Structural Investigation of the Carbohydrate Element of Human Pituitary Follicle-Stimulating Hormone by Methylation Analysis

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A preparation of human follicle-stimulating hormone has been subjected to methylation analysis by using the methyl sulphanyl carbanion-dimethyl sulphoxide-methyl iodide method. The hydrolysis products of the methylated glycoprotein were reduced, acetylated, analysed by gas-phase chromatography and mass spectrometry and identified by comparison with standards. Methylation analysis demonstrated that (1) the D-galactose, mannose, fucose and 2-amino-2-deoxyglucose units exist in the pyranose forms, (2) the 2-amino-2-deoxyglucose units are *N*-acetylated, (3) the fucopyranose units occupy terminal non-reducing positions, (4) the D-galactopyranose units are linked in the 1- and 2-positions, (5) the mannopyranose units exist in three forms, some as terminal non-reducing residues, some as 1,6-linked residues and some as 1,3,4-linked branch points, and (6) the 2-acetamido deoxyglucopyranose units are 1,6-linked. These structural assignments are compared with other data previously obtained for the carbohydrate moieties of follicle-stimulating hormone.

The carbohydrate compositions of several preparations of human pituitary follicle-stimulating hormone have been reported (Barker *et al.*, 1969; Papkoff *et al.*, 1967; Papkoff & Ekblad, 1970; Reichert *et al.*, 1968; Roos, 1968), and it is known that the hormone contains D-galactose, D-mannose, L-fucose, 2-amino-2-deoxy-D-glucose, *N*-acetylneuraminic acid and possibly 2-amino-2-deoxy-D-galactose. The configurations are assumed except for D-galactose, where it has been proved by the use of D-galactose oxidase. However, little information has been obtained on the arrangement of these carbohydrate residues in the molecular structure.

Since the *N*-acetylneuraminic acid units of the hormone are easily removed by the action of neuraminidase, it is presumed that they occupy terminal non-reducing positions (Chaplin *et al.*, 1970). The asialo hormone was active immunologically (Butt *et al.*, 1970) and exhibited biological activity *in vitro* (Ryle *et al.*, 1970) but was biologically inactive *in vivo*. Thus it appears that at least some sections of the carbohydrate structure are essential for activity. Morell *et al.* (1971) have presented evidence indicating a generalized role for the terminal sialic acid residues of circulating glycoproteins including FSH.* The desialized molecules were found to be removed promptly from the circulation in rats and were recovered from the liver. Removal of the *N*-acetylneuraminic acid units also rendered FSH a substrate for D-galactose oxidase (Chaplin *et al.*,

1970), suggesting that the D-galactose residues are adjacent to the *N*-acetylneuraminic acid units. That L-fucose units also occupy terminal positions in the carbohydrate structure seemed likely since L-fucosidase partially removed L-fucose from FSH. The results of periodate-oxidation studies of FSH narrowed the possibilities for the linkage positions of the carbohydrate units (Kennedy & Butt, 1969). Further structural detail of the carbohydrate element of FSH is now reported, based on the results of methylation analysis under the conditions devised by Hakomori (1964).

Materials and Methods

Activities and analyses

Biological and immunological activities. Biological activity was determined *in vivo* by the ovarian augmentation method (Brown, 1955). Immunological activity was determined by the single-antibody solid-phase radioimmunoassay (Butt & Lynch, 1968).

Infrared spectral analysis. Infrared spectra were obtained by analysis of the test material as a potassium bromide disc with a Perkin-Elmer 472 spectrometer.

Amino acid analysis. After hydrolysis at 108°C in 5.9M-HCl for 24h, samples were analysed for amino acids with a Technicon AutoAnalyser.

Mass-spectral analysis. Mass spectrometry was carried out with an AEI MS9 double-focusing mass spectrometer fitted with a heated inlet system and a

* Abbreviation: FSH, follicle-stimulating hormone.

direct insertion probe, with a source temperature of 180–190°C.

Preparation of standard methyl carbohydrates

Samples of partially methylated monosaccharides were prepared by partial methylation of the parent sugars by using the method of Purdie & Irvine (1903). After hydrolysis of the glycosidic methyl groups and reduction, the mixtures of the partially methylated carbohydrates were separated as their acetyl derivatives by gas-phase chromatography, and analysed by mass spectrometry, as described under 'Analysis of methylated FSH' below. Other methyl monosaccharide standards were kindly made available by Professor S. A. Barker and Dr. J. M. Webber.

Preparation of FSH

Human pituitary follicle-stimulating hormone, preparation FSH-CP150, was prepared by gel filtration of FSH-CP1 on Bio-Gel P-150 as previously described (Barker *et al.*, 1969). Fractions corresponding to maximum biological activity *in vivo* were bulked, dialysed against distilled water (4 × 2 litres) at 4°C and freeze-dried (yield approx. 75 mg). The biological and immunological activities of the product *in vivo* were determined, and its infrared spectrum was recorded.

Methylation studies

Methylation of FSH. Dimethyl sulphanyl carbanion was prepared by an adaptation of the method of Sandford & Conrad (1966). FSH-CP150 (75 mg) was dissolved with stirring in dry redistilled dimethyl sulphoxide (5 mg/ml) contained in a serum-capped flask. An equal volume of 2M-dimethyl sulphanyl carbanion in dimethyl sulphoxide was added through the cap, an atmosphere of dry oxygen-free nitrogen being used. After the mixture was stirred for 6 h in the dark, dry redistilled methyl iodide (15 ml) was added with cooling and the mixture stirred at 20°C for 1 h. Excess of methyl iodide was evaporated off under reduced pressure through the cap and the FSH methylated again with further similar volumes of 2M-dimethyl sulphanyl anion in dimethyl sulphoxide and methyl iodide as before. Finally, excess of methyl iodide was evaporated off.

Isolation of methylated FSH. The dimethyl sulphoxide solution of methylated FSH was diluted with 3 vol. of water and extracted twice with equal volumes of redistilled chloroform. The combined chloroform extracts were washed twice with water and the washings added to the aqueous solution. Both the aqueous and organic solutions were evaporated by rotary evaporation *in vacuo* until only residue remained.

The residue from the organic solution was further evaporated with a small volume of ethanol–benzene (4:1, v/v), leaving a brown oily solid (methylated FSH-O, 22 mg; 29% yield). The residue from the aqueous solution, which contained dimethyl sulphoxide, was fractionated in two parts on a column (150 cm × 2.5 cm diam.) of coarse Sephadex G-25 at 4°C with water as eluent. Fractions corresponding to excluded materials were combined, concentrated to a small volume by rotary evaporation *in vacuo*, and refractionated on the same column. The fractions containing the excluded material were bulked and freeze-dried to yield a white flocculent solid (methylated FSH-A, 45 mg; 60% yield). The biological and immunological activities of the freeze-dried methylated FSH-A were determined and an infrared spectrum was obtained. The amino acid contents of the methylated fractions were also determined.

Analysis of methylated FSH. Samples of methylated FSH-A and methylated FSH-O were hydrolysed with 90% (w/v) formic acid for 1.5 h at 100°C (8 mg/ml). The acid was then removed by rotary evaporation, and the residue redissolved in 0.125M-sulphuric acid (4 mg/ml) and hydrolysis continued for 14 h at 100°C. The hydrolysates were neutralized by the addition of barium carbonate, and the solid was centrifuged off. After removal of the supernatant, the solid was washed with water (2 × 2 ml) and ethanol (2 × 2 ml). The combined washings and supernatant were evaporated by rotary evaporation.

Reduction and acetylation of the hydrolysates was accomplished by the method of Albersheim *et al.* (1967). The residues containing the methyl sugars were treated with sodium borohydride (10 mg/ml) in aq. 0.5M-ammonia soln. for 1 h at 20°C. Excess of borohydride was decomposed by the dropwise addition of redistilled acetic acid followed by rotary evaporation to dryness. Boric acid was removed by repeated treatment (five times) with redistilled methanol (1 ml) and rotary evaporation to dryness to remove methyl borate and excess of methanol.

The residues, containing the methyl alditols, were acetylated with redistilled acetic anhydride (1 ml) in sealed tubes at 100°C for 3 h. Water (1 ml) was added and the solution evaporated to dryness, final traces of water being removed by azeotropic distillation with ethanol–benzene (4:1, v/v); the alditol acetates were redissolved in redistilled chloroform (100 µl). Analytical gas chromatography of portions (1 µl) of the solutions was carried out on columns (250 cm × 0.4 cm diam.) of 3% ECNSS-M on Gas-Chrom Q (Phase Separations Ltd.; 100–120 mesh) with a Series 104 gas chromatograph (Pye Unicam Ltd.). Nitrogen at a flow rate of 40 ml/min was used as carrier gas; runs were made isothermally at 170°C or 185°C, and peaks were recorded by using a flame-ionization detection system and a 0–10 mV recorder (Honeywell). The retention time of 1,5-di-*O*-acetyl-2,3,4,6-

tetra-*O*-methyl-D-glucitol was used as a standard, the value being determined before and after each analytical run.

By using the results obtained to anticipate the elution of peaks from the column, preparative gas chromatography was carried out similarly but with a carrier gas flow rate of 100 ml/min and a 10:1 stream-splitter. The major proportion of the gaseous outlet did not pass to the detector but was led out via a side arm into a U-tube containing glass beads and cooled by liquid air. Individual components were separately trapped, their elution positions being anticipated by reference to the corresponding analytical run and to the recorder chart. The U-tubes were flushed out with redistilled chloroform, which was then evaporated off, and the residues were redissolved in chloroform (10 μ l).

Results

Assay and analyses

Biological and immunological activities. The biological activity *in vivo* and the immunological activity of the FSH-CP150 were both found to be 1500 i.u. of FSH/mg; the corresponding values for methylated FSH-A were 0 and <10 i.u./mg.

Infrared spectral analysis. The infrared spectra of FSH-CP150 and methylated FSH-A are shown in Fig. 1.

Amino acid analysis. The amino acid analyses of the methylation products of FSH gave a yield of only 8% of the weight of the methylated glycoprotein as detected amino acids, presumably owing to partial methylation of the amide nitrogen atoms.

Methylation analysis

The chromatographic retention times of the alditol acetates derived from the neutral sugars of methylated FSH-A and methylated FSH-O were corrected for minor variations between runs by reference to the elution position of the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol standard. The components of the chromatograms were identified by careful comparisons of the corrected retention times with those obtained for a range of standard methylated monosaccharides (Table 1). The identifications were confirmed by mass-spectral analysis of the components from the preparative runs, and checking the prominent peaks with those obtained for the standards and with published data (Björndal *et al.*, 1970).

The deviation of the relative retention times for the fucose derivatives (component 1) from unity is

Fig. 1. Infrared spectra of (a), FSH-CP150 and (b) methylated FSH-CP150

Table 1. *Methylation analysis of FSH*

Retention times (t_R) are expressed relative to that for the appropriate standard. Values in columns 3 and 5 refer to the percentage of total methylated components recovered, as measured from peak areas.

Component no.	G.l.c. results				Identification (based on g.l.c. and mass-spectral data)
	Methylated FSH-A		Methylated FSH-O		
	<i>t</i> _R	%	<i>t</i> _R	%	
1	0.88	8	0.90	4	1,5-Di- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl-L-fucitol
2	1.03	6	1.06	8	1,5-Di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl-D-mannitol
3	0.86	14	1.03	14	1,2,5-Tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methyl-D-galactitol
4	0.97	18	0.96	8	1,5,6-Tri- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl-D-mannitol
5	0.93	11	0.93	4	1,3,4,5-Tetra- <i>O</i> -acetyl-2,6-di- <i>O</i> -methyl-D-mannitol
6	*	25	*	4	1,5,6-Tri- <i>O</i> -acetyl-3,4-di- <i>O</i> -methyl-2-(<i>N</i> -methyl)acetamido-2-deoxy-D-glucitol
7	*	4	*	32	1,5,6-Tri- <i>O</i> -acetyl-3,4-di- <i>O</i> -methyl-2-acetamido-2-deoxy-D-glucitol

* See the Results section.

attributed to the difficulties encountered in making precise measurements for low retention times, it being necessary to operate the chromatograph at temperatures suited to elution of the basic sugar derivatives as discrete peaks (see below). However, that the identification was correct was confirmed by mass spectrometry. The low retention time for the 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-galactitol (component 3) derived from methylated FSH-A was considered to have been influenced by the adjacent elution position of an unidentified peak, which appeared to be of proteinaceous origin. Again the identification was confirmed by mass spectrometry.

Two further peaks (components 6 and 7) arising from both methylated FSH-A and methylated FSH-O possessed retention times much greater than those of the neutral sugar derivatives, and occurred in the positions of derivatives of 2-amino-2-deoxyhexoses (Hellerquist & Lindberg, 1971; B. Lindberg, personal communication). Although simple mass spectrometry will not differentiate between the configurations of monosaccharides of a particular type, it appeared that the two peaks could have arisen from 2-amino-2-deoxy-D-glucose, since this is by far the predominant 2-amino-2-deoxyhexose of FSH. That the peaks had arisen from such residues was confirmed by consideration of the mass spectra.

Component 6 gave prominent peaks at $m/e = 233$, 189, 158, 116 and 43, these values being consistent with the expected breakdown pattern of 1,5,6-tri-*O*-acetyl-3,4-di-*O*-methyl-2-(*N*-methyl)acetamido-2-deoxy-D-glucitol. Analogous mass spectra have been reported as preliminary results for similar

derivatives of other methylated 2-amino-2-deoxy-D-glucose (Björndal *et al.*, 1970).

Although it is reported that the methylation procedure of Hakomori (1964), when applied to pure carbohydrates, always gives *N*-methylated 2-acetamido-2-deoxyhexoses (Björndal *et al.*, 1970), appearance of component 7 together with its mass spectrum suggested that with FSH only some of the 2-acetamido-2-deoxyhexose residues were *N*-methylated. Thus the mass spectrum of component 7 gave prominent peaks at $m/e = 233$, 189, 128 and 43 and comparison of these values with those for 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-2-acetamido-2-deoxy-D-glucitol confirmed the component to be 1,5,6-tri-*O*-acetyl-3,4-di-*O*-methyl-2-acetamido-2-deoxy-D-glucitol. It may be that the presence of protein and the tertiary structure of FSH modulates the *N*-methylation, giving rise to the two derivatives of 2-acetamido-2-deoxy-D-glucose units.

Certain other minor peaks observed in the chromatograms were considered to have arisen from the relatively unstable *N*-acetylneuraminic acid units of FSH. This was substantiated by virtue of their mass spectra, which could be fitted to derivatives of 2-acetamido-2-deoxy-D-mannose, a constituent structure of *N*-acetylneuraminic acid.

Discussion

The methyl sulphiny carbanion-dimethyl sulphoxide-iodide technique of Hakomori (1964) has been found by Hellerqvist *et al.* (1968) and Björndal & Lindberg (1969) to methylate fully some poly-

saccharides in one step. When applied to glycoproteins, the alkaline conditions at the methylation stage can give rise to β -elimination at any glycosylserine or glycosylthreonine linkages, releasing the polysaccharide units, which may have molecular weights less than 10000. For this reason dialysis, which is often used for isolation of methylated polysaccharides, was avoided. However, materials having molecular weights lower than 5000 would not be recovered in the excluded fraction in the gel-filtration purification, and a chloroform-extraction step was therefore included to remove lower-molecular-weight methylated oligosaccharides before gel filtration.

The use of [^{14}C]methyl iodide to determine when methylation had reached a maximum was not applicable to the methylation of a glycoprotein since the amide linkages can become methylated but do so at rates slower than methylation of free hydroxyl groups. Similarly, infrared spectral analysis is not so conclusive for the glycoprotein owing to masking of any residual hydroxyl absorption by amide absorption. However, comparison of the infrared spectra of FSH and methylated FSH (Fig. 1) shows that the $-\text{OH}$ and $-\text{NH}-$ absorptions (3360cm^{-1}) are markedly decreased, shifted in frequency (3460cm^{-1}) and sharpened on methylation, indicative of loss of hydrogen bonding. The decrease in the monosubstituted amide absorption (1545cm^{-1}) indicated that some of the amide nitrogen had been methylated, the absorption (1650cm^{-1}) corresponding to both mono- and di-substituted nitrogen being retained. The peak at 1740cm^{-1} appearing on methylation might be due to methyl carboxyl ester groups produced by esterification of neuraminic acid, aspartic acid and glutamic acid residues. The increase in absorption at 2820cm^{-1} and particularly 1115cm^{-1} demonstrates the production of methoxyl groups.

In view of the lability of *N*-acetylneuraminic acid units, the loss of biological activity on methylation is not surprising. The loss of immunological activity, in contrast to retention during periodate oxidation (Kennedy & Butt, 1969), is possibly due to modification of the protein structure by *N*-methylation of the amino acids.

The presence of amino acids in the chloroform extract would suggest that the occurrence of carbohydrate in methylated FSH-O cannot be due to β -elimination alone. It may also have arisen, for example, from more extensive breakdown of the peptide chain, by partial extraction of higher-molecular-weight material, or by cleavage of e.g. weaker bonds involved in linking of the subunits of the glycoprotein, subunit formation by other glycoprotein hormones being well established (Butt & Kennedy, 1971). Nevertheless, it is not possible to say from analysis for normal amino acids whether or

not the amino acid content of methylated FSH-O is appreciable.

The total yield of methylated FSH (89%) compares very favourably with yields reported for a urinary glycoprotein (15%) and ovomucoid (25%) obtained with fairly similar methylation procedures but less rigorous isolation procedures (Hakomori, 1964).

From the results of analysis of the two methylated FSH fractions, methylated FSH-A and methylated FSH-O (Table 1), it is possible to make structural assignments to the carbohydrate units of FSH. Reference to the general identity of the components 1-7 reveals that in each case the 5-position of the compound is *O*-acetylated. This corresponds to the involvement of the hydroxyl at C-5 in ring formation, and thus the results demonstrate that the L-fucose, D-galactose, D-mannose and 2-amino-2-deoxy-D-glucose units of the hormone all exist in the pyranose forms. The pyranose forms have hitherto been assumed.

Component 1, 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-fucitol would arise from methylation, hydrolysis, reduction and acetylation of a terminal non-reducing L-fucopyranose unit. This identification is in agreement with the periodate-oxidation studies (Kennedy & Butt, 1969). Acid-hydrolysis studies (J. F. Kennedy & M. F. Chaplin, unpublished work) have indicated that the fucose units of FSH, together with the neuraminic acid units, are easily liberated with little breakdown of the remainder of the carbohydrate moieties of FSH.

Component 2, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol, would be derived from a terminal non-reducing D-mannopyranose unit. Periodate-oxidation studies (Kennedy & Butt, 1969) similarly indicated that some D-mannopyranose residues could be terminal non-reducing. Component 4, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-mannitol, corresponds to 1- and 6-substituted D-mannopyranose units in FSH, again as predicted by the periodate-oxidation studies. Component 5 was identified as 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-D-mannitol. The existence of branch points involving D-mannose units was indicated by the periodate-oxidation studies, and the present identification demonstrates the involvement of a 1,3,4-linked D-mannopyranose residue.

The identity of component 3, 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-galactitol, indicates the presence of 1- and 2-linked D-galactopyranose units in FSH, as did the periodate-oxidation studies.

The appearance of components 6 and 7, 1,5,6-tri-*O*-acetyl-3,4-di-*O*-methyl-2-(*N*-methyl)acetamido-2-deoxy-D-glucitol and 1,5,6-tri-*O*-acetyl-3,4-di-*O*-methyl-2-acetamido-2-deoxy-D-glucitol, demonstrates the existence of 1- and 6-linked 2-acetamido-2-deoxy-D-glucopyranose units, in full agreement with the periodate results. The mass-spectral data

Table 2. Comparison of carbohydrate component and methylation results for FSH

Methylation results				
Carbohydrate unit	% of identified products*		Composition	
	Linkage type	Unit type	Carbohydrate unit	%*
1-linked L-fucopyranose	8	8	L-Fucose	4
1- and 2-linked D-galactopyranose	17	17	D-Galactose	17
1-linked D-mannopyranose	8	36	D-Mannose	36
1- and 6-linked D-mannopyranose	18			
1-, 3- and 4-linked D-mannopyranose	10			
1- and 6-linked 2-acetamido-2-deoxy-D-glucose	39	39	2-Acetamido-2-deoxy-D-glucose	41
			2-Acetamido-2-deoxy-D-galactose	2

* % of the total of the units given in the table.

for components 6 and 7 also show that the 2-amino-2-deoxy-D-glucose units are *N*-acetylated, a fact which has hitherto been assumed by analogy with other glycoproteins.

In addition to substantiating the results of periodate-oxidation studies, these results also resolve a number of ambiguities arising from such. Thus, whereas the periodate-oxidation results could not distinguish between L-fucose units linked in the 1- and 2-positions and those linked in the 1-position only, the methylation results eliminate the former possibility. Similarly, the possibilities that some of the D-mannose units were 1- and 2-substituted and 1- and 3-substituted are now eliminated. The present results also demonstrate the linkage points of the mannose residues involved as branch points. Since only one type of D-galactopyranose residue was indicated by the methylation results, the former possibilities of 1- and 3-linked and 1- and 6-linked units no longer exist. The 41% D-galactose units recovered intact after periodate oxidation would therefore appear to represent inefficient oxidation, a situation likely to be due to the adjacent position of the acidic *N*-acetylneuraminic acid units in the FSH structure. It appears that all the 2-acetamido-2-deoxy-D-glucopyranose residues are 1- and 6-linked, and the 13% found to be unoxidized by periodate could again represent inefficient oxidation.

The amounts of the various sugar residues present were calculated from the results of the methylation analysis (Table 2), an equimolar response in the flame-ionization-detection system being assumed. The values show moderate agreement with those expected from the component analysis of the FSH-CP150. The differences in the methyl sugar contents

of methylated FSH-A and methylated FSH-O suggest that the latter fraction could not have arisen entirely by partial extraction of the higher-molecular-weight material. Nor do these differences necessarily imply that cleavage of the peptide chains or degradation of the carbohydrate chains has occurred. Indeed it can be argued that, for carbohydrate degradation, the more substituted residues are more resistant to alkaline degradation and therefore that a fraction which contains alkaline degradation products would contain a higher proportion of such residues than the fraction containing the undegraded material. Because of the large number of non-reducing end groups and the terminal non-reducing position of neuraminic acid (Chaplin *et al.*, 1970), the results suggest that, unless FSH contains one very highly branched carbohydrate moiety, there must be more than one carbohydrate moiety in the molecule. The latter situation would be in agreement with the ability of the glycoprotein hormones to form two subunits, both containing carbohydrate (Butt & Kennedy, 1971). It should be noted, however, that a limitation of the technique presently employed is that the results would not indicate if the carbohydrate part of the FSH preparation is heterogeneous.

The earlier results obtained with galactose oxidase (Chaplin *et al.*, 1970) would indicate that the galactose residues are linked to the neuraminic acid residues, which then protect against enzymic oxidation. If these were linked to the 3- and 4-positions of the mannose branch point, in turn linked 1→6 to a comparatively long carbohydrate chain, this would help explain the lack of response of FSH to enzymic hydrolysis by glycosidases (Chaplin *et al.*, 1970), owing to steric hindrance.

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