT, several other glycopeptides are likely to be present in the glycopeptide mixture, based on the Edman data. All of the reasonable compositions obtained for hFSH are shown in Figure 2a.

Even though the Edman data identify a list of possible sequences, the mass spectral data cannot be used to distinguish among sequences that are isomeric. For instance, glycans that originate from αN^{56}IT and βN^{7}IT of eFSH cannot be distinctively identified. Therefore, there was a need to separate the α and the β subunits of eFSH to uniquely identify glycans present on these two glycopeptides. Panels b and c of Figure 2 show the Edman data for the separated subunits of eFSH α and β subunits.

As described in Experimental Procedures, Edman data were combined with the mass spectral data to obtain glycopeptide compositions for both equine and human FSH. Figures 4−6 represent the mass spectra obtained for both hormones in the negative ion mode. The peak list corresponding to the numbers on the spectra can be found in Supporting Information, Tables 1−3. More than 35 glycopeptides were identified for each of these two hormones. Pictorial description of the glycans can be found in Table 1. This is further illustrated in Figure 7, which provides a comparative description of the glycans present on each of the glycosylation sites in eFSH and hFSH. This study exclusively compares the anionic species found in FSH since it had been found previously that 90% of the total glycans in human FSH are sialylated or sulfated (*2*), and the heterogeneity of these hormones is primarily determined by the variability of the anionic species (*30*).

Pictorial Representation of Glycans. While others have previously reported the glycan compositions and structures for FSH (*2*, *18*, *20*), this is the first report that provides information about which glycans are present at all four eFSH glycosylation sites and three of four hFSH sites. While MS data only provide compositions for each of the glycopeptides, these compositions can be converted to glycan structures by comparing the data in this study to previous carbohydrate analyses (*2*, *18*). Table 1 contains pictorial descriptions of each of the observed glycans, and it shows which glycans were found at which glycosylation site for each of the hormones. Baenziger and co-workers (*2*) had identified all but seven of the structures that we observed in pituitary FSH from their glycosidase digestion and glycan chromatography data. [Those seven are A ($n = 1$), C, G, I, K ($n = 0$, $m = 1$), K ($n = 1$, $m = 0$), and L.] Four of these species, A ($n = 1$), C, G, and L, are virtually identical to four other glycans in the table, A ($n = 0$), B, E ($n = 1$), and M ($n = 1$), with the exception that the former glycans contain an additional GlcNAc moiety. Two other structures, K ($n = 0$, $m = 1$) and K ($n = 1$, $m = 0$), are identical to K ($n = 1$, $m = 1$), except they contain only a sulfate or a sialic acid group instead of both groups. The composition of one of the structures, I, matches a tetraantennary glycan previously reported by Renwick et al. (*18*), which was identified from another FSH isolate.

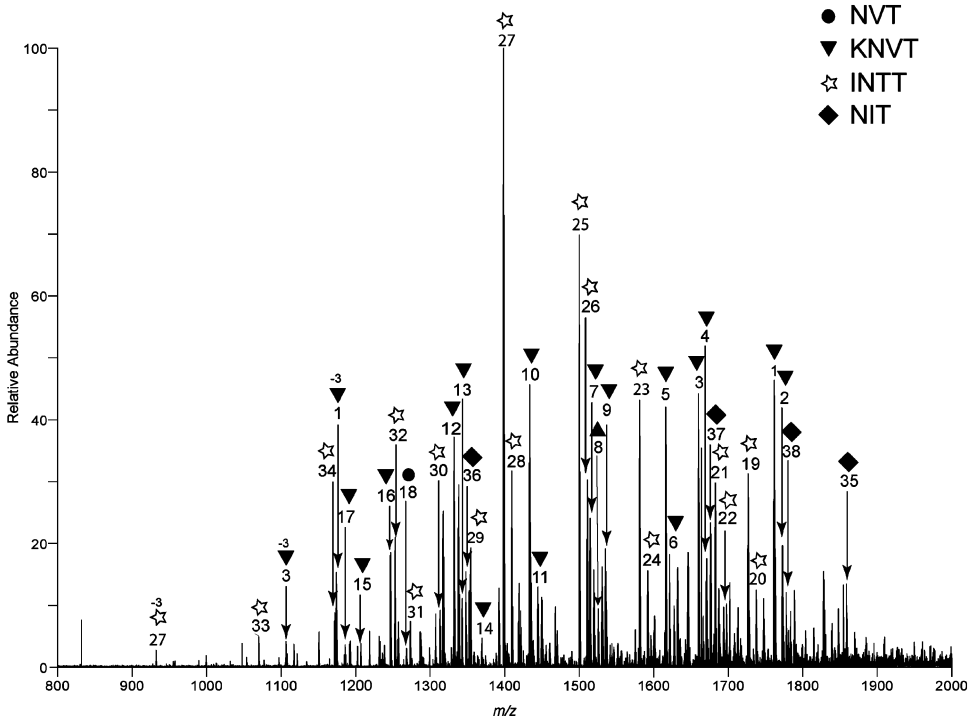


FIGURE 4: FT-ICR-MS spectrum obtained for hFSH in the negative mode. Different symbols have been used to identify glycopeptides from different glycosylation sites from α and *β* subunits. Symbols: (◆) NIT; (☆) INTT; (●) NVT; (▼) KNVT. All peaks are numbered according to Table 1 in Supporting Information. Peaks without a charge are all doubly charged. The same number is used to identify the composition having different charge states on the spectrum.

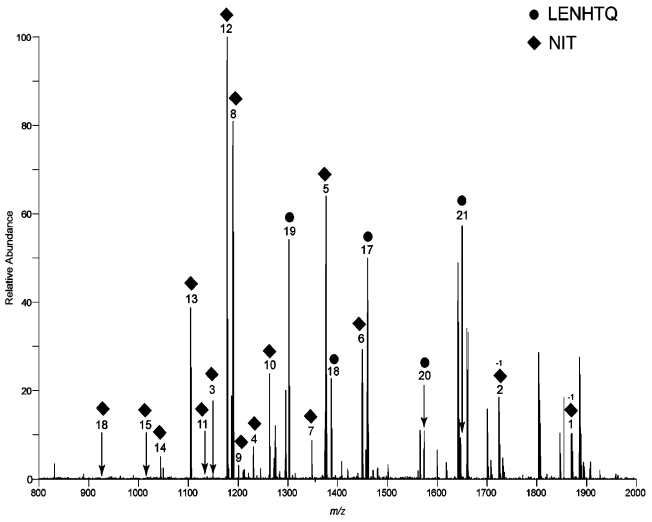


FIGURE 5: FT-ICR-MS spectrum obtained for the eFSH α subunit in the negative mode. Different symbols have been used to identify glycopeptides associated with different glycosylation sites from the α subunit. Symbols: (◆) NIT; (●) LENHTQ. All peaks are numbered according to Table 2 in Supporting Information. Peaks without a charge are all doubly charged.

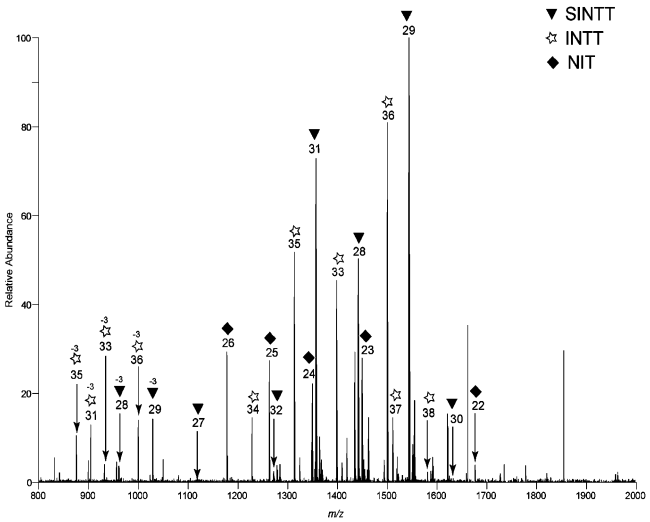


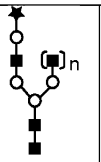
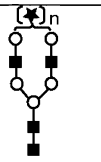
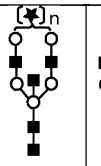
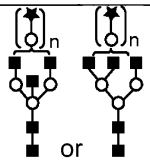
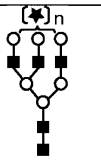
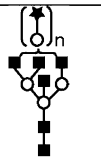
FIGURE 6: FT-ICR-MS spectrum obtained for the eFSH *β* subunit in the negative mode. Different symbols have been used to identify glycopeptides associated with different glycosylation sites from the *β* subunit. Symbols: (☆) INTT; (▼) SINTT; (◆) NIT. All peaks are numbered according to Table 3 in Supporting Information. Peaks without a charge are all doubly charged.

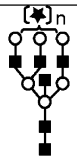
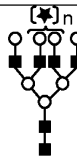
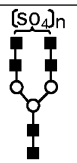
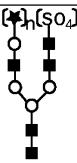
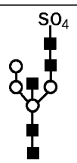
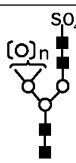
DISCUSSION

Characteristic Patterns of Glycans in eFSH at Different Sites. It can be seen from Table 1 and Figure 7 that a majority of the glycans associated with the peptide NIT are attached to α Asn^{56}. This is consistent with the fact that the glycans attached to NIT in the α subunit exhibited a large degree of variation (*20*). Biantennary sialylated and sulfated glycans were present. These were predominantly of the complex type, but some hybrid forms were also observed. The glycans attached to N^{7}IT in the *β* subunit consisted of fewer structures, suggesting a lesser degree of heterogeneity. The extent of sulfation was less than at α Asn^{56}, and the structures were all complex type and mostly biantennary.

Glycopeptides derived from the α subunit Asn^{82} site of eFSH can be definitively identified from the unique peptide sequence LEN^{82}HTQ. These possess mainly biantennary glycans having disialylated or sulfated structures as well as glycans containing both a sulfate and a sialic acid group. Since SIN^{24}TT is uniquely mapped to the *β* Asn^{24} glycosylation site, glycans at *β* Asn^{24} can also be identified definitively, and these belong to either bi- or triantennary classes of oligosaccharide structures [A, B, D or E, F, J

Table 1: Glycan Structures of Equine and Human Pituitary FSH[a]

Peptide	Site on protein						
		A	B	C	D or E	F	G
		n=0 n=1	n=1 n=2	n=1	n=1 n=2 n=1n=2	n=1 n=2 n=3	n=1
hFSH							
KNVT	α^{52}		12 13		15 10 11	14 7 3 8 4	9
NVT	α^{52}		18				
INTT	β^{24}	33* 34*	32* 27* 28*	29*	31* 25* 26*	23* 19* 24* 20*	
NIT	β^{7}		36*			37*	
eFSH							
NIT	α^{56}		7*	4	5,6*		
	β^{7}		24*		23*	22*	
LENHTQ	α^{82}				20 21*		
SINTT	β^{24}	27*	28*		29*	30*	
INTT	β^{24}/β^{7}		33*		36* 37*	38*	

Peptide	Site on protein						
		H	I	J	K	L	M
		n=1 n=2 n=3	n=3	n=1 n=2	n=1 n=0 n=1 m=1 m=1 m=0		n=0 n=1 n=2
hFSH							
KNVT	α^{52}	5,6 1,2			16 17		
NVT	α^{52}						
INTT	β^{24}	21* 22*			30*		
NIT	β^{7}	38*	35*				
eFSH							
NIT	α^{56}			11* 12* 13	8,9 3 10*	14	1*,2 16 15
	β^{7}			26*	25*		
LENHTQ	α^{82}			19	17*,18		
SINTT	β^{24}			32*	31*		
INTT	β^{24}/β^{7}			34*	35*		

[a] Numbers appearing in the table correspond to peaks on the mass spectra in Figures 4−6. hFSH glycans correspond to data in Figure 4, while eFSH glycans correspond to data from Figures 5 and 6. Structures having an asterisk are all fucosylated.

($n = 2$), and K ($n = 1$, $m = 1$)], which are mainly sialylated. Except for a single glycan attached to the peptide sequence SIN[24]TT, all other glycans associated with SINTT are also present as glycans attached to the related peptide INTT. When pairs of ions corresponding to the glycopeptides SINTT and INTT are present, one can infer that these ions come from the same glycosylation site in the original protein, in this case the β Asn[24] site. However, since TN[7]IT was also identified as a potential peptide from Edman sequencing, and mass spectrometry could not discriminate between TN[7]IT and IN[24]TT, it is possible that some or all of the glycans that were identified from Asn[24], with the sequence IN[24]TT, were also present at Asn[7].

All glycans attached to both glycosylation sites in the β subunit were fucosylated, in contrast to the α subunit where only a fraction of glycans showed fucosylation. Similar observations were reported by Renwick et al. for ovine and human lutropin (*31*, *32*), human thyrotropin (*33*), and human choriogonadotropin (*34*). The function of a fucose residue in glycoprotein hormone glycans is not very well understood (*35*), but according to some investigators, addition of fucose can be as important as the addition of sulfate or sialic acid, since it ensures the proper binding to the appropriate receptor (*36*). While fucosylated glycans enabled hTSH to activate more than one intracellular signaling pathway (*29*, *37*), creating hybrid hormone preparations based on fucosylation

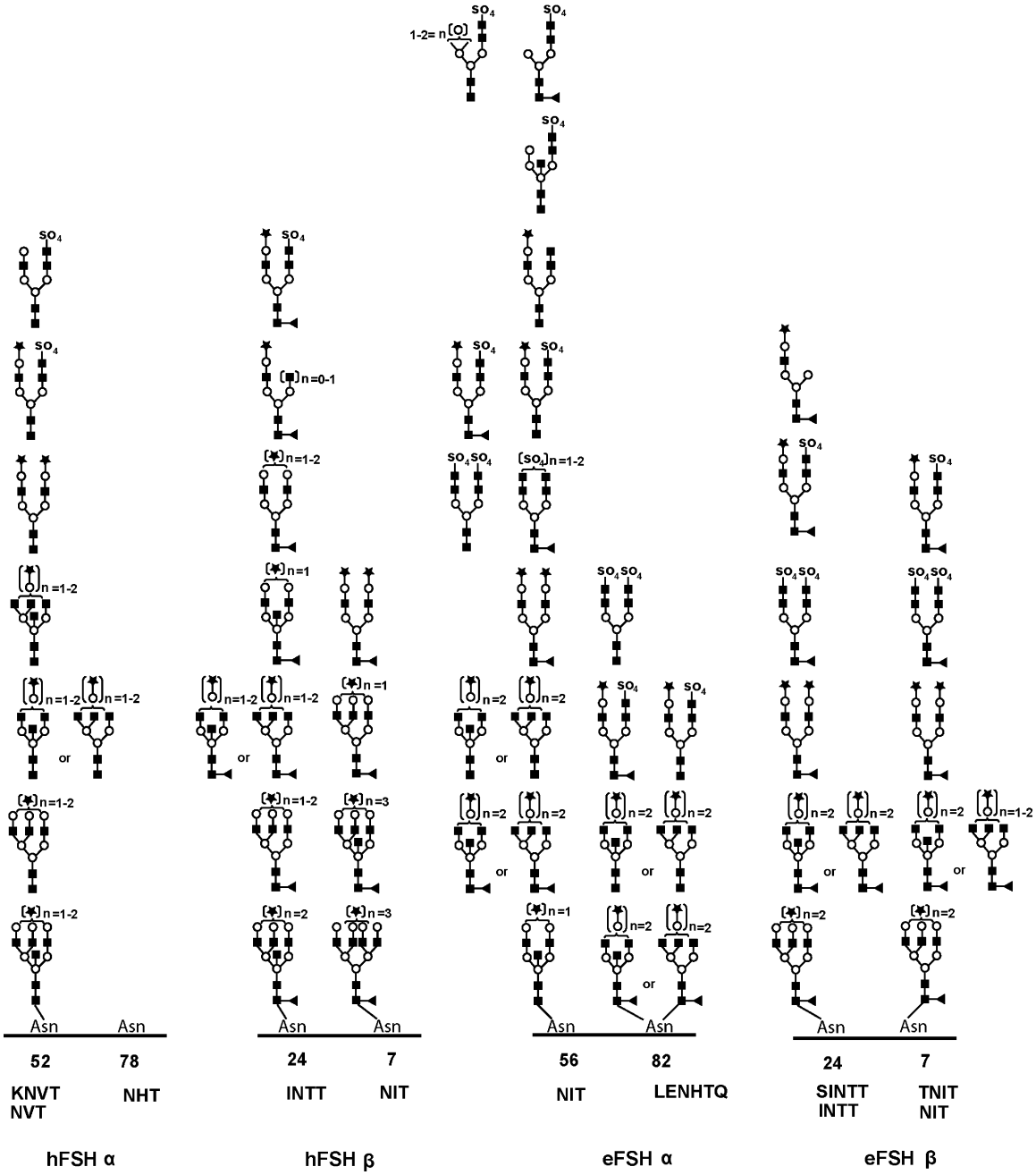


FIGURE 7: Comparative description of the glycans present on each of the glycosylation sites in eFSH and hFSH. Symbols: (■) HexNAc; (○) hexose; (▲) fucose; (★) sialic acid. Where bisecting, biantennary and triantennary glycans are possible, both structures are shown. Triantennary structures could be branched at the 3-linked or 6-linked mannose, but only one structure was depicted for simplicity.

of α Asn[56], glycans had no effect on LH activation of the PLC pathway in cultured ovarian luteal cells (*1*, *38*). The differences in fucosylation correlate with the fact that glycans on the α subunit are buried within the dimer while those on the *β* subunit are solvent exposed (*9*).

The eFSH data we present herein represent a major advance in site-specific glycosylation analysis. Only a single eFSH glycosylation site had been characterized previously. Neutral and negatively charged glycan structures released by selective PNGase hydrolysis were identified by MALDI-MS (*20*). The present study identified 16 of these negatively charged glycans, which represented most of the major negatively charged glycans found in the earlier study. Phosphorylated high-mannose glycans were the only forms not identified as glycopeptide ions. While the low-abundance glycans were not detected herein, this work is highly complementary to previous analyses because it also presents glycosylation site-specific information about the other three eFSH glycosylation sites.

Characteristic Patterns of Glycans in hFSH at Different Sites. Glycans attached to both KNVT and INTT can be easily mapped to their specific glycosylation sites because of the unique peptide sequences. The KNVT glycopeptides were derived from the α Asn[52] site, while INTT-containing glycopeptides mapped to the *β* Asn[24] site. Both sites possessed almost the same glycan population, which were exclusively sialylated, mainly bi- and triantennary structures. The second glycosylation site on the *β* subunit (*β* Asn[24]) had a significantly different oligosaccharide population than what was observed at the first site, *β* Asn[7]. The glycans

at the former site were largely biantennary with only two to three triantennary glycans and a single monoantennary glycan. While most glycans were terminated with sialic acid, a sulfated glycan was present in this population. The glycans at the latter site were mostly triantennary, with only a single biantennary glycan structure. The only tetraantennary structure identified in the present study came from this site. One commonality between glycosylation at β Asn^{24} and β Asn^{7} was that all glycans were fucosylated, whereas the glycans from the α subunit had no fucose. Therefore, in general, the hFSH β subunit contains fucosylated, bi-, tri-, and tetraantennary glycans terminated with sialic acid.

There were no mono- or disulfated complex glycans or sulfated hybrid-type glycans observed for hFSH in either subunit. The few sulfated glycans present also contained sialic acid. However, Green et al. (*2*) had identified monosulfated, hybrid-type glycans that constituted 2% of the total FSH carbohydrate population. This difference may be attributed to variation between two different hFSH preparations.

The present study represents a significant advance in characterizing hFSH glycosylation. We were able to characterize three of four N-glycosylation sites and account for all but two glycan structures reported in two separate studies by other investigators (*2*, *18*). The site that this analysis was unable to detect, Asn^{78}, must possess glycans that are also present at the other three glycosylation sites, since the carbohydrate structures detected in two total oligosaccharide analyses of hFSH were essentially the same as those that we detected from glycopeptides derived from all sites, excluding those at Asn^{78}. The fact that the Asn^{78} glycopeptides were not detected is not a significant limitation of this study, because glycans associated with the *other* three sites, Asn^{52}, Asn^{7}, and Asn^{24}, appear to regulate hFSH biological activity (*13*−*15*). Therefore, the data presented here represent a major step toward to our goal of correlating glycan structure with hFSH function.

Comparison of Glycans on Equine and Human FSH. From Table 1 it is evident that the hFSH α subunit contains bi- and triantennary, complex-type glycans, which are almost all sialylated, whereas the α subunit of eFSH possesses only biantennary glycans, some of which are sulfated and others of which are sialylated. (While some triantennary structures might exist, in every case an alternative biantennary glycan with a bisecting HexNAc is also possible.) Hybrid-type glycans are only present in the α subunit of eFSH. The absence of larger glycans specifically at the Asn^{56} of the α subunit could imply that the eFSH α subunit is less sterically hindered by smaller glycans present at this glycosylation site compared to larger glycans encountered at the corresponding site of the hFSH α subunit; therefore, the former could bind more tightly to the receptor.

While all glycans in the β subunit are fucosylated in both eFSH and hFSH, several other structural differences are apparent. The β subunit of eFSH contains mostly biantennary glycans that are mainly sialylated, whereas in hFSH bi-, tri-, and tetraantennary glycans are present. In eFSH, both sites in the β subunit contain sulfated glycans, whereas only the β Asn^{24} site contains sulfated glycans in hFSH. This comparison between human and equine FSH glycosylation on the β subunit demonstrates that glycosylation can vary dramatically, even when comparing proteins from two mammalian sources, whose amino acid sequence is ∼94% identical.

This work also complements other emerging evidence that demonstrates structural differences between human and horse FSH. Human and equine FSH preparations analyzed in this study represent two patterns of mammalian FSH glycosylation. In the former, which may be representative for primate FSH (*39*), the glycans are larger, consist of two to four complex branches, are terminated with sialic acid, and contain very little sulfation. In contrast, smaller, one to two branch, sulfated glycans are identified at every glycosylation site of eFSH. This places the horse FSH at the upper end of the continuum for glycan sulfation (*2*), and this structural difference is important because higher degrees of sulfation are associated with more rapid hormone clearance (*6*).

While we have demonstrated that the individual glycans on the β subunit of human and equine FSH are different, other studies have also demonstrated that the global pattern of glycosylation on this subunit also varies for these two hormones. It has been recently shown that the hFSH β subunit is glycosylated in an all-or-none manner (*39*), whereas eFSH β is glycosylated either exclusively at Asn^{24} or at both Asn^{7} and Asn^{24} (*1*). In humans, the relative abundance of the glycosylated and nonglycosylated FSH β subunit appears to be physiologically regulated (*39*). Both the information presented here, which provides information about glycan composition at specific glycosylation sites, and earlier work, which documents differences in the presence and absence of glycosylation on the β subunit, are important studies needed to fully understand the glycoprotein structure. However, to clearly understand how the structure of these hormones induces specific biological effects, thoroughly characterized isoforms, with defined glycan composition, will be needed for use in functional assays. These functional studies are a future emphasis of our research, and the site-specific glycosylation analysis method presented in this report is a key component to making such structure−function studies possible.

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SUPPORTING INFORMATION AVAILABLE

Tables 1−3 providing the peak list corresponding to the numbers on the spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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