Identification of post-translational modifications resulting from LH β polymorphisms by matrix-assisted laser desorption time-of-flight mass spectrometric analysis of pituitary LH β core fragment

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Abstract

Metabolism of the human chorionic gonadotrophin (hCG)- and LH β -subunits (hCG β , LH β) terminates with the urinary excretion of core fragment (hCG β cf, LH β cf) molecules that retain antigenic shape and constituent *N*-linked carbohydrate moieties. We have previously demonstrated the resolved mass spectra of hCG β cf, from which the carbohydrate moieties present at two *N*-linked glycosylation sites were identified. LH β cf was subjected to the same mass spectrometric analysis. As LH β shares 82% homology with hCG β but possesses only one glycosylation consensus site a simpler spectral fingerprint of LH β cf glycoforms was expected. LH β cf was reduced with dithiothreitol and analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Glycoforms were predicted by subtracting the peptide mass from the *m/z* values of the observed peaks and then sequentially subtracting the masses of the monosaccharide residues of hCG β *N*-linked carbohydrates reported in the literature. The mass spectra of LH β cf revealed a broad single peak ranging from *m/z* 8700 to 10 700. Following reduction, this peak was replaced by a set of partially resolved peaks between *m/z* 4130 and 5205 corresponding to glycosylated forms of the peptide LH β 6–40. A peak at *m/z* 4252.2 corresponded to the non-glycosylated peptide LH β 55–93. Remaining peaks indicated that the pooled sample comprised a wide set of glycoforms, contained LH β cf with two *N*-linked carbohydrate moieties and indicated evidence of further glycosylation due to amino acid substitution in polymorphic variants. This is evidence that a single nucleotide polymorphism alters the post-translational modification of a protein and hence its structural phenotype.

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Introduction

Luteinizing hormone (LH) is a dimeric glycoprotein that is synthesised and secreted by the anterior pituitary gland. LH is an essential requirement in the stimulation of follicular growth, oocyte maturation, ovulation and in promoting spermatogenesis. Its mode of action is via stimulation of steroidogenesis in both testicular and ovarian gonadal tissues. The structure of LH is analogous to other members of the glycoprotein hormone family that includes

chorionic gonadotrophin, thyroid-stimulating hormone and follicle-stimulating hormone. Each consists of an α -subunit that is common to all members and a unique β -subunit that confers specific hormonal activity (Pierce & Parsons 1981). Although there are no defining crystallography data describing the LH β -subunit (LH β) it is assumed that its molecular structure is essentially similar to that of the human chorionic gonadotrophin β -subunit (hCG β), with which it shares a high degree of linear sequence homology and a

Figure 1 *N*-linked carbohydrates attributed to intact LH by Green & Baenziger (1988a,b) and subsequently found to be present on LHβ by Weisshaar *et al.* (1991). *N*-acetylglucosamine (□); mannose (○); *N*-acetylgalactosamine (■); galactose (●); sialic acid (▲); *n*=1 or 2.

target receptor (Carlsen *et al.* 1973, Shome & Parlow 1973, Lapthorn *et al.* 1994, Wu *et al.* 1994). As with other glycoprotein hormones, LH is characterised by carbohydrate moieties present on both subunits that act to determine receptor affinity and metabolic clearance (Ryan *et al.* 1987). The *N*-linked carbohydrates (shown in Fig. 1) found on both the LHα-subunit (Asn 52 and Asn 78) and the β-subunit (Asn 30) confer a high degree of microheterogeneity being composed of a wide population of complex and hybrid sugars (Green & Baenziger 1988a,b, Weisshaar *et al.* 1991). (All *N*-linked glycans possess the pentasaccharide Manα1–6(Manα1–3)Manβ1–4 GlcNAcβ1–4 GlcNAc (Kornfeld & Kornfeld 1985) commonly termed the trimannosyl core. Based on the residues added to the trimannosyl core, *N*-linked glycans are further classified into three subgroups: complex, when no mannosyl residues are present in subsequent branching; high mannose, when mannosyl residues only are attached to the core; and hybrid, which contain both complex and high mannose branches.) *N*-linked glycans in LH

possess terminal sulphate residues in the case of the hybrid carbohydrates and either terminal sulphate or sialic residues acid or a combination in the case of the complex sugars (Green & Baenziger 1988a,b).

The terminal core fragment of the LHβ-subunit (LHβcf) was first isolated as an LH degradation product in human pituitary glycoprotein extracts (Birken *et al.* 1993). Indeed LH, its free subunits and LHβcf are all found in the pituitary (Birken *et al.* 1996), indicating that some proteolytic degradation of the free LHβ-subunit takes place there. Due to the high structural homology with hCGβ it is likely that circulating LHβ (O'Connor *et al.* 1998) shares a similar metabolic fate being largely degraded to the terminal core fragment in the kidneys (Markkanen *et al.* 1979). Evidence supporting the existence of urinary LHβcf is based on the detection of immunoreactive material resembling human chorionic gonadotrophin β subunit (hCGβcf) in mid-menstrual and postmenopausal urine (Iles *et al.* 1992, 1999, Neven *et al.* 1993).

Figure 2 Schematic diagram of the primary sequence of LH β cf peptides showing disulfide bonds and *N*-linked glycosylation positions in the wild-type metabolite (a) and in that of the common polymorphic variant (b) where the amino acid substitutions are also indicated.

The terminal urinary metabolite of hCG β cf is composed of two peptides: β 6–40 which possesses two *N*-glycosylation consensus sequences and β 55–92 which is not glycosylated (Birken *et al.* 1988). The dipeptide is stabilised by a tight grouping of four cystine residues and has a mass of approximately 10 kDa (Fig. 2a). Structural analysis suggests that LH β cf is more heterogeneous in the length of its constituent peptides than hCG β cf. Matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) mass spectrometry (MS) of reduced, carboxymethylated and deglycosylated LH β cf has revealed the presence of two non-glycosylated peptides spanning not only amino acids β 55–93 but also β 49–93 (Birken *et al.* 2001). The primary structure of the glycosylated peptide, not subjected to MS, has previously been determined to be β 6–40 (Birken *et al.* 1993, O'Connor *et al.* 1998).

Up to ten mutations and polymorphisms in the LH β gene have been described including six silent polymorphisms in both exons and introns (reviewed in Huhtaniemi *et al.* 1999). Following the failure to detect serum LH in some patients by two-site immunoassays that utilised highly specific monoclonal antibody pairs (Pettersson *et al.* 1991), mutations altering the antigenic structure of the

LH β -subunit were identified. Of particular interest is a genetic variant of LH (vLH) that arises from two single point mutations in the LH β gene that each alters the primary amino acid sequence: Trp8→Arg (TGG→CGG) and Ile15→Thr (ATC→ACC) (Furui *et al.* 1994, Pettersson *et al.* 1994, Nilsson *et al.* 1998). An extra glycosylation consensus sequence (Asn-X-Thr/Ser) is thereby introduced into the LH β -subunit by the second mutation which, it may be speculated, might permit the attachment of an *N*-linked oligosaccharide moiety at Asn13.

Recently, we utilised MALDI-TOF MS to elucidate the carbohydrates residual on hCG β cf (Jacoby *et al.* 2000) following reports of an increase in human chorionic gonadotrophin (hCG) hyperglycosylation in the urine of individuals with Down's pregnancies or with gynaecological cancers (Elliott *et al.* 1997, Cole *et al.* 1998, 1999). Direct analysis of hCG β cf was not possible as the technique did not prove discriminating enough to identify individual glycoforms because of the lack of resolution between them. To circumvent this problem the four cystine residues between the two polypeptides were reduced with dithiothreitol (DTT) prior to MS analysis (Kusmann *et al.* 1997). This separated the non-glycosylated β 55–92 chain

from the glycosylated β6–40 chains while increasing the resolution between all peaks and producing a distinctive ‘fingerprint’ spectrum (Jacoby *et al.* 2000). It was then possible to determine the mass of each glycosylation variant on the β6–40 chain by calculating the difference in mass between each observed peak to the mass of its corresponding non-glycosylated primary amino acid sequence. This study set out to investigate the glycosylation profile of a pool of LHβcf following treatment with DTT. The expectation was that as the wild-type LHβcf retains a single *N*-linked carbohydrate moiety at Asn 30 then a simpler fingerprint spectrum would be obtained by contrast with that acquired from hCGβcf.

Materials and methods

Materials

The LHβcf from pooled pituitary tissue used in this study, batch no. 697, was previously isolated by Birken *et al.* (1993) and was a kind gift from Dr Steven Birken, Columbia University, New York, NY, USA. This, rather than that isolated from urine, was utilised as sufficient was readily available in the pure form. DTT, for the reduction of disulfide bonds, and NH_4HCO_3 were obtained from Sigma-Aldrich Co. Ltd, UK. The MALDI MS matrix 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid: SA) was similarly purchased from Sigma-Aldrich Co. Ltd and used without further purification. Acetonitrile and trifluoroacetic acid (TFA) were of analytical grade and obtained from VWR International Ltd., UK.

MALDI-TOF MS

To acquire spectra of LHβcf, 10 pmol (0.5 µl of 20 µmol/l in distilled and deionised H_2O (dH_2O)) was applied to a stainless steel MALDI MS target and mixed together with 1.0 µl SA (20 g/l in 70:30 (v/v) acetonitrile–1 ml/l TFA in dH_2O) and air dried. The method by which spectra of reduced glycoforms were obtained has been previously reported (Jacoby *et al.* 2000). Briefly 10 pmol LHβcf (0.5 µl of 20 µmol/l in dH_2O) and 0.5 µl DTT (100 mM/l in 100 mM/l NH_4HCO_3 , pH 8.8) were mixed together on a MALDI MS target and incubated at room temperature. When this mixture was virtually dry 0.5 µl 1 ml/l TFA in dH_2O was

added to the remaining droplet. The mixture was again allowed to partially dry before adding 1.0 µl SA (20 g/l in acetonitrile–0.1% TFA, 70:30 v/v) and air dried as before. The instrument was calibrated externally using horse heart cytochrome C (M_r 12 360.1; Sigma-Aldrich Co. Ltd). Mass spectrometric analysis was carried out on a VG TOF Spec E (Micromass Ltd, UK) with a 1.5 m flight tube operating at an accelerating voltage of 20 kV in positive linear mode. A pulsed nitrogen laser ($\lambda_{\text{max}}=337$ nm) was utilised to desorb ions from the samples which were detected by a microchannel plate detector at a sampling rate of 500 MHz. Spectra were generated by summing 30–40 laser shots. Data were acquired from the instrument operating in the positive linear mode.

Treatment of results

Initially the peak relating to the non-glycosylated LHβcf peptide (β55–93) was internally calibrated to the average molecular mass of its given primary sequence: $[\text{M}+\text{H}]^+$ 4256.0 (calculated using the computer software MassLynx 3.2; Micromass Ltd). This facilitated the automatic correction of the mass values of the remaining peaks to their correct values.

In order to determine the inferred mass values of the LHβcf carbohydrate moieties, the average molecular mass ($[\text{M}+\text{H}]^+$ 3768.4 Da) of the primary sequence of the glycosylated LHβcf polypeptide (β6–40) was subtracted from the mass ($[\text{M}+\text{H}]^+$) values of the acquired peaks (Table 1). The carbohydrate content of these peaks was then deduced by the sequential subtraction of the masses of the sugar residues present in LHβ-subunit carbohydrates (Table 2). Carbohydrate structures (Table 3) were then predicted from the deduced monosaccharide content, taking into account the small difference between the calculated and the observed masses ($\leq 0.40\%$). When subtraction of the average molecular mass of the wild-type glycosylated LHβcf polypeptide did not produce a value from which a carbohydrate could be determined then the same procedure was used, subtracting the average molecular mass of the variant glycosylated LHβcf polypeptide containing either Trp 8→Arg ($[\text{M}+\text{H}]^+$ 3744.4 Da) and Ile15→Thr ($[\text{M}+\text{H}]^+$ 3762.4 Da) mutations or both mutations ($[\text{M}+\text{H}]^+$ 3732.4 Da). Conformational information describing the anomeric glycosidic linkages within the LHβ

Table 1 Peptides of LHβcf released after DTT reduction, detected by MALDI-TOF MS

Peak	Observed mass ([M+H] ⁺)	Predicted LHβ variant	Mass of inferred 1° sequence ([M+H] ⁺) ^a	Mass -1° sequence	Predicted mass of residual CHO	Δ ^b	Δ%
1	4151.6	mut8	3744.4	407.2	406.4	0.8	0.02
2	4321.9	wt	3744.4	547.5	552.5	-5.0	-0.12
3	4360.1	mut15	3762.4	597.7	609.6	-11.9	0.27
4	4395.1	—	—	—	—	—	—
5	4424.7	dbl mut	3732.4	692.4	698.7	-6.3	-0.14
6	4466.7	dbl mut	3732.4	734.4	730.7	3.7	0.08
7	4505.7	wt	3774.4	731.3	730.7	0.6	0.01
8	4537.1	mut15	3762.4	774.7	771.7	3.0	0.07
9	4590.6	mut15	3762.4	828.3	812.8	15.5	0.34
10	4648.5	wt	3774.4	874.1	876.8	-2.8	-0.06
11	4681.0	dbl mut	3732.4	948.6	958.9	-10.3	-0.22
12	4718.6	mut15	3762.4	956.2	958.9	-2.7	-0.06
13	4760.5	dbl mut	3732.4	1028.1	1038.9	-10.8	-0.23
14	4808.3	dbl mut	3732.4	1075.9	1080.0	-4.1	-0.09
15	4893.9	mut15	3762.4	1131.5	1137.0	-5.5	-0.11
16	5006.9	mut15	3762.4	1244.5	1242.1	2.4	0.05
17	5101.5	mut15	3762.4	1339.1	1340.2	-1.1	-0.02
18	5146.2	mut15	3762.4	1383.8	1388.3	-4.5	-0.09
19	5204.6	mut8	3744.4	1460.2	1461.3	-1.1	-0.02

^aMass of the primary sequences of the glycosylated LHβcf peptide: β6–40.

^bDifference between the calculated mass of the expected total carbohydrate (CHO) and the inferred mass following subtraction of the mass of the primary sequence from the peak mass.
mut, mutation; wt, wild-type; dbl, double mutant.

N-linked carbohydrate structures have been reported previously (Green & Baenziger 1988a,b, Weisshaar *et al.* 1991) and were taken into account in our analysis.

Results

A typical spectrum of LHβcf obtained by MALDI-TOF MS using the matrix SA is shown in Fig. 3. The spectrum is characterised by a single

Table 2 Relative molecular mass (RMM) of the monosaccharides and terminal groups expected to be present in the LHβ-subunit *N*-linked carbohydrates

	RMM	RMM-H ₂ O
Monosaccharide		
SO ₄	96.02	—
Sialic acid (SA)	309.27	291.25
Galactose (Gal)	180.16	162.14
<i>N</i> -acetylgalactosamine (GalNAc)	221.21	203.19
<i>N</i> -acetylglucosamine (GlcNAc)	221.21	203.19
Mannose (Man)	180.16	162.14
Fucose (Fuc)	164.16	146.14

broad peak at *m/z* 8700–10 700. The discernible peaks present at *m/z* 9320 and 9475 are presumably partially resolved glycoforms but were not always as well defined on all the spectra acquired of the intact core fragment. Following reduction with DTT the broad peak was replaced by a set of partially resolved peaks between *m/z* 4130 and 5205 (Fig. 4). Resolution was improved by allowing the data analysis software to determine the *m/z* values of the centroid positions of the spectral peaks (Fig. 5) and these masses were used to predict the carbohydrates present. The peak at *m/z* 4252.3 was deemed to arise from the non-glycosylated peptide chain (β55–93) because the *m/z* value lies within the 0.5% error margin allowed for the linear operating mode of the MALDI-TOF instrument. The low percentage errors between the expected and the observed mass values of the acquired peaks indicated the likelihood that real glycoforms were detected despite the fact that LHβcf asparagine-linked glycoforms were not observed directly (Table 1).

Table 3 Proposed structures of the oligosaccharides in the glycoforms contributing to the microheterogeneity of wild-type LHβcf (peaks 2, 7 and 10) and LHβcf containing a Trp 8→Arg substitution (peaks 1 and 19). The composition of these structures (as well as those in Tables 4 and 5) are presumed from the masses acquired for the glycopeptides while the indicated linkages have been adopted from previous extensive characterisation of those oligosaccharides (Green & Baenziger 1988*a,b*, Weisshaar *et al.* 1991)

Peak	Proposed structure	Inferred sugar mass (<i>m/z</i>)
2	<div> Fucα1→⁶ GlcNAcβ1→⁴GlcNAc-Asn </div>	552.5
7	<div> ⁶ Manα1→⁴Manβ1→⁴GlcNAcβ1→⁴GlcNAc-Asn ³ </div>	730.7
10	<div> ⁶ Manα1→⁴Manβ1→⁴GlcNAcβ1→⁴GlcNAc-Asn ³ <div style="margin-left: 150px;">Fucα1→⁶</div> </div>	876.8
1	GlcNAcβ1→ ⁴ GlcNAc-Asn	406.4
19	<div> Galβ1→⁴GlcNAcβ1→²Manα1→⁶ GlcNAcβ1→²Manα1→³Manβ1→⁴GlcNAcβ1→⁴GlcNAc-Asn </div>	1461.3

N-linked carbohydrate moieties, shown in Tables 3–5, were determined as described above. Where the number of monosaccharide residues calculated for the carbohydrate moieties associated with the mass spectral peaks precluded the determination of a single glycoform or a single pair of glycoforms then possible alternative structures are suggested. Initially the mass of the sugars was calculated assuming that the glycosidic linkage was to a peptide arising from a wild-type peptide (LHβcf_{wt}) with no polymorphic variation (Table 3). This enabled identification of the glycosylation of the glycoforms at peaks 2, 7 and 10. Peak 2 (*m/z* 4321.9) arises from a polypeptide possessing a single truncated and fucosylated monoantennary oligosaccharide (GlcNAc₂Fuc) with a predicted mass of 552.5, whereas the polypeptide giving rise to peak 7 (*m/z* 4505.7) has a single truncated non-fucosylated monoantennary oligosaccharide (GlcNAc₂Man₂) with a predicted mass of 730.7. Similarly peak 10 (*m/z* 4648.5) arises from a polypeptide possessing a single truncated monoantennary oligosaccharide that is additionally fucosylated (GlcNAc₂Man₂Fuc) with a predicted mass of 876.8. Carbohydrates associated with the remaining peaks could not be determined for a

wild-type LHβcf. However, subtracting the average molecular mass for the glycosylated peptide with a Trp 8→Arg mutation permitted the determination of the glycosylation for peaks 1 and 19 (Table 3). The predicted mass of 406.4 for the carbohydrate moiety of the glycoform at peak 1 (*m/z* 4151.6) indicates a disaccharide (GlcNAc₂). The predicted mass of the carbohydrate moiety attached to peak 19 (*m/z* 5204.6) suggests a biantennary oligosaccharide. This peak arises from a polypeptide possessing a non-fucosylated trimannosyl core attached to two additional *N*-acetylglucosamine residues as well as a galactose residue (GalMan₃GlcNAc₄) with a predicted carbohydrate mass of 1461.3. Subtracting the average molecular mass for the glycosylated peptide with an Ile15→Thr substitution then permitted the determination of the glycosylation for peaks 3, 8, 9, 12, 15, 16, 17 and 18 (Table 4). Peak 3 (*m/z* 4360.1) appears to arise from a polypeptide with two monoantennary carbohydrate moieties (GlcNAc and [GlcNAc]₂) with a predicted mass of 609.6. The predicted mass of the carbohydrate moiety attached to peak 8 (*m/z* 4537.1), 771.7, suggests a polypeptide possessing a single truncated monoantennary carbohydrate (ManGlcNAc₃) or two

Figure 3 MALDI-TOF mass spectrum of purified LH β cf in SA matrix generated by summation of 50 laser shots and calibrated externally using horse heart cytochrome C (M_r 12 360.1).

monoantennary carbohydrate moieties (GlcNAc and ManGlcNAc₂). Peak 9 (m/z 4590.6) and peak 12 (m/z 4718.6) arise from a polypeptide possessing two single and truncated monoantennary disaccharides ([GlcNAc₂]₂) of which one, in peak 12, is fucosylated. The predicted carbohydrate mass of the glycoforms seen at peaks 9 and 12 is 812.8 and 958.9 respectively. The masses derived for the glycosylation moieties attached to the polypeptides giving rise to peaks 15, 16 and 17 allow the possibility for several carbohydrate permutations on each protein. The glycoform at peak 15 (m/z 4893.9) has a predicted carbohydrate mass of 1137.0 indicating either a single non-fucosylated monoantennary oligosaccharide (GalNAcMan₂GlcNAc₃) or alternatively pairs of monoantennary carbohydrates ([ManGlcNAc₂]₂ or [Man₂GlcNAc₂ and GlcNAc₂] or [Man₂GlcNAc₃ and GlcNAc]). Peak 16 (m/z 5006.9) arises from a glycoform consisting of a fucosylated trimannosyl core possessing an additional *N*-acetylglucosamine residue as a bi- or tri-antennary extension or linked to a second asparagine residue. Alternatively, the carbohydrate, with a predicted mass of 1242.1, may be present as a single fucosylated monoanten-

nary structure. Peak 17 (m/z 5101.5) arises from a polypeptide possessing two truncated non-fucosylated monoantennary sugars, with a total predicted carbohydrate mass of 1340.2, with several possible structural permutations. The glycosylation moiety of the polypeptide giving rise to peak 18 (m/z 5146.2) can only occur as a fucosylated monoantennary and a fucosylated trimannosyl core (GlcNAcFuc and Man₃GlcNAc₂Fuc) with a total predicted mass of 1388.3. The carbohydrate moieties of the glycoforms that give rise to remaining peaks 5, 6, 11, 13 and 14 were determined assuming that both mutations, Trp 8→Arg and Ile15→Thr, were present in the reduced LH β cf molecule (Table 5). In peak 5 (m/z 4424.7) the carbohydrate moiety appears to consist of two fucosylated *N*-acetylglucosamine residues ([GlcNAcFuc]₂) with a predicted mass of 698.7, whereas the glycoform giving rise to peak 6 (m/z 4466.7) contains a single monoantennary sugar (GlcNAc₂Man₂) with a predicted mass of 730.7. Peak 11 (m/z 4681.0) arises from a glycoform that contains a pair of di-saccharides of which one is fucosylated (GlcNAc₂ and GlcNAc₂Fuc) with a predicted mass

Figure 4 MALDI-TOF mass spectrum of LH β cf after 'on-target' reduction and using SA matrix. The spectrum was generated by summation of 50 laser shots and calibrated internally using fragment mass 4256.0.

of 958.9. The glycoform at peak 13 (m/z 4760.5) indicates a polypeptide with a predicted carbohydrate mass of 1038.9 suggesting a single fucosylated trimannosyl core (Man₃GlcNAc₂Fuc). Finally, the polypeptide giving rise to peak 14 (m/z 4808.3) possesses a glycosylation moiety, with a predicted mass of 1080.0, which occurs either as two monoantennary carbohydrates (Man₂GlcNAc₂ and GlcNAc) of which one is fucosylated or a single fucosylated monoantennary structure (Man₂GlcNAc₃Fuc). There were a number of peaks remaining in the mass spectrum of DTT-reduced LH β cf for which carbohydrates were not able to be determined. These include a number of low intensity peaks for which mass values could not be acquired by the software as well as a peak of moderate intensity (peak 4) whose mass (m/z 4395.1) could not be correlated with the mass of the LH β_{wt} or vLH β peptide primary sequences.

Discussion

Mass spectral and ion chromatography analyses of the structure of pituitary LH β cf have predicted

that, although the LH β -subunit is acted on by proteases in the pituitary, a lack of glycosidases there might leave its carbohydrate moieties intact (Birken *et al.* 1996, O'Connor *et al.* 1998). This premise was based on the acquisition of a broad unresolved MALDI-TOF mass spectrum of pituitary LH β cf with an average mass of $\sim 10\,000$ Da that, when the mass of the protein had been subtracted, gave the impression that a single intact *N*-linked carbohydrate moiety had been retained. We have found that the unresolved, broad nature of MALDI-TOF mass spectra of glycoproteins is an indication of a population of molecules possessing wide microheterogeneity in terms of its carbohydrate content. This becomes apparent when the carbohydrates are investigated following the sequential use of glycanases or when the protein is broken down, as here with DTT, to its constituent peptides to improve spectral resolution.

The use of DTT to obtain a MALDI-TOF MS spectrum of reduced hCG β cf produced a mass spectrum which enabled us to readily identify many of the *N*-linked carbohydrates arising from the glycosylation moieties present on the metabolite (Jacoby *et al.* 2000). Of nine glycosylated peptides

Figure 5 MALDI-TOF mass spectrum of DTT-reduced LH β cf showing peaks in the m/z range 4000–5300 (top) and the equivalent averaged centroids of those peaks (bottom) to facilitate peptide and carbohydrate identification.

detected we calculated that seven possessed two carbohydrate moieties, one a single carbohydrate moiety and the last either one or two carbohydrate moieties. It was expected that, as the primary sequence of the LH β -subunit possesses only a single consensus sequence permitting *N*-linked glycosylation, an analogous but simple fingerprint spectrum of LH β cf would be acquired using the same technique. By contrast with hCG β cf, we identified eighteen glycosylated peptides in the pituitary LH β cf sample of which seven were determined to possess single carbohydrate moieties, six with two carbohydrate moieties and five with either one or two carbohydrate moieties.

Amino acid sequence analysis of LH β cf isolated from pituitary tissue revealed the presence of two amino-terminal sequences in equal amounts starting at residues β 6 and β 55 (Birken *et al.* 1993). The primary structure of the glycosylated peptide has been determined to be β 6–40 by MALDI-TOF MS following *N*-glycanase digestion (O'Connor *et al.* 1998, Birken *et al.* 2001). Subsequent analysis suggested that LH β cf is more heterogeneous in the length of its constituent peptides than hCG β cf.

MALDI-TOF MS of reduced and carboxymethylated or alkylated LH β cf revealed the presence of two non-glycosylated peptides spanning not only amino acids β 55–93 but also β 49–93 (Birken *et al.* 2001). Following DTT reduction, our MALDI-TOF MS data revealed the presence of only a single non-glycosylated peptide, β 55–93, in our sample of pooled LH β cf. A peak corresponding to the peptide β 49–93, with a predicted mass of 4900.8, was not demonstrated in this study. However, following the same purification procedure (Birken *et al.* 1993), different batches of LH β cf have shown variability in the proportional content of non-glycan peptides although both β 55–93 and β 49–93 peptides have usually been detected (S Birken, personal communication). It is possible that the signal from this peptide may not have been recognised by our MALDI detector, which may have been 'swamped' by greater concentrations of smaller peptides. A simpler explanation would be that the β 49–93 was not present in the batch of LH β cf examined here.

In our previous analysis of hCG β cf glycosylation, the prior reduction of the metabolite with DTT

Table 4 Proposed structures of the oligosaccharides in the glycoforms contributing to the microheterogeneity of LHβcf an Ile15→Thr substitution (peaks 3, 8, 9, 12, 15, 16, 17 and 18). Where the masses found give rise to a variety of carbohydrate combinations on any one peptide, these are shown

Peak	Proposed structure	Inferred sugar mass (<i>m/z</i>)
3	GlcNAc-Asn + GlcNAcβ1→4GlcNAc-Asn	609.6
8	GlcNAc-Asn + Manβ1→4GlcNAcβ1→4GlcNAc-Asn GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc-Asn	771.7
9	GlcNAcβ1→4GlcNAc-Asn + GlcNAcβ1→4GlcNAc-Asn	812.8
12	GlcNAcβ1→4GlcNAc-Asn + GlcNAcβ1→4GlcNAc-Asn	958.9
15	GalNAcβ1→4GlcNAcβ1→2Manα1→ Manβ1→4GlcNAcβ1→4GlcNAc-Asn Manβ1→4GlcNAcβ1→4GlcNAc-Asn + Manβ1→4GlcNAcβ1→4GlcNAc-Asn Manα1→ Manβ1→4GlcNAcβ1→4GlcNAc-Asn + GlcNAcβ1→4GlcNAc-Asn GlcNAcβ1→2Manα1→ Manβ1→4GlcNAcβ1→4GlcNAc-Asn + GlcNAc-Asn	1137.0
16	One of the pair of glycoforms possessing a fucose residue	1242.1
17	GlcNAcβ1→4GlcNAc-Asn + GlcNAcβ1→2Manα1→ Manβ1→4GlcNAcβ1→4GlcNAc-Asn Manβ1→4GlcNAcβ1→4GlcNAc-Asn + GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc-Asn GlcNAcβ1→4GlcNAc-Asn + GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc-Asn	1340.2
18	+	1388.3

Table 5 Proposed structures of the oligosaccharides in the glycoforms contributing to the microheterogeneity of LHβcf containing both Trp 8→Arg and Ile15→Thr substitutions (peaks 5, 6, 11, 13 and 14). Where the masses found give rise to a variety of carbohydrate combinations on any one peptide, these are shown

Peak	Proposed structure	Inferred sugar mass (<i>m/z</i>)
5	$\text{Fuca1} \rightarrow \text{GlcNAc-Asn}$ + $\text{Fuca1} \rightarrow \text{GlcNAc-Asn}$	698.7
6	$\text{Man}\alpha 1 \rightarrow \text{Man}\beta 1 \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$	730.7
11	$\text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$ + $\text{Fuca1} \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$	958.9
13	$\text{Man}\alpha 1 \rightarrow \text{Man}\beta 1 \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$ $\text{Fuca1} \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$ $\text{Man}\alpha 1 \rightarrow \text{Man}\beta 1 \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$	1038.9
14	GlcNAc-Asn + $\text{Man}\alpha 1 \rightarrow \text{Man}\beta 1 \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$ $\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow \text{Man}\beta 1 \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$ $\text{Fuca1} \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$ $\text{Man}\alpha 1 \rightarrow \text{GlcNAc}\beta 1 \rightarrow 4\text{Man}\beta 1 \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$ $\text{Fuca1} \rightarrow \text{GlcNAc}\beta 1 \rightarrow \text{GlcNAc-Asn}$	1080.0

enhanced the resolution of each glycosylated peptide to the extent that all peaks were well separated (Jacoby *et al.* 2000). When LHβcf was reduced with DTT many more peaks corresponding to glycosylated peptides were seen in comparison with hCGβcf and these were not as well resolved. The more complicated spectrum of DTT-reduced LHβcf, we realised, could not solely be accounted for by the hormone's increased glyco-microheterogeneity. However, the complex fingerprint could be accounted for by the presence of a second *N*-linked glycosylation moiety. More importantly, a specific DNA polymorphism, which has previously been described, gives rise to a glycosylation signal sequence that may lead to the insertion of a second *N*-linked glycosylation moiety (Furui *et al.* 1994, Pettersson *et al.* 1994, Suganuma *et al.* 1996, Nilsson *et al.* 1998). The commonly occurring vLH molecule possesses amino acid substitutions at β8 and β15. The substitution at β15 of an isoleucine residue by a threonine residue results in the formation of a glycosylation consensus sequence theoretically permitting the addition of an

additional *N*-linked carbohydrate to the asparagine residue at β13. In contrast to the studies of hCG, there has been little investigation of LH oligosaccharide composition. The asparagine-linked oligosaccharides on intact human LH have previously been elucidated by Green & Baenziger (1988*a,b*) using conventional methodologies including radiolabelling reducible groups with NaB[³H]₄, release of oligosaccharides by *N*-glycanase digestion, sequential digestions with endo- and exoglycosidase enzymes followed by HPLC and ion-exchange separations. In a second study by Weisshaar *et al.* (1991), the dissociation of the dimeric hormone was followed by the release of oligosaccharides by hydrazinolysis from LHβ and from tryptic glycopeptides of LHα. The subsequent characterisation of the *N*-linked sugars largely by one and two dimensional protein nuclear magnetic resonance spectroscopy (¹H-NMR) permitted the attribution of the oligosaccharides to individual attachment sites. These studies, carried out before the genetic variants were known, found no evidence of an extra glycosylation site. Our data, by

contrast, while finding evidence of fragments of many of the sugars described by Green & Baenziger (1988a,b) and Weisshaar *et al.* (1991), could not have been analysed without the assumption that a second carbohydrate moiety was present on the molecule. We found that virtually all the glycosylation on peptides bearing a mutation at β 15 arises from either two separate sugars or from a strong possibility of there being two sugars on that peptide. It should be pointed out that due to the small quantity of pure sample available for this investigation it was not possible to obtain MS/MS or MALDI-TOF evidence of peptide mass following exo- or endoglycosidase digestion that would confirm the presence of one or two mutations. Furthermore, in the earlier studies the separation techniques used largely confined the detection of LH *N*-linked oligosaccharides to charged species possessing at least one terminating sialyl or sulfate group (Green & Baenziger 1988a,b, Weisshaar *et al.* 1991). The fact that only degraded neutral species lacking terminating sialyl or sulfate groups were found here is therefore of interest even though MS has a bias towards the detection of neutral species. It may be that the MALDI-TOF used here detected only glycopeptides containing inefficiently compiled oligosaccharides or sugars degraded within the pituitary. However, as the lack of glycosidase activity within the pituitary is thought to leave LH carbohydrate moieties intact (Birken *et al.* 1996), it is more likely that degradation of the sugars occurred during an isolation or purification process or as a result of prompt fragmentation within the MALDI-TOF source.

The utilisation of the mass of the primary sequence of the LH β cf glycosylated peptide with no substitutions, with a substitution at either β 8 or β 15 or at both positions to determine each peak's glycosylation profile, showed that all those species were present in our LH β cf pooled sample. It has been suggested from molecular biology studies that the two point mutations occur together in the vLH molecule (Nilsson *et al.* 1997). The sites of the mutations within the LH β gene occur within close proximity of each other and may not necessarily be expected to separate as a result of crossover events during meiosis. However, most of the population studies have been based on differences in immunoassay reactivity measurements of LH rather than detection of the point mutations within

the gene by molecular biology techniques (Haavisto *et al.* 1995, Nilsson *et al.* 1997, 1998). Thus it is possible that the two mutations can occur separately. Because of the mass differences conferred by these amino acid substitutions our results suggest that this is the case. This would have to be confirmed using molecular biology techniques but is strongly indicated by the data analysis presented here.

Clinically, abnormal LH secretion has been associated with a range of menstrual and fertility disorders such as polycystic ovary syndrome (PCOS) (Homburg *et al.* 1988, Regan *et al.* 1990, Balen *et al.* 1993, Risma *et al.* 1995). However, investigation into the function of vLH in PCOS and other fertility disorders have produced conflicting results (Rajkhowa *et al.* 1995, Suganuma *et al.* 1995, Takahasi *et al.* 1998, Tapanainen *et al.* 1999). MALDI-TOF MS of LH β cf produces a complex but distinctive fingerprint spectrum. Analysis of the oligosaccharides remaining on LH β cf will determine the *N*-linked sugars remaining on the metabolite and also determine which sugars are present on LH β peptides encoded by LH β gene variants. By doing so it may, at a later date, be possible to show that this technique has a diagnostic value by linking the genetic polymorphism of the LH β gene with the fertility disorders to which vLH has been associated. Ideally, sampling should be non-invasive and could take place from the urine. In individuals, the major breakdown product of LH in the urine is the core fragment LH β cf. Direct MALDI-TOF MS analysis in our laboratories of unextracted non-pregnant female urine samples taken shortly after the LH surge demonstrates a spectral peak corresponding to LH β cf. This indicates that its concentration in urine is at a suitable level for detection purposes. Currently, ultrafiltration techniques are being investigated which will allow sample concentration and desalting as well as the removal of low molecular weight peptides that interfere with the MALDI-TOF mass spectrum following DTT reduction. By doing so, this clinical utility will potentially allow the facile identification of LH polymorphic variation in individuals prior to diagnosis by more complicated genetic tests which will be carried out as fresh stocks of the LH β cf become available following in-house purification.

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