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THE STRUCTURE OF A SULFATED GLYCOPROTEIN OF CHICK ALLANTOIC FLUID: METHYLATION AND PERIODATE OXIDATION*

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ABSTRACT

The structure of an antigenic, sulfated glycoprotein from chick chorioallantoic fluid has been investigated by exogalactosidase digestion, methylation and mass spectral analyses, periodate oxidation, and Smith degradation. The main carbohydrate chains are composed of D-galactosyl residues linked at C-3 and 2-acetamido-2-deoxyglucose residues linked at C-4. Fucose and *N*-acetylneuraminic acid residues are nonreducing terminal groups, and the *N*-acetylneuraminic acid groups are linked to the D-galactose residues at C-3. Most of the sulfate groups (91% of the sulfate) are located on C-6 of the 2-acetamido-2-deoxyglucose residues, and the rest on C-6 of the D-galactose residues. A large number of the D-galactose residues (36.9% of the total) are present as nonreducing terminal groups and another 21.7% of the D-galactose residues are in penultimate position to the nonreducing terminal *N*-acetylneuraminic acid residues. Although mild periodate oxidation indicates the presence of D-galactose in furanoside form (5.5% of total D-galactose), no 5-*O*-methyl derivative of D-galactose was observed on methylation.

INTRODUCTION

A sulfated glycoprotein (CAFS), isolated from the chick allantoic fluid infected by influenza virus, has been studied in our laboratory[1–3] as well as in others[4–6]. It has been shown[2] that the carbohydrate chains in the antigenic, sulfated glycoprotein (mol. wt. 26,000) are highly heterogeneous, ranging from an average mol. wt. of 1,100 to 2,000. The chains are linked to the peptide backbone through an alkali-labile *O*-glycosyl linkage of a 2-acetamido-2-deoxygalactose residue[1,2]. The sulfate groups are located on the 2-acetamido-2-deoxyglucose residues, and the *N*-acetylneuraminic acid residues are the nonreducing terminal groups[2]. Fatty acids are attached to the hydroxyl groups of various carbohydrate units in ester linkage[2]. The present paper reports further structural studies of the glycoprotein by methylation and mass spectroscopy, exogalactosidase digestion, periodate oxidation, and Smith degradation.

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*Dedicated to Professor Michael Heidelberger, in honor of his 87th birthday.

RESULTS AND DISCUSSION

As has been reported previously, the exo-α- and β-D-galactosidases of jack bean and *E. coli* did not liberate D-galactose from the CAFS significantly, even after incubation of up to 10 days[2]. However, an exo-D-galactosidase preparation from *Coccobacillus*, which contained both α- and β-D-galactosidase activities as tested with p-nitrophenyl α- and β-D-galactopyranosides as substrates, hydrolyzed under similar conditions 30% of the total D-galactose residues from the native CAFS after 3 days, thus indicating ~1/3 of the D-galactose residues to be located at the nonreducing terminal.

Electrostatic charges in polymers are known to hinder the rate of periodate oxidation by repulsing the oncoming periodate ion. To reduce this effect, an inert electrolyte was added to the oxidation system[7,8]. The rate of the oxidation of the CAFS (in 25mM sodium periodate containing 0.2M sodium perchlorate, at 30°, in the dark) leveled off after 4 h, and no observable overoxidation was detected (Fig. 1). The oxidation of the glycoprotein was stopped after 5 min, and followed by sodium borohydride reduction. Acid hydrolysis of the product (2M hydrochloric acid for 5 h at 100°) gave arabinose, equivalent to 5.5% of the total galactose, among other products. This indicates that at least some of the D-galactose residues in the glycoprotein exists in furanoside form, and this could be the explanation for the resistance of the glycoprotein to the exogalactosidases of jack bean and *E. coli*. The exogalactosidase preparation from *Coccobacillus* might have different specificity permitting hydrolysis of the furanoside and pyranoside forms.

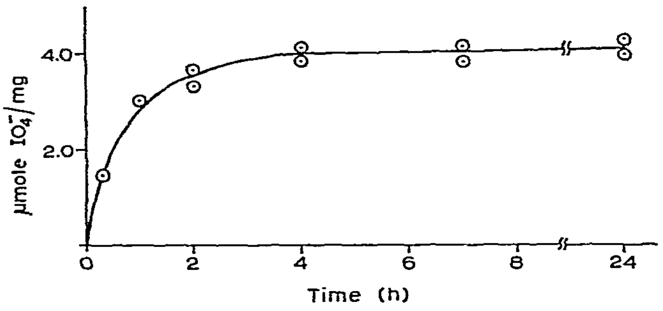

Fig. 1. Rate of oxidation of CAFS by periodate.

For the Smith degradation, the periodate oxidation was carried out routinely for 16 h. The analytical results from each step of the Smith degradation are presented in Table I. As expected, *N*-acetylneuraminic acid and fucose were completely destroyed, and 38% of the D-galactose of the native CAFS was degraded after the first periodate oxidation. Removal of *N*-acetylneuraminic acid from the CAFS increased the proportion of D-galactose residues oxidized to 65.5%. The second Smith degradation did not, however, change the D-galactose content, and the amount of

TABLE I

COMPOSITION OF THE PRODUCTS OF THE SMITH DEGRADATION OF CAFS BEFORE AND AFTER REMOVAL OF *N*-ACETYLNEURAMINIC ACID

CAFS	*Products of degradation*[a]				
	Gal	*GalNAc*	*GlcNAc*	*NeuNAc*[b]	*Fuc*
Native					
Untreated	100	100	100	100	100
After 1st degradation	62.0	98.3	96.5	0	0
After 2nd degradation	60.8	95.4	98.1	0	0
After removal of NeuNAc					
Untreated	100	100	100		100
After 1st degradation	34.5	96.1	99.1		0
After 2nd degradation	33.9	94.9	98.1		0

[a] In %. [b] Abbreviation: NeuNAc, *N*-acetylneuraminic acid.

hexosamine was not affected either by the first or the second Smith degradation. These results indicate that the glycoprotein has a considerable proportion of D-galactose residues as nonreducing terminal groups and that *N*-acetylneuraminic acid residues are linked to C-3 of the D-galactose residues. However, a puzzling result in this experiment was the resistance of CAFS to periodate oxidation during the second Smith degradation. A possible explanation may be that the periodate ions were not accessible to the sugar molecules of the glycoprotein, owing to the closeness of the carbohydrate chains to each other on the peptide backbone, in addition to the shielding effect of the sulfate groups.

Infrared spectroscopic analysis indicated the presence of a primary axial sulfate group. Its location was identified by methylation analyses of the glycoprotein before and after desulfation. The desulfation product, which was obtained in 77% of the theoretical yield by heating at 100° in dimethyl sulfoxide solution containing 2%

TABLE II

CARBOHYDRATES COMPONENTS OF NATIVE AND DESULFATED CAFS USED FOR METHYLATION AND OXIDATION

Components	*CAFS*	
	Native	*Desulfated*
Galactose (%)	30.1	31.2
Hexosamine (%)	29.2	29.5
Sulfate (%)	6.2	0.0
N-Acetylneuraminic acid (%)	13.0	7.0
Fucose (%)	1.3	1.1
Ratio (%) of 2-amino-2-deoxyglucose to 2-amino-2-deoxygalactose	56.3:43.7	56.1:43.9

pyridine[9] for 9 h, was soluble in water, formamide, *N*,*N*-dimethylformamide, and dimethyl sulfoxide. The analysis of the product indicated that a small amount of fucose and a considerable proportion of *N*-acetylneuraminic acid residues (65%) were cleaved off from the glycoprotein, but that no 2-amino-2-deoxygalactose was lost during the desulfation (see Table II). A study[10] of the mol. wt. of a desulfated keratan sulfate of porcine nucleus pulposus by gel filtration on a Bio-Gel P-100 column and elution with formamide indicated that the desulfation method used in the present work resulted in a higher degree of depolymerization (due to the cleavage of glycosidic bonds) than desulfation effected by the methanolic acid procedure[11,12].

Methylation of CAFS was obtained by Hakomori's procedure[13,14] in dimethyl sulfoxide with three additions of methylsulfinyl carbanion and methyl iodide, alternatively, followed by Purdie's technique modified by Kuhn[15]. Because of the low solubility of native CAFS in dimethyl sulfoxide, CAFS was acetylated[16] prior to methylation, but not the desulfated CAFS which was readily soluble in dimethyl sulfoxide. Overall yield of methylated CAFS was 74.5% with a methoxyl content of 21.8% for native CAFS. The yield for the desulfated CAFS was 79.2% with a methoxyl content of 23.2%. The relatively low value of the methoxyl group was due to the high content of amino acid (12.7%) in the CAFS preparations. The methylated CAFS was hydrolyzed either by formolysis followed by acid hydrolysis[17], or by acetolysis followed by acid hydrolysis[18], and the partially methylated monosaccharides in the hydrolyzate were fractionated into neutral and amino sugar fractions through a column of cation-exchange resin.

The *O*-methyl ethers of the 2-amino-2-deoxy sugars were further separated by g.l.c. and identified by g.l.c.–m.s. analysis as their alditol acetates. In addition, they were tested for susceptibility to periodate oxidation and analyzed by paper chromatography for the identification of the amino sugars. G.l.c. on a 3% ECNSS column yielded two peaks, of which Peak 2 (see Fig. 2) was readily identified as 1,4,5,6-tetra-*O*-acetyl-2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)glucitol (**1**) by the retention time, together with the mass spectra of the corresponding marker, since its retention time is easily distinguishable from that of the galactitol isomer. However, Peak 1 (see Fig. 2) was not readily identifiable by the retention time, because the retention times of 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol (**2**) and its galactitol isomer are very close to each other, and the mass spectra of these two isomers are identical. The co-injection of the two markers produced a single peak. Three possibilities have to be considered for the identification of Peak 1. In a previous communication[2], it was reported that almost all 2-acetamido-2-deoxygalactose residues of the CAFS were located at the reducing end of the carbohydrate chain linked to the serine and threonine residues of the peptide backbone, since up to 86% of 2-acetamido-2-deoxygalactose was lost by treatment with aqueous alkali with concomitant formation of a Kuhn's chromogen. Since the methylation reaction took place in a strong alkaline-organic medium, it is possible that Peak 1 was that of a methylated Kuhn's chromogen. On the other hand, if the chromogen did not survive the alkaline medium during methylation and acid hydrolysis, Peak 1 was possibly

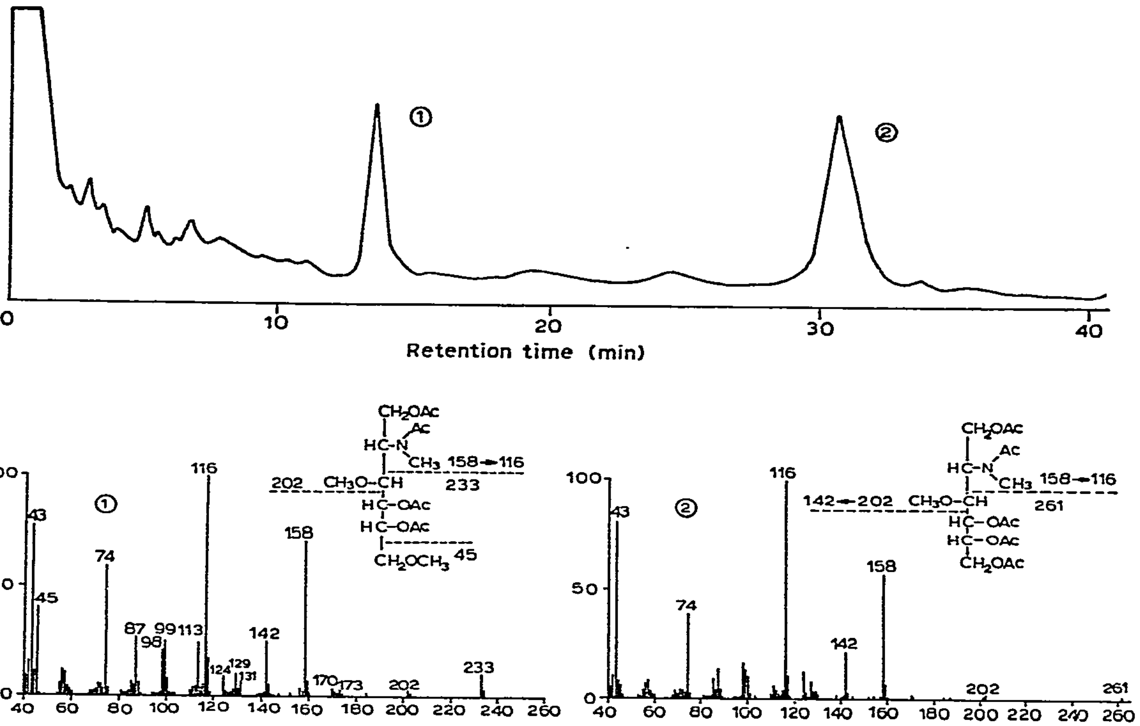

Fig. 2. G.l.c. and m.s. analysis of the fraction containing *O*-methyl 2-deoxy-2-(*N*-methylacetamido) compounds from the methylated CAFS, as alditol acetates.

derived from **2**. A third possibility was that Peak 1 was simply that of 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol (**3**), provided no elimination reaction took place. The mass spectrum of Peak 1 (see Fig. 2) is almost identical with that of synthetic **2** and **3**, thus eliminating the possibility of Peak 1 being that of a methylated Kuhn's chromogen. To investigate the possibility of Peak 1 being that of either **2** or **3**, unsuccessful attempts were made to demethylate the methylated amino sugar fraction with boron tribromide[18] or hydrogen iodide[19]. Apparently, the rate of destruction of the hexosamines was faster than the demethylation reaction.

When placed on a Whatman No. 1 paper and developed in Solvent A, the fraction of methylated amino sugar, obtained from the hydrolyzate of methylated CAFS, yielded two spots; one had the mobility of 2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)glucose (R_F 0.61) and the other had a mobility of R_F 0.92, which is close to the mobility of synthetic 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucose (R_F 0.89). The mobility of R_F 0.92 was easily distinguishable from that of synthetic 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactose (R_F 0.81). Additional evidence that Peak 1 (see Fig. 2) was that of **2** came from g.l.c. analysis. Although the 3% ECNSS column under the condition used failed to resolve the peaks of **2** and **3** when injected together, there was a slight difference in their retention times.

This difference could be utilized for the distinction between the two methylated isomers by use of two internal standards, 1,2,3,4,5-penta-*O*-acetyl-D-arabinitol (**4**) and 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucitol (**5**). The ratios of the difference in the retention times calculated according to $R_{T_3}-R_{T_2}/R_{T_3}-R_{T_1}$ were reproducible: Peak 1 (see Fig. 2) had a ratio of 0.60, **2** of 0.60, and **3** of 0.62; R_{T_1}, retention time of **4** (6.22 min); R_{T_2} of **5** (14.79 min); R_{T_3} of Peak 1 (27.58 min), or of **2**, or of **3**. The data strongly indicate that the material of Peak 1 was **2**.

The identification of the two methylated amino sugars as **1** and **2** indicates that the main carbohydrate chain of CAFS contains 2-acetamido-2-deoxyglucose residues linked at C-4 (Table III). The methylation analysis of the CAFS before and after the removal of *N*-acetylneuraminic acid did not change the contents of the methylated amino sugars, thus indicating that the *N*-acetylneuraminic acid groups were not linked to the 2-acetamido-2-deoxyglucose residues (Table III). The presence of a single peak of **2** after the desulfation of the CAFS, followed by the methylation analysis, indicates that approximately 64% of the 2-acetamido-2-deoxyglucose residues were sulfated, accounting for 91.3% of the total sulfate groups (Table III). The rest of the sulfate groups was located on C-6 of the D-galactose residues, as indicated by the presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol in the reduced, acetylated hydrolyzate of methylated CAFS, which disappeared after desulfation of CAFS, followed by methylation (see Table IV). This is in agreement with the previous report[1] that a small amount of galactose 6-sulfate was obtained by partial acid hydrolysis, followed by paper chromatography, and electrophoresis. The presence of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol indicates that the small amount of sulfated galactose residues are nonreducing terminal-groups (see Table IV).

TABLE III

G.L.C. ANALYSIS OF *O*-METHYL 2-DEOXY-2-(*N*-METHYLACETAMIDO) SUGARS FROM THE METHYLATED, NATIVE, DESULFATED, AND *N*-ACETYLNEURAMINIC ACID-FREE CAFS, AS THE ALDITOL ACETATES[a]

*2-Deoxy-2-(*N*-methyl-acetamido)glucitol peracetate*	$(R_T)^b$	*Proportions (%)*		
		Native	*NeuNAc[c] removed*	*Desulfated*
3,4,6-Tri-*O*-methyl-	1.00	0.0	0.0	0.0
3,6-Di-*O*-methyl-	1.87	36.1	35.8	100.0
3-*O*-Methyl-	4.16	63.9	64.2	0.0

[a] No 2-amino-2-deoxygalactose derivative was observed. [b] R_T, relative retention time. [c] For abbreviations, see Table I.

G.l.c. of the methylated, neutral alditol acetates on the 3% ECNSS column yielded two major and three minor peaks. The peaks were identified by their relative

TABLE IV

G.L.C. ANALYSIS OF *O*-METHYL NEUTRAL SUGARS FROM THE METHYLATED, NATIVE, DESULFATED, AND *N*-ACETYLNEURAMINIC ACID-FREE CAFS, AS THE ALDITOL ACETATES[a]

Compound peracetates	R_T[b]	*Proportions* (%)		
		Native	*NeuNAc removed*[c]	*Desulfated*
2,3,4-Tri-*O*-methylfucitol	0.64	2.7	2.3	2.0
2,3,4,6-Tetra-*O*-methylgalactitol	1.00	35.9 (36.9)	63.1 (64.6)	75.7 (77.2)
2,4,6-Tri-*O*-methylgalactitol	1.90	61.4 (63.1)	30.5 (31.2)	21.6 (22.3)
2,3,6-Tri-*O*-methylgalactitol	2.42	1.0 (1.0)	1.1 (1.1)	0.7 (0.7)
2,3,4-Tri-*O*-methylgalactitol	3.06	2.6 (2.7)	3.0 (3.1)	0.0 (0.0)

[a] The proportions (%) are based on the total neutral sugar alditol acetates; the values (%) in the parentheses are based on the total galactose content. [b] R_T, relative retention time. [c] For abbreviations, see Table I.

g.l.c. retention times, mass spectra, and susceptibility to periodate oxidation as sugar alditols (see Table IV). The identity and the amount of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol indicate that the main carbohydrate chains of CAFS contain D-galactose residues linked at C-3. The presence of fucose as 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol indicates that the fucose residues are located at nonreducing end-groups. The point of attachment of the fucose residues is unknown at present. Neuraminidase digestion and mild acid hydrolysis established the location of the *N*-acetylneuraminic acid residues as nonreducing terminal-groups[2], and the methylation analyses of CAFS before and after removal of the *N*-acetylneuraminic acid residues indicate that all of the residues are linked to C-3 of the D-galactose residues. The formation of a large proportion of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol (36.9% of the total *O*-methyl galactitol peracetate from native CAFS) indicates that a considerable amount of the D-galactose residues are nonreducing terminal-groups of the carbohydrate chains, and another 27.7% of the D-galactose residues are in penultimate position to the nonreducing terminal *N*-acetylneuraminic acid groups. The desulfation reaction increased the amount of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol to 75.7% of the total galactose content, apparently owing to cleavage of the galactosyl bonds during the desulfation reaction. There was, however, no indication that hexosaminyl bonds were cleaved under the same condition (see Table III).

The presence of galactofuranose residues in the glycoprotein has been investigated[2,20] by g.l.c.–m.s. analyses of the reduced and acetylated hydrolyzate of the methylated glycoprotein. An ion of low intensity at *m/e* 89, which is a diagnostic mass number of a furanose ring[21,22], was observed only for the peaks of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol. Furthermore, even though the 3% ECNSS column was capable to resolve the peaks of

1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl- and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol, and 1,3,4-tri-*O*-acetyl-2,5,6-tri-*O*-methyl- and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol, no separate peaks of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl- and 1,3,4-tri-*O*-acetyl-2,5,6-tri-*O*-methyl-galactitol were present in the reduced, acetylated hydrolyzate of methylated CAFS. Thus, no real evidence for the presence of galactofuranose residues in a significant amount was observed by the methylation and g.l.c.–m.s. analyses, though the possibility still remains that some of the degraded galactosyl groups could be lost during the methylation procedure.

Although structures that account quantitatively for the composition and molecular size of the carbohydrate chains can be proposed, the sequence of the glycosyl units remains equivocal.

EXPERIMENTAL

Material. — The preparation of glycoprotein from chick allantoic fluid infected with influenza virus (Japan 170/62 A_2) was described in a previous communication[2].

Analytical procedures. — General analytical procedures and g.l.c. analysis of alditol acetates and 2-acetamido-2-deoxyglycitol acetates were performed as described previously[2]. Neutral sugars were also analyzed on a borate column with a Technicon automatic sugar analyzer using an accelerated system[23]. Paper chromatograms of partially methylated amino sugars were developed by the descending technique on Whatman No. 1 paper with 1-butanol–ethanol–water (4:1:5, upper layer) (A). Sugars were detected with the aniline phthalate spray or alkaline silver nitrate reagent. Mobility (R_F) of the spot was expressed relative to methyl 2-deoxy-2,3,4,6-tetra-*O*-methyl-2-*N*-(methylacetamido)-β-D-glucopyranoside. G.l.c. analysis of partially methylated sugar alditol acetates and *N*-methylhexosaminitol acetates was achieved on a glass column (1.8 m), packed with 3% cyanoethylsilicone-1,2-ethanediol succinate (ECNSS) on Gas Chrom Q, operated isothermally at 170° and 190°, respectively. The peaks were analyzed by mass spectrometry by use of a Finnigan GC-MS model 1015 with all glass separator and Du Pont GC-MS model 21-492. Identification of the partially methylated alditol and *N*-methylhexosaminitol acetates was accomplished by comparison with the retention times and mass spectra established by Björndal *et al.*[21,22,24] for partially methylated alditol acetates, and the retention times and mass spectra established by Stellner *et al.*[25] for partially methylated *N*-methylhexosaminitol.

*Exo-*D*-galactosidase from* Coccobacillus. — The exo-D-galactosidase preparation used was one of the fractions obtained by carboxymethylcellulose column chromatography of a cell-free extract of *Coccobacillus*, prepared by Dr. Y. Kikuchi in our laboratory by a modified method of Hirano and Meyer[26]. The enzyme preparation had both exo-α- and β-D-galactosidase activities as tested with *p*-nitrophenyl α- and β-D-galactopyranosides as substrates, but it did not contain endo-galactosidase activity. The native CAFS (200 μg) was incubated with the enzyme preparation (0.33 mg protein) in pH 7.0 buffer (0.1M disodium hydrogenphosphate containing potassium dihydrogenphosphate) at 37° for 3 days, and the digest was analyzed on a Technicon automatic sugar analyzer.

Desulfation. — Solvolytic desulfation in dimethyl sulfoxide containing pyridine[9] was applied as follows: CAFS (10 mg) was converted to its pyridinium salt by passage through a small column (5 ml) of AG 50-X8 (H^+) ion-exchange resin, elution with water (30 ml), and collection in a container with pyridine (0.1 ml), cooled in an ice-bath. The effluent was evaporated and dried thoroughly. The residue was dissolved in dimethyl sulfoxide containing 2% pyridine by heating for 15 min to 60°, and the solution was heated for 9 h at 100°. The organic solvent was removed by distillation *in vacuo*. Inorganic sulfate ions were removed by adition of an equivalent amount of barium acetate solution, followed by centrifugation, or by an anion-exchange column.

Periodate oxidation and Smith degradation. — Oxidation of CAFS (10 mg) was achieved in 25mM sodium periodate (1.0 ml) containing 0.2M sodium perchlorate, at 30° in the dark[7,8]. The reaction was followed by measuring[19] the consumption of periodate spectrometrically at 222.5 nm, and the reaction was stopped by addition of 1,2-ethanediol. The oxidized glycoprotein was isolated from the reaction mixture by gel filtration through a Bio-Gel P-2 column, elution in 10% ethanol, and assay by the anthrone reaction. In some experiments, the oxidized glycoprotein was isolated by dialysis against distilled water. Aliquots of the retentate and dialyzate were taken for analysis.

A sample of the oxidized glycoprotein was dissolved in water (1.0 ml), which contained 20 mg of sodium borohydride, and kept for 4 h at room temperature. After removal of the excess of sodium borohydride as methyl borate, the residue was treated with 0.5M hydrochloric acid (1 ml) for 24 h at 24°. The solution was neutralized with 5M sodium hydroxide (0.10 ml) and desalted through the Bio-Gel P-2 column with elution in 10% ethanol. The glycoprotein was eluted at the void volume as an anthrone positive peak. In some experiments, the hydrolyzed glycoprotein was isolated by dialysis. An aliquot (1.5 mg) was taken for analysis. For the 2nd Smith degradation, the three steps just described were repeated. The aliquots from each step were analyzed for neutral sugars either by g.l.c. or by the Technicon automatic sugar analyzer, and for amino sugars either by g.l.c. or by a Beckman amino acid analyzer, as described previously.

Methylation. — On account of the low solubility, the glycoprotein was acetylated prior to methylation. For acetylation[16], in a typical experiment, CAFS (20 mg) was dissolved in freshly-distilled, warm formamide (2.0 ml) by shaking for 30 min in a tube with Teflon-lined screw-cap, and freshly distilled acetic anhydride (0.2 ml) was added. The tube was covered with aluminum foil and shaken at room temperature overnight. After addition of acetic anhydride (0.2 ml), shaking was continued for another 24 h at room temperature. The reaction mixture was poured into cold ethanol (15 ml) containing potassium acetate with stirring, and kept for 2 h at 4°. The precipitate was collected by centrifugation, dissolved in water (3 ml), and reprecipitated by addition of ethanol (30 ml). The precipitate was collected again by centrifugation, washed three times with ethanol (20 ml each), dissolved in water, and lyophilized to yield 22.3 mg (97.0%) of the acetylated product.

For methylation, a stream of nitrogen was constantly applied throughout the procedure. A methylsulfinyl carbanion solution (0.2 meq/ml) was prepared by stirring dry sodium hydride (150 mg) in freshly distilled (over calcium hydride) dimethyl sulfoxide (30 ml) at 65° until liberation of hydrogen gas ceased and the solution turned to clear green (40 min). The solution was cooled and stored in a deep freezer under nitrogen until needed (as long as 2 months).

The acetylated glycoprotein (22.3 mg) was dissolved in dimethyl sulfoxide (2 ml) by warming gently to 50° for 30 min and cooled. The methylsulfinyl carbanion solution (4 ml) was added and stirred for 6 h at room temperature. Methyl iodide (1 ml, freshly distilled in the presence of silver oxide) was added dropwise, keeping the reaction mixture at room temperature and stirring for 17 h. The additions of the carbanion and methyl iodide were repeated twice at the same intervals. The methylated glycoprotein was isolated, after addition of water (5 ml), by passage through Bio-Gel P-2 column (1.0 × 90 cm), prewashed with dimethyl sulfoxide, and elution with 10% aqueous ethanol. The elution was monitored by the anthrone reaction. The methylated glycoprotein, eluted at the void volume, was pooled and lyophilized to yield 17.8 mg (84.5%) of methylated glycoprotein (Found: OCH_3, 18.3%).

For further methylation by Purdie's method, the partially methylated CAFS was dissolved in *N*,*N*-dimethylformamide (2.0 ml, freshly distilled in the presence of potassium hydroxide) in a test tube with Teflon-lined screw-cap. Freshly prepared silver oxide (0.5 g) and distilled methyl iodide (0.5 ml) were added. The tube covered with aluminum foil was shaken for 24 h at room temperature. The addition of silver oxide and methyl iodide was repeated once more, and the mixture shaken for 24 h at the same temperature. After addition of water (20 ml), the reaction mixture was centrifuged, and the precipitate was washed twice with water and twice with methanol, until the precipitate gave a negative anthrone reaction. The washings and supernatant were combined and concentrated to a small volume. The methylated CAFS was isolated by passage through a Bio-Gel P-2 column (prewashed with *N*,*N*-dimethylformaimde) and elution with 10% aqueous ethanol. The anthrone-positive peak, eluted at the void volume, was pooled and lyophilized to yield 15.7 mg of the methylation product.

Further methylation by both the methylsulfinyl carbanion and Purdie's reagent did not increase significantly the methoxyl content. The over-all yield was increased (up to 80%) by extracting the methylated product with chloroform prior to the gel-filtration procedure. After Hakomori methylation, water (3 vol.) was added and the solution extracted with chloroform (2 vol.) twice. The chloroform layer was dried (sodium sulfate), filtered, and evaporated. The aqueous solution and washings were combined, evaporated to a small volume, and the product was isolated *via* gel filtration, as described before. The isolated products from the chloroformic and aqueous layers were combined and methylated by Purdie's method, after which the same extractions and gel-filtration procedures were applied. G.l.c. analyses of the hydrolyzate of the methylated products obtained by both isolation procedures did not differ significantly.

The desulfated CAFS (10 mg) was methylated directly in dimethyl sulfoxide solution, as just described, after solubilizing the glycoprotein in warm dimethyl sulfoxide (2 ml), without prior acetylation. After methylation, the product was isolated by the chloroform extraction procedure, as just described.

Hydrolysis of methylated CAFS and g.l.c. — The permethylated CAFS (1 mg/ml) was hydrolyzed with 90% (w/w) formic acid for 3 h at 100°. The acid was removed *in vacuo* in a rotary evaporator at 30°, 0.3M hydrochloric acid (1 mg/ml) was added, and the solution was heated[17] for 18 h at 100°. The acid was removed either by lyophilization after dilution with water (3 vol.) or in a rotary evaporator at 30°. For analysis of the partially methylated neutral sugar, the residue was passed through a small column (1.0 × 5.0 cm) of AG 50-X8 (H^+) ion-exchange resin and washed with water (5 bed vol.). The eluate and washings were combined, evaporated *in vacuo* to a small volume (1.0 ml), and treated with sodium borohydride (10 mg/ml) for 24 h at room temperature. The excess of borohydride was destroyed by addition of glacial acetic acid (3 drops), and removed by repeated additions and evaporations of methanol. The residue, dried *in vacuo*, and containing the partially methylated alditols, was acetylated with redistilled acetic anhydride (2 ml) in a tube with Teflon-lined screw-cap for 3 h at 120°. Acetic anhydride was removed by co-evaporation with toluene *in vacuo* below 45°. The methylated alditol acetates were redissolved in dichloromethane and analyzed by g.l.c. on a 3% ECNSS-M column. The methylated hexosamines were eluted from the column of AG 50-X8 ion-exchange resin by M hydrochloric acid (5 bed vol.), evaporated to dryness at 30°, reduced and acetylated as described before, and analyzed by g.l.c.

Oxidation of partially methylated sugar alditols of methylated CAFS. — The partially methylated sugar alditols (2 mg), prepared from the permethylated CAFS as described before, was oxidized with aqueous 0.25M sodium periodate (0.5 ml) for 18 h at room temperature. The excess of periodate ions was destroyed, and the product reduced with sodium borohydride (20 mg) for 3 h at room temperature. The excess of sodium borohydride was destroyed by addition of glacial acetic acid, and removed as methyl borate as described before. The iodate ions in the reaction mixture were removed by passing the mixture through a column (5 ml) of AG 1-X8 (AcO^-) ion-exchange resin, and elution with water (30 ml). The product in the effluent was recovered by evaporation and dried thoroughly before acetylation for g.l.c. analysis, as described previously.

Demethylation. — A partially methylated amino sugar fraction of the hydrolyzate of methylated CAFS was divided into three portions. Two portions were subjected to demethylation by treating with cold boron tribromide[18] and with hydrogen iodide[19], respectively, and the third portion was used as a control. After demethylation, they were reduced, acetylated, and studied by g.l.c., as described previously.

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