O-Glycosylation Mimics N-Glycosylation in the 16-kDa Fragment of Bovine Pro-opiomelanocortin

The major O-glycan attached to Thr-45 carries SO₄-4GalNAcβ1-4GlcNAcβ1-, which is the archetypal non-reducing epitope in the N-glycans of pituitary glycohormones.

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The NH₂-terminal domain of pro-opiomelanocortin, designated as the 16-kDa fragment, is highly conserved throughout the vertebrate family and is likely therefore to have an important functional role. Bovine 16-kDa fragment is a 77-residue glycopeptide, which has been found to be glycosylated at threonine 45 and asparagine 65. Available evidence suggests that glycoforms lacking glycans at the O-linked site are processed in the intermediate pituitary at -Arg⁴-Lys⁵- to give the residue 1-49 amino-terminal peptide and a carboxyl-terminal glycopeptide referred to as Lys¹-Thr as the glycoprotein (Eipper and Mains, 1980). It was Seidah and co-workers (Seidah et al., 1981) who first discovered that the amino-terminal biosynthetic fragment of POMC bore both O- and N-linked glycans at threonine 45 and asparagine 65, respectively (Fig. 1). In addition, a second N-linked glycosylation site was localized to the carboxyl-terminal region of rat and mouse ACTH (Mains and Eipper, 1976; Eipper and Mains, 1978). Glycosylation of ACTH is confined to these species since the structural cue for N-glycosylation (i.e.-Asn-X-Ser/Thr) is missing from the ACTHs of other mammalian species (Kawauchi, 1983).

Within the anterior pituitary, the 16-kDa or amino-terminal fragment is a major product of POMC processing and is not subject to further cleavage (Seidah et al., 1981; Keutmann et al., 1981). Structural analysis of human 16-kDa fragment derived from the posterior pituitary revealed that it is glycosylated at both O- and N-sites with high efficiency (Bennett et al., 1986). In the bovine intermediate pituitary, the 16-kDa fragment consists of 77 residues and has been found to be partially cleaved at an -Arg⁴-Lys⁵- processing site to yield an amino-terminal 49-residue peptide and a carboxyl-terminal peptide consisting of residues 50-77 (James and Bennett, 1985). This latter peptide, known as Lys¹-Thr as the glycoprotein (Eipper and Mains, 1980), has been shown to be glycosylated at asparagine 65 (Bennett, 1986a). In contrast, structural analysis of the bovine residue 1-49 amino-terminal peptide yielded no evidence for O-glycosylation at threonine 45 (Bennett, 1984). The homologous peptides isolated from rat and mouse intermediate pituitaries have also been found to lack O-glycans (Bennett, 1986b; Seger and Bennett, 1986). Characterization of the 16-kDa fragment, which remains unprocessed in the bovine intermediate pituitary, indicated that this glycopeptide is fully O-glycosylated at threonine 45 (James and Bennett, 1985). These observations prompted the suggestion that cleavage of the processing site at -Arg⁴-Lys⁵- is dependent upon the absence of O-glycosylation at the adjacent threonine 45. Since cleavage at this site does not occur in the anterior pituitary, it was suggested that this putative regulatory mechanism might account at least in part for the observed tissue specific processing of 16-kDa fragment (Seger and Bennett, 1985).

The abbreviations used are: POMC, pro-opiomelanocortin; ACTH, adrenocorticotropic; FAB, fast atom bombardment; GC, gas chromatography; Hex, hexose; HexNAc, N-acetylgalactosamine; HPLC, high performance liquid chromatography; 16-kDa fragment, the amino-terminal fragment of pro-opiomelanocortin; MSH, melanotropin stimulating hormone; MS, mass spectrometry; NeoGc, N-glycolylneuraminic acid; PCC, pro-hormone converting enzyme.

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*The major pro-opiomelanocortin (POMC), the common precursor for corticotropin (ACTH), endorphin, and related peptides secreted by this pituitary, has been known since its discovery to be a glycoprotein (Eipper and Mains, 1980). It was Seidah and co-workers (Seidah et al., 1981) who first discovered that the amino-terminal biosynthetic fragment of POMC bore both O- and N-linked glycans at threonine 45 and asparagine 65, respectively. In addition, a second N-linked glycosylation site was localized to the carboxyl-terminal region of rat and mouse ACTH (Mains and Eipper, 1976; Eipper and Mains, 1978). Glycosylation of ACTH is confined to these species since the structural cue for N-glycosylation (i.e., Asn-X-Ser/Thr) is missing from the ACTHs of other mammalian species (Kawauchi, 1983).
1986). However, the use of site-directed mutagenesis technology has indicated that O-glycosylation is most likely not a determinant in the regulation of processing of the 16-kDa fragment in the anterior pituitary corticotroph. Neither wild-type POMC nor POMC bearing an alanine for threonine substitution at position 45 was cleaved at -Arg⁴⁵-Lys⁴⁶- by mouse AtT-20 tumor cells, which are known to process POMC in a manner similar to that of the anterior pituitary (Noel et al., 1991). Therefore, it is more likely that O-glycosylation plays a critical role in the regulation of 16-kDa processing within the intermediate pituitary rather than its anterior counterpart. Indeed, a putative pro-hormone converting enzyme (PCE), an aspartyl protease isolated from bovine pituitary tissue, has been shown in vitro to have the appropriate specificity with respect to cleavage of the bovine 16-kDa fragment. The peptide lacking the O-glycan structure was found to be a substrate for PCE, whereas the fully glycosylated peptide was unprocessed (Birch et al., 1991). PCE is thought to be the mammalian homologue of the YAP-3 aspartyl protease found in yeast, which has been shown to process POMC at pairs of basic residues in vitro (Azaryan et al., 1993).

Whether PCE is involved in POMC processing in vivo remains an open question, especially since recent studies of the properties of pro-hormone convertase 2, a member of the mammalian family of subtilisin-like proteases, have produced evidence that pro-hormone convertase 2 could be responsible for all the POMC cleavages occurring specifically within the intermediate lobe including the processing of the 16-kDa fragment (Zhou et al., 1993).

O-Glycosylation of the 16-kDa fragment may also play a role in determining its fate following secretion. It has been proposed that cleavage of the 16-kDa fragment by an adrenal cortical protease generates a peptide corresponding to the amino-terminal residue 1-49 sequence. This peptide in turn is processed by the adrenal cortical protease generating a peptide corresponding to the 16-kDa fragment (Zhou et al., 1993).

The acquisition of structural data on the O-glycans is an essential prerequisite to studies aimed at clarifying the role O-glycosylation plays in 16-kDa processing and function. We have recently used strategies based on fast atom bombardment mass spectrometry (FAB-MS) to characterize the N-glycans present in bovine 16-kDa fragment (Siciliano et al., 1993). The major acidic N-glycans are biantennary structures having SO₄-4GalNAcβ1-4GlcNAcβ1- on the 3- and α-branch and a neutral 6-branch that is fucosylated in some glycoforms. The sulfated epitope is characteristic of pituitary glycoprotein hormones N-glycans (Baenziger and Green, 1988). We now present results from a similar mass spectrometric study of the O-linked oligosaccharides of bovine 16-kDa fragment and provide rigorous evidence for the most abundant O-glycan being a tetrasaccharide bearing the same SO₄-4GalNAcβ1-4GlcNAcβ1- epitope. This structure has not been previously detected in the O-glycans of any glycoprotein. We discuss the possibility that the same enzymes could be responsible for the synthesis of the sulfated GalNAc moiety in both the N- and O-glycans of the 16-kDa fragment.

EXPERIMENTAL PROCEDURES

Preparation of 16-kDa Fragment—To determine the O-glycan structure of the 16-kDa or amino-terminal fragment of bovine POMC, two preparations of glycopeptide were used. The first was a portion of that used to determine the N-glycan structure linked to asparagine 65 (Siciliano et al., 1993). For this preparation, 600 bovine posterior pituitary extracts were extracted in acid and the solubilized peptides subjected to both reversed-phase and anion-exchange batch fractionation. Removal of the sulfated epitope was achieved using preparative and analytical HPLC (Siciliano et al., 1993). To complete the O-glycan structure, a second preparation of 16-kDa fragment was used. 100 lyophilized bovine posterior pituitary (PelFreez Biologicals, Rogers, AR) were extracted, and the solubilized glycopeptide was purified as described previously (James and Bennett, 1986; Bennett, 1989a) with the exception that the batch anion-exchange step was eliminated.

Reduction and Carboxymethylation—Reduction of the 16-kDa fragment was carried out in Tris buffer (0.6 M pH 8.4) for 1 h at 37°C using a 4 M excess (over cysteine residues) of dithiothreitol. Carboxymethylation was carried out in the same buffer using a 5-fold molar excess over total thiol) for 30 min at room temperature. Desalting was achieved using a C₄ Sep-Fak (Waters) previously primed with methanol, propan-1-ol, and 5% acetic acid and eluted with 40% propan-1-ol in 5% acetic acid.

V8 Protease Digestion—Reduced and carboxymethylated 16-kDa fragment was digested with V8 protease (1:25 enzyme:substrate) in 100 mM ammonium bicarbonate buffer (50 mM, pH 8.4) for 16 h at 37°C using 2.5 μl of enzyme solution. The reaction was stopped by freeze-drying, and the enzyme was inactivated by boiling the sample for 5 min.

Peptide N-Glycosidase F Digestion—Peptide N-glycosidase F digestion was carried out in ammonium bicarbonate buffer (50 mM, pH 8.4) for 16 h at 37°C using 2.5 μl of enzyme solution. The reaction was stopped by freeze-drying, and the enzyme was inactivated by boiling the sample for 5 min.

Reductive Elimination—The 40% propan-1-ol fraction was treated with 400 μl of 1 M sodium borohydride (NaBH₄) 0.05 M NaOH at 45°C overnight. The sample was then neutralized with glacial acetic acid and desalted on a Dowex (500W × 8H) column, and the borates were removed by co-evaporation under N₂ with 10% acetic acid in methanol.

Permethylation—Oligosaccharides were permethylated using the sodium hydroxide procedure as previously described (Dell, 1990). Briefly 0.5-1.0 ml of dimethyl sulfoxide/NaOH slurry (prepared by grinding 5 pellets of sodium hydroxide with approximately 3 ml of dry MeSO) was added, followed by about 0.5 ml of methyl iodide. The mixture was shaken for 10 min at room temperature and then quenched with 1 ml of water, and the products were extracted into chloroform, which was washed several times with water. The chloroform layer was dried under a stream of nitrogen, and the residue was dissolved in methanol for FAB-MS analysis.

Peptide carboxymethylation—Base-catalyzed peracetylation was performed using 1-methyl imidazole/deuteroacetic anhydride (1:5 (v/v) ratio, 30 μl total volume) at room temperature for 1 h. The reagents
were dried down under nitrogen. Sulfated oligosaccharides were eluted in the 30% fraction from a C18 Sep-Pak, and neutral and sialylated oligosaccharides were eluted in the 50% acetonitrile fraction.

**Periodate Oxidation**—The reduced 0-linked glycans were treated with 20 μl of 10 mM sodium metaperiodate (NaIO4) in acetic buffer (0.1 M, pH 5.5) at room temperature for 4 h in the dark. The reaction was quenched with 2 μl of ethylene glycol and left to stand for 30 min. The glycans were reduced with 10 mg/ml NaBH4 in 2 M NH4OH at room temperature for 2 h before being subjected to perdeuteracetylation and desialylating using a C18 Sep-Pak.

**Removal of Sulfate Groups**—Removal of sulfate groups was carried out in 0.5 M HCl in methanol at room temperature for 3 h. The reagents were dried down under nitrogen.

**Exoglycosidase Digestions**—Digestion with exo-β-D-galactosidase (EC 3.2.1.23, 0.01 unit) from bovine testes was carried out in 200 μl of 5 mM sodium citrate-phosphate buffer, pH 4.6, containing 10 μl of toluene at 37 °C for 48 h. Digestion with exo-β-N-acetyl-0-glucosaminidase (EC 3.2.1.30, 0.1 unit) from bovine kidney was carried out in the same buffer at 37 °C for 48 h. After each digestion, aliquots (10%) were taken. The remaining oligosaccharides produced by the enzymatic reactions were deuteracetylated and desialylated with C18 Sep-Pak. In sequential digests, enzymes were desiccated by boiling the sample for 5 min prior to addition of the next enzyme.

**HPLC Fractionation of the Neutral Glycans**—The neutral glycans eluted in the 50% fraction were fractionated by reverse phase HPLC using a Shiseido C8 column attached to a Kontron HPLC system (model 450 data system, model 420 HPLC pump, model 430 detector, and 320 integrator). The sample was loaded in 10% acetonitrile (v/v) and eluted with a solvent system of MilliQ water (A) and acetonitrile (B) (Rathburn) at a flow rate of 1 ml/min, isocratically with 10% B for 5 min, then with linear gradients of 10–30% B in 5 min, 30–80% B in 60 min, and 80–100% B in 5 min. The elution was monitored at 214 and 280 nm, and 1-ml fractions were collected, dried, and redissolved in methanol for FAB-MS screening.

The tetra- and pentasaccharides were detected, suggesting that a complete separation of the sulfated glycans from the neutral ones had been achieved. Signals on the low mass side of the spectra that are reproduced in this paper. The matrix was thiglycolyl, and the derivatized oligosaccharides were dissolved in methanol prior to loading on the target.

**Deuterization for Linkage Analysis**—Partially permethylated alditol acetates were acquired using a VG analytical high field ZAB-HF mass spectrometer fitted with an M-Scan gun operated at 10 K. Spectra were recorded on a oscillographic chart paper and counted manually, giving nominal masses. The negative ion mass spectra and FAB spectra and the positive ion mode high mass region spectra were acquired using a ZAB-2SE FD mass spectrometer fitted with a cesium gun. Spectra from this instrument were computer-processed, giving accurate mass assignments, which are rounded to the nearest whole number in most of the spectra that are reproduced in this paper. The matrix was thiglycolyl, and the derivatized oligosaccharides were dissolved in methanol prior to loading on the target.

**RESULTS**

**Isolation of O-Glycans**—Reduced carboxymethylated 16-kDa fragment was digested sequentially with V8 protease and peptide N-glycosidase F, and the released N-glycans were separated from the peptides and O-linked glycopeptide using a Sep-Pak. The included fraction, which contained the O-glycopeptide, was rehydrolyzed, eluted, and the released oligosaccharides were purified by Dowex chromatography and converted to their deuteracetyl derivatives using N-methylimidazole/deuteracetic anhydride (Khoo et al., 1993). Upon Sep-Pak purification, derivatized sulfated oligosaccharides were recovered in the 30% acetonitrile fraction, while the remaining components eluted in the 50% acetonitrile fraction. Sugar analysis of the 30% and 50% fractions indicated that at least 80% of the oligosaccharides were recovered in the 30% fraction (data not shown).

**Characterization of the Sulfated Fraction by FAB-MS and Linkage Analysis**—The positive and negative FAB mass spectra of the 30% acetonitrile fraction are reproduced in Fig. 2. The negative spectrum (Fig. 2a) is dominated by an (M – H)^+ cluster centered at m/z 1365 corresponding to S1HexNAcHexNAc. The Gaussian-like appearance of the molecular ion cluster is caused by a portion of the deuteracetylated reagent being incompletely deuterated, resulting in the signals on the low mass side of the m/z 1365 cluster. Those on the high mass side are predominantly due to natural abundance 13C, although it is possible that there is a minor amount of sulfated Hex,HexNAc, HexNAcitol (m/z 1369 (M – H)^+) in this preparation because the isotopic spread is wider than expected. In the low mass region, fragment ions corresponding to sulfated HexNAc are present at m/z 325, 327, 372, 388, and 491. The origin of these ions has been reported previously (Dell et al., 1991). Interestingly, the presence of the signal at m/z 491, which arises from ring cleavage of the penultimate sugar residue with retention of carbons 4–6 on the fragment ion, is indicative of S1HexNAc being linked to position 4 of the next sugar. In the positive FAB spectrum (Fig. 2b), the major peaks at m/z 1411 and 1309 are assigned to S1Hex,HexNAcHexNAcitol ((M + 2Na – H)^+) and (OH)Hex,HexNAcHexNAcitol (loss of sodium sulfate from m/z 1411). In accord with the negative ion data, it appears that Hex1HexNAcHexNAcitol is present as a minor component (m/z 1415 and 1313). No peaks due to neutral glycans were detected, suggesting that a complete separation of the sulfated glycans from the neutral ones had been achieved. At a fragmentation gave signals at m/z 343 (Hex^+), 374/294 (S1HexNAc'/OH),HexNAc^+), and 667/587 (S2HexNAc'/OH,HexNAc^+), thus defining two non-reducing sequences Hex^+ and S1HexNAcHexNAc^+ for the major component. The β-cleavage ions at m/z 723 and 1016, attributed to sodium adducts of (OH)Hex,HexNAc and (OH)Hex,HexNAcHexNAcitol provide further evidence for the attachment of the sulfate group to a terminal HexNAc. The signal at m/z 683 corresponds to elimination of the sulfated HexNAc moiety to give "dehydrated" Hex,HexNAcitol, which is observed as a protonated species. Signals at m/z 671 and 591 are assigned to S2HexNAc and (OH)Hex,HexNAc^+ and are probably derived from the minor sulfated component S1Hex,HexNAcHexNAcitol.

**GC-MS Analysis**—GC-MS was carried out using a Hewlett Packard model 5890 gas chromatograph connected to a VG Trio 1 quadrupole mass spectrometer. The partially permethylated alditol acetates were dissolved in dichloromethane prior to injection on a DB-5 (25 m × 0.32 mm internal diameter, J&W Scientific) column at room temperature. The temperature was then increased to 100 °C over 1 min and held for 1 min before increasing to 290 °C at 6 °C/min. The @-cleavage ions at m/z 1411, 1309, 1210, 30%, 50%, 723, and 1016 were detected, suggesting that linkage analysis experiments were carried out on the Sep-Pak-purified perdeuteracetylated oligosaccharides. Aliquots of the 30% and 50% acetonitrile fractions were permethylated using mild Hakomori and sodium hydroxide permethylation conditions, respectively; the products were hydrolyzed, re-duced, and acetylated, and the resulting partially permethylated alditol acetates were analyzed by GC-MS. In order to define the linkage position of the sulfated group, a second aliquot of the 30% acetonitrile fraction was desulfated with methanol-HCl prior to permethylation and linkage analysis. The desulfated permethylated sample was analyzed by FAB-MS before...
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**FIG. 2.** a, negative FAB spectrum of the deuteroacetylated O-linked glycans that eluted from Sep-Pak in the 30% acetonitrile fraction. Signals between m/z 500 and 1150 are magnified five times compared with the molecular ion region, while signals below m/z 500 are magnified three times. The very minor signals between m/z 600 and 700 are a HexNAc increment above those corresponding to sulfated HexNAc (assigned in the text). The signal at m/z 1220 corresponds to (M - H)⁺ of glycans lacking one deuteroacetyl group. The signal at m/z 1051 is derived from ring cleavage of non-reducing Hex residue. The minor cluster at m/z 944 is not assigned. b, positive FAB spectrum of the deuteroacetylated O-linked glycans that eluted from Sep-Pak in the 30% acetonitrile fraction. Signals between m/z 500 and 1100 are magnified five times compared with the rest of the spectrum. Carbohydrate-derived signals are assigned in the text.

conversion to the partially methylated alditol acetates. The FAB data (not shown) confirmed that no significant hydrolysis of glycosidic linkages had occurred under the conditions used for the desulfation. Results from linkage analysis of the sulfated fraction are reported in Tables I (before desulfation) and II (after desulfation). Data from the 50% fraction are reported in Table IV. The following conclusions can be drawn from the data in Tables I and II. (i) The presence of 3,6-linked GalNAcitol, but not 3- or 6-linked GalNAcitol, is indicative of a branched core. (ii) Both 4-linked GlcNAc and 4-linked GalNAc are detected prior to desulfation, but 4-linked GalNAc is absent in the desulfated fraction, suggesting that the sulfate is attached at the 4-position of terminal GalNAc. (iii) Terminal GalNAc is present in the desulfated sample but not in the sulfated sample, thus confirming the conclusion in (ii). (iv) All the detected galactose is terminal.
Taken together, the GC-MS and FAB-MS data are consistent with the major component comprising a Type 2 core substituted at the 4-position of the 6-linked GlcNAc by a 4-O-sulfated GalNAc residue. However, the data do not rule out an alternative novel core in which the attachment sites of the two branches to the GalNAcitol are reversed. This ambiguity was resolved by periodate oxidation.

Characterization of the Core of the Major Sulfated Glycan by Periodate Oxidation and FAB-MS—The mixture of glycans obtained by reductive elimination of the O-linked glycopeptide was subjected to mild periodate oxidation, and the products were reduced, deuterocacylated, and analyzed by negative FAB-MS after Sep-Pak purification. The negative FAB spectrum of the 30% acetonitrile fraction (Fig. 3) gave a strong signal at $m/z$ 772, which is the correct mass for $S_1$HexNAc$_2$ attached to a two-carbon fragment derived from GalNAcitol.

**Table I**

<table>
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<th>Elution time (min)</th>
<th>Assignment</th>
<th>Characteristic fragment ions</th>
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<td>14.76</td>
<td>t-Gal</td>
<td>102, 118, 129, 145, 161, 162, 205</td>
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<td>18.91</td>
<td>3,6-GalNAc-itol</td>
<td>130, 246, 318</td>
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<tr>
<td>19.40</td>
<td>4-GalNAc</td>
<td>117, 159, 233</td>
</tr>
<tr>
<td>19.55</td>
<td>4-GlcNAc</td>
<td>117, 159, 233</td>
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**Table II**

<table>
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<th>Elution time (min)</th>
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<tr>
<td>19.40</td>
<td>4-GlcNAc</td>
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This is the result expected when periodate cleaves between carbons 4 and 5 of 3,6-linked GalNAcitol carrying the sulfated branch on position 6, i.e. the common Type 2 core structure of O-glycans.

**Determination of Anomeric Configurations in the Major Sulfated Glycan**—Anomeric configurations were determined by a combination of hexosaminidase digestion, chromium trioxide oxidation, and FAB-MS. After deuterocacylation, the sulfated glycan was separated from non-sulfated components by Sep-Pak purification, and sulfate and deuterocacyl groups were removed by mild methanolation. A portion of the product was reacetylated with non-deuterated acetic anhydride and analyzed by FAB-MS to check for completeness of O-deacylation and to establish whether any glycosidic cleavages had occurred. The FAB spectrum (not shown) contained signals at $m/z$ 1296, 1299, 1302, and 1305 (approximate ratio 1:2:3:2) consistent with intact Hex$_1$HexNAc$_2$HexNAcitol retaining zero, one, two, and three deuterocacyl groups, respectively. We considered that this level of O-acylation would not seriously prejudice the hexosaminidase experiment, and the remainder of the methanolyzed sample was therefore incubated with bovine kidney exo-$\beta$-N-acetyl-$\alpha$-glucosaminidase for 48 h. The products were fully deuterocacylated and analyzed by FAB-MS (Fig. 4). Molecular ion clusters at $m/z$ 746, 1039, and 1332 correspond to $(M + H)^+$ for Hex$_1$HexNAc$_2$HexNAcitol, Hex$_1$HexNAc$_2$HexNAcitol, and Hex$_1$HexNAc$_2$HexNAcitol, respectively, showing that the $\beta$-hexosaminidase is able to sequentially remove GalNAc and GlcNAc from the GalNAc$\_1$-4GlcNAc branch.

The hexosaminidase experiment was corroborated by chromium trioxide oxidation, which rapidly oxidizes $\alpha$-linkages while $\beta$-linkages remain largely unaffected under the conditions used (Angyal and James, 1970). The degree of oxidation can be readily monitored by FAB-MS because each oxidized sugar residue is shifted in mass by 14 mass units (Khoo and Dell, 1990). The mixture of $O$-glycans was desulfated by mild methanolation, and the products were deuterocacylated and

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**Fig. 3.** Negative FAB spectrum of the products of mild periodate cleavage. The sample was reduced, deuterocacylated, and purified by Sep-Pak prior to FAB analysis. The data shown were obtained from the 30% acetonitrile fraction.
**915**

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FIG. 4. Positive FAB spectrum of the deuterioacetylated products of β-hexosaminidase digestion of the desulfated glycan. Digestion was not complete because a portion of the sample had retained acyl groups incorporated during purification (see “Results”).

**TABLE III**

<table>
<thead>
<tr>
<th>m/z value</th>
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<td>1374</td>
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<tr>
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<td>760</td>
<td>14</td>
<td>1 β-linkage</td>
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</table>

**FIG. 5.** Structure of the major O-glycan attached to threonine 45 in bovine 16-kDa glycopeptide.

purified using a Sep-Pak. Fractions were screened by positive FAB-MS, which showed that the Hex2HexNAcHexNAcitol (m/z 1336) component was recovered in the 50% acetonitrile fraction, together with some Hex3HexNAcHexNAcitol (m/z 1332), Hex2HexNAcHexNAcitol (m/z 1039), and Hex3HexNAcitor (m/z 746). The FAB data reproduced in Table III were obtained after chromium trioxide oxidation of this fraction. These data show that Hex2HexNAcHexNAcitol, Hex3HexNAcHexNAcitol, and Hex3HexNAcitor have three, three, two, and one β-linkages, respectively, i.e., all linkages in these oligosaccharides are β.

**Structure of the Major Sulfated O-Glycan—** Taken together, the above data show that the major sulfated O-glycan attached to the bovine 16-kDa fragment has the structure shown in Fig. 5. This is an oligosaccharide with a Type 2 core structure, which is elongated on the 6-branch by the sulfated epitope previously observed on N-linked oligosaccharides of pituitary glycoproteins including POMC (Baenziger and Green, 1988; Siciliano et al., 1993).

**Analysis of Non-sulfated O-Glycans—** Non-sulfated glycans comprised less than 20% of the O-glycan population, and complete characterization was difficult because of heterogeneity and because there were significant differences among the minor oligosaccharides isolated from two preparations of 16-kDa fragment. The two samples of 16-kDa were prepared using similar, but not identical, protocols and were derived from different batches of pituitaries. O-Glycan variability appears to be confined to the non-sulfated components, since the glycan shown in Fig. 5 was the major component in both preparations.

After deuterioacetylation and Sep-Pak purification of O-glycans from the first 16-kDa sample, the non-sulfated glycans, eluting in the 50% acetonitrile fraction, gave the positive FAB data reproduced in Fig. 6. The spectrum is dominated by the signal at m/z 1336, which corresponds to (M + H)+ for Hex2HexNAcHexNAcitol. Less abundant molecular ions are present at m/z 746 (Hex2HexNAcitor) and 1039 (Hex3HexNAcitor) and major A-type fragment ions at m/z 343 and 636 are assigned to Hex and HexHexNAc', respectively. The chloride ion adducts of each of the above species were observed in the negative ion mode and the absence of any (M − H)− signals attributable to sulfated components indicated that the Sep-Pak procedure had efficiently separated the sulfated glycans from their non-sulfated counterparts (data not shown). In order to search for very minor components, which are more readily observed as their permethyl derivatives, the remainder of the perdeuteroacetylated sample was permethylated and 10% of the products were analyzed in the positive ion mode. The major signals in the FAB spectra were fully consistent with the results from the deuterioacetyl derivative but there were additional very minor signals corresponding to (M + H)+ for NeuAcHexHexNAcitor, NeuGcHexHexNAcitor, NeuAcHexHexNAcitor, and NeuGcHexHexNAcitor, and NeuGcHexHexNAcitor (data not shown). Linkage analysis data acquired on the remainder of the permethylated sample are reported in Table IV. Terminal Gal, 4-linked GlcNAc, 3-linked GalNAcitor, and 3,6-linked GalNAcitor were observed as prominent constituents, to-
FIG. 6. Positive FAB spectrum of the deuterocetylated O-linked glycans that eluted from Sep-Pak in the 50% acetonitrile fraction. The (M + H)+ signals of fully deuterocetylated components are assigned in the text. Signals are also present for (M + Na)+ species 22 mass units above each (M + H)+ and for components lacking one or more deuterocetyl groups that occur at 45 mass unit intervals below fully derivatized species.

TABLE IV
GC-MS analysis of partially methylated alditol acetates derived from neutral O-linked glycans (50% fraction)

<table>
<thead>
<tr>
<th>Major components are indicated by an asterisk.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Elution time</strong></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>12.90</td>
</tr>
<tr>
<td>14.75*</td>
</tr>
<tr>
<td>15.96</td>
</tr>
<tr>
<td>16.50</td>
</tr>
<tr>
<td>16.71</td>
</tr>
<tr>
<td>16.83</td>
</tr>
<tr>
<td>17.02*</td>
</tr>
<tr>
<td>17.28</td>
</tr>
<tr>
<td>18.83*</td>
</tr>
<tr>
<td>18.43</td>
</tr>
<tr>
<td>18.92</td>
</tr>
<tr>
<td>19.35*</td>
</tr>
<tr>
<td>20.23</td>
</tr>
<tr>
<td>20.68</td>
</tr>
</tbody>
</table>

The non-sulfated deuterocetylated O-glycans from the second 16-kDa preparation gave data (Fig. 7a) that were significantly different from those described above (Fig. 6). Although Hex,HexNAcitol (m/z 746), Hex,HexNAc,HexNAcitol (m/z 1039), and Hex,HexNAc,HexNAcitol (m/z 1336) are still present, the last is no longer the major component. Instead, the most abundant high mass molecular ion corresponds to a fucosylated glycan of composition FuclHexlHexNAc2HexNAcitol (m/z 1568), while a less intense, but nevertheless quite prominent, signal is observed at m/z 1322 (HexlHexNAc,HexNAcitol) corresponding to its non-fucosylated counterpart. The A-type fragment ion at m/z 876 is indicative of a HexNAc(Fuc)HexNAcC branch in the fucosylated glycan. The mixture of deuterocetylated glycans was purified by HPLC, and fractions were monitored by off-line FAB-MS. Linkage analysis was carried out on the major fucosylated component, which eluted in fraction 27. Partially methylated alditol acetates were observed for terminal Fuc, terminal Gal, terminal galNAc, 3,4-linked GlcNAc, and 3,6-linked GalNAcitol together with additional minor components (Table V). These data suggest that the fucosylated glycan has the same structure as the major sulfated glycan (Fig. 5), except that the sulfate is absent and the GlcNAc is substituted at position 3 by fucose. However, we cannot rule out the alternative structure in which the positions of GalNAc and Fuc are exchanged. We attempted to resolve this ambiguity by permuting the mixture of O-glycans and searching for linkage specific fragment ions in the FAB spectra. Unfortunately, fragment ions from the fucosylated component were insufficiently abundant for a firm conclusion to be made. In accord with the data obtained from the first 16-kDa sample, permethylation enabled the facile identification of sialylated components (Fig. 7b), which gave molecular ions at m/z 873 (NeuAc,HexlHexNAcitol), 903 (NeuGc,HexlHexNAcitol), 1234 (NeuAc,HexlHexNAcitol), 1265 (NeuAc,NeuGc,HexlHexNAcitol), 1286 (NeuGc,HexlHexNAcitol), 1323 (NeuAc,HexlHexNAcitol), and 1353 (NeuGc,HexlHexNAcitol). The disialylated oligosaccharides were not observed in the first sample, but this may not be a significant observation because higher quality data were obtained from the second batch.

**DISCUSSION**

Using a FAB-MS/derivatization strategy, complemented by chemical and enzymatic degradation, we have shown that
the major O-glycan attached to threonine 45 in bovine 16-kDa fragment is a sulfated tetrasaccharide (Fig. 5). This structure is novel because of the sulfated β-linked GalNAc residue attached to the 6-arm of the Type 2 core giving the SO₄⁻GalNAc³¹-GlcNAc⁵¹ moiety, which is normally found in the N-glycans of pituitary glycohor-mones (Baenziger and Green, 1988). Although recent studies have suggested that this epitope is more widespread in N-glycans than previously thought (Smith et al., 1992; Dell and Khoo, 1993), there is no published evidence for its presence in O-glycans. The GalNAc transferase responsible for the addition of β-linked GalNAc to the N-glycans in the pituitary glycohor-mones recognizes a tripeptide motif, Pro-X-Arg/Lys (where X is usually a hydrophobic residue), located 6–9 residues NH₂-terminal of the glycosylation site (Smith and Baenziger, 1992). In the 16-kDa fragment the proteolytic processing site, Pro-Arg-Lys, which is 15 residues upstream from the N-glycosylation site (Fig. 1), is believed to be the recognition motif for GalNAc addition to the N-glycans (Skelton et al., 1992; Siciliano et al., 1993). Thus, some flexibility in both the...
location of the tripeptide motif and the nature of X can be accommodated by the β-GalNAc transferase, although its activity is probably compromised, as exemplified by the fact that none of the N-glycans in the 16-kDa fragment contains more than one sulfated β-GalNAc epitope (Siciliano et al., 1993), in contrast to lutropin and thyrotropin, which have major glycoforms containing two sulfated β-GalNAc antennae. We consider it likely that the Pro-Arg-Lys motif regulates β-GalNAc addition at both the N- and O-glycosylation sites in bovine 16-kDa fragment, with a single β-GalNAc transferase being responsible for synthesis at both sites. Precedent exists for a capping β-GalNAc transferase being capable of accepting both N- and O-glycans as substrates. Conzelmann and Kornfeld (1984b) have isolated a β1,4-GalNAc transferase from a cytotoxic T lymphocyte cell line, which is similar, if not identical, to the enzyme that is responsible for synthesis of the Sd4 and Cad blood group determinants, both of which contain β-linked GalNAc as a capping group attached to a β-Gal residue (Blanchard et al., 1983, 1985). Using glycoporphin and Tamm-Horsfall glycoprotein as O- and N-glycan acceptors, respectively, Conzelmann and Kornfeld (1984b) showed that the purified enzyme was able to transfer GalNAc to Gal acceptors on both O- and N-glycans. A similar broad specificity might be exhibited by the pituitary β-GalNAc transferase, provided the putative O-glycan acceptor is appropriately located with respect to the tripeptide recognition site. In the 16-kDa fragment, the O-glycosylation site is very close to the tripeptide motif that is believed to be recognized by the GalNAc transferase (Fig. 1).

A number of other protein hormones have been shown to be O-glycosylated. These include the α-subunit of lutropin (Parsons and Pierce, 1984), the β-subunit of human chorionic gonadotropin (Kessler et al., 1979), placental lactogen (Shimomura and Bremel, 1985), human erythropoietin (Lai et al., 1987), human urokinase (Bergwerff et al., 1992), and proteases from snake venom (Pfeiffer et al., 1992; Tanaka et al., 1992). In the O-glycan family, GalNAcβ1→4Gal is a constituent of the very rare blood group determinant called Cad (discussed earlier) and has been observed on non-reducing termini of oligosaccharides present in a cloned mureine cytotoxic T lymphocyte cell line (Conzelmann and Kornfeld, 1984a). β-Linked GalNAc has also been found in cervical glycoproteins from the bonnet monkey (Nasir-ud-Din et al., 1990), in fish egg glycoproteins (Shimamura et al., 1983; Iwasaki et al., 1984), and as a minor constituent of porcine zona pellucida glycoproteins (Hirano et al., 1993). In none of these instances is it known to be sulfated. Sulfate is, nevertheless, a relatively common substituent on O-glycans, especially the heavily glycosylated mucins, where it usually occurs attached in various linkages to Gal and GlcNAc (see, for example, Capon et al. (1989) and Mawhinney et al. (1992)). The biological significance of sulfation is poorly understood. Sulfated epitopes might be important for anti-adhesion by masking binding epitopes and providing a negatively charged surface on the glycoprotein. On the other hand, they appear to constitute an important part of the binding epitope in the L-selectin ligand (Lasky et al., 1992) and are an effective alternative to sialic acid in the E-selectin epitope (Yuen et al., 1992). The sulfated antennae of the pituitary glycohormone N-glycans are recognized by a specific hepatic reticuloendothelial cell receptor, which is believed to be responsible for their rapid clearance from the circulation (Fiete et al., 1991). However, studies of the metabolic clearance rate and half-life disappearance rate of human 16-kDa fragment have suggested that the hepatic receptor is unlikely to play a critical role in determining the metabolic fate of this glycopeptide (Lu et al., 1983; Siciliano et al., 1993). Carbohydrate composition analysis of the 16-kDa fragment and its biosynthetic derivatives isolated from pituitary tissue has indicated that processing at the -Arg6-Lys10 site is dependent upon the absence of O-glycosylation at threonine 45 (Brown et al., 1981; Bennett, 1984; James and Bennett, 1985; Seger and Bennett, 1986). This is consistent with the known predilection of O-glycans to inhibit the action of proteases (Jentoft, 1990). Whether the novel sulfated structure identified in the present study is critical for masking the cleavage site within the 16-kDa fragment has yet to be determined. The non-sulfated structures that are present as minor constituents might be equally capable of inhibiting processing. Alternatively, non-sulfated glycoforms could be processed to yield glycosylated forms of the amino-terminal residue 1-49 fragment that have remained undetected because of their low abundance.

The present study shows that less than 20% of the O-glycans in the 16-kDa fragment are non-sulfated and that they constitute a highly heterogeneous population of neutral and sialylated glycans, the latter containing both N-acetyl and N-glycolyl neuraminic acid. In two separate preparations of bovine pituitaries, considerable differences were detected in the neutral O-glycans despite minimal differences being observed in the acidic glycans. Although the neutral O-glycan variability could be due to differences in the purification

### Table V

<table>
<thead>
<tr>
<th>Elution time</th>
<th>Assignment</th>
<th>Characteristic fragment ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.87*</td>
<td>t-Fuc</td>
<td>118, 131, 162, 175</td>
</tr>
<tr>
<td>14.73*</td>
<td>t-Gal</td>
<td>102, 118, 129, 146, 161, 162, 205</td>
</tr>
<tr>
<td>15.96</td>
<td>3-Gal</td>
<td>118, 129, 161, 254</td>
</tr>
<tr>
<td>16.43</td>
<td>3,6-GalNAc</td>
<td>117, 159, 203, 205</td>
</tr>
<tr>
<td>18.82*</td>
<td>3,6-GalNAcitol</td>
<td>130, 246, 318</td>
</tr>
<tr>
<td>18.92*</td>
<td>t-GalNAc</td>
<td>117, 159, 203, 205</td>
</tr>
<tr>
<td>19.35</td>
<td>4-GlcNAc</td>
<td>117, 159, 233</td>
</tr>
<tr>
<td>20.23*</td>
<td>3,4-GlcNAc</td>
<td>117, 159, 346</td>
</tr>
</tbody>
</table>

GC-MS linkage analyses of HPLC fraction 27, which gave a major molecular ion corresponding to Fuc3HexHexNAc3 in the FAB spectrum.
protocols for the two preparations (see “Experimental Procedures”), which could conceivably have separated some glycoforms into fractions that were not characterized, we believe this to be unlikely because the same N-glycans (neutral as well as acidic) were observed in both preparations (data not shown). Furthermore, the sizes of the O-glycans observed in each preparation are similar and they are expected to have comparable chromatographic behavior. In future work we intend to explore the possibility that the structures of the non-sulfated O-glycans are affected by the metabolic status of the animals from which the pituitaries are obtained.

The expression of the various pressor, stereodifferent, and mitogenic actions of derivatives of the 16-kDa fragment is dependent upon correct processing of POMC at the -Arg4'-LysSo- processing site. Whether or not the previously prepared fragment, are cleaved at the foundation for future research directed toward establishing procedures), which could conceivably have separated some glycoforms, prepared by desulfation and controlled exoglycosidase digestion of the 16-kDa fragment, are cleaved at the -Arg4'-LysSo- processing site, which in turn is dependent upon correct processing of POMC at the -Arg4'-LysSo- processing site, which in turn is dependent upon the O-glycosylation status. Several hormonal functions have been proposed for the 16-kDa fragment and the products resulting from the action of pro-hormone convertases (Seger and Bennett, 1986; Estivariz et al., 1989). The amino-terminal fragment encompassing the first 49 residues has been shown to be a mitogen for adrenal cortical cells (Estivariz et al., 1982; Lowry et al., 1993). The carboxyl-terminal fragment comprising the γ-MSH sequence has been shown to potentiate the stereodirectional actions of ACTH on adrenal cells both in vivo and in vitro (Pederson and Brownie, 1980; Al-Dujaili et al., 1981). The secretion of both glucocorticoids and mineralocorticoids by the adrenal cortex is enhanced by peptides bearing the γ-MSH sequence (Pederson and Brownie, 1980; Pederson et al., 1980; Al-Dujaili et al., 1981; Seidah et al., 1981). The latter class of steroid is critical in maintenance of electrolyte homeostasis and blood pressure. In keeping with these observations, γ-MSHs have also been shown to have pressor and cardioaccelerator activity following intravenous administration to rats (Callahan et al., 1984; Klein et al., 1985; Sun et al., 1992; DeWilde et al., 1993). These hemodynamic effects have been shown to be mediated primarily through the sympathetic nervous system (Callahan et al., 1984; DeWilde et al., 1993). The effects of γ-MSH related peptides may also be mediated centrally through the autonomic nervous system (Gruber and Callahan, 1989). POMC is synthesized in the brain primarily in the arcuate nucleus of the hypothalamus. The biosynthetic processing of POMC in the rat hypothalamus is reminiscent of that observed in the intermediate lobe of the pituitary including extensive cleavage of the -Arg4'-LysSo- processing site within the 16-kDa fragment (Emeson and Eipper, 1986). The O-glycosylation status of the 16-kDa fragment is likely to be important for processing in the hypothalamus as well as the pituitary. It will therefore be interesting to establish whether the novel sulfated O-glycan is also present in POMC biosynthesized in the hypothalamus.

The structural studies reported in this paper provide a firm foundation for future research directed toward establishing the role of O-glycosylation in the control of POMC processing and for addressing other structure/function issues. For example, it will be interesting to examine whether truncated O-glycans, prepared by desulfation and controlled exoglycosidase digestion of the 16-kDa fragment, are cleaved at the -Arg4'-LysSo- processing site. Whether or not the previously identified pituitary GalNAC transferase (Smith and Baenzig, 1988) is responsible for addition of the capping GalNAC in the 16-kDa O-glycan can conceivably be resolved by assessing whether appropriately truncated glycoforms are substrates for this enzyme.

Acknowledgment—H. P. J. B. is grateful for the skilled technical assistance of Susan James.

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Epper, B. A., and with these observations, γ-MSHs have also been shown to have pressor and cardioaccelerator activity following intravenous administration to rats (Callahan et al., 1984; Klein et al., 1985; Sun et al., 1992; DeWilde et al., 1993). These hemodynamic effects have been shown to be mediated primarily through the sympathetic nervous system (Callahan et al., 1984; DeWilde et al., 1993). The effects of γ-MSH related peptides may also be mediated centrally through the autonomic nervous system (Gruber and Callahan, 1989). POMC is synthesized in the brain primarily in the arcuate nucleus of the hypothalamus. The biosynthetic processing of POMC in the rat hypothalamus is reminiscent of that observed in the intermediate lobe of the pituitary including extensive cleavage of the -Arg4'-LysSo- processing site within the 16-kDa fragment (Emeson and Eipper, 1986). The O-glycosylation status of the 16-kDa fragment is likely to be important for processing in the hypothalamus as well as the pituitary. It will therefore be interesting to establish whether the novel sulfated O-glycan is also present in POMC biosynthesized in the hypothalamus.

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O-Glycosylation of Bovine POMC

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