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Structural requirements for sulfation of asparagine-linked oligosaccharides of lutropin

(posttranslational modification/pituitary/placenta/gonadotropins/glycoproteins)

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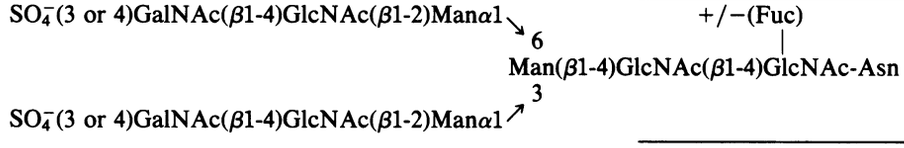
ABSTRACT **Human and bovine pituitary glycoprotein hormones (lutropin, follitropin, and thyrotropin) contain varying amounts of *N*-acetylgalactosamine and sulfate. The sulfate on asparagine-linked oligosaccharides of bovine lutropin (bLH) is present exclusively on GalNAc in the sequence GalNAc(β1-4)GlcNAc(β1-2)Manα. We have examined the structural requirements for sulfation of bLH oligosaccharides by using a reconstituted cell-free system. After cleavage from the protein, oligosaccharides containing the sequence GalNAc(β1-4)GlcNAc(β1-2)Manα were sulfated by enzymes in pituitary membranes. Addition of one or two sulfates was observed, depending upon the number of GalNAc acceptor sites on the oligosaccharide. Neither GalNAc alone nor oligosaccharides devoid of GalNAc were sulfated. Membranes from placenta or liver did not sulfate oligosaccharides released from bLH, indicating that the sulfating activity is pituitary-specific. The lack of peptide dependence for sulfation, in conjunction with the oligosaccharide specificity, suggests that the sequence GalNAc(β1-4)GlcNAc(β1-2)Manα contains the recognition signal for the sulfotransferase(s).**

The glycoprotein hormones from human and bovine pituitary bear unusual asparagine-linked oligosaccharides that contain *N*-acetylgalactosamine (GalNAc) and sulfate. We recently characterized (1) the structures of the sulfated oligosaccharides of bovine lutropin (bLH). The most highly sulfated species, S-2, has the following structure.



Monosulfated (S-1) oligosaccharides also contain the sequence SO_4-GalNAc-GlcNAc-Man but differ in the structure of their nonsulfated branches (1–3). Not all glycoprotein hormones contain GalNAc and sulfate. The asparagine-linked oligosaccharides of placental human chorionic gonadotropin (hCG) have the sequence sialic acid (Sia)-Gal-GlcNAc-Man at their nonreducing termini (4, 5). This is true for the oligosaccharides on both the α subunit of hCG, which contains the same amino acid sequence as α subunits of the pituitary glycoprotein hormones, and the hormone-specific β subunit. The absence of sulfate on hCG may reflect the lack of a placental enzyme capable of sulfation (2, 3) and/or the absence of GalNAc on the asparagine-linked oligosaccharides.

The pituitary glycoprotein hormones are not equally sensitive to sulfation, either *in vivo* or in a cell-free sulfation system (2). For example, purified human and bovine LH are extensively sulfated (1–3, 6, 7), whereas human follitropin (follicle-stimulating hormone, or FSH) contains relatively little if any sulfate but is more extensively sialylated (2, 8). Unlike human and bovine LH, human FSH is not sulfated in the cell-free system unless first digested with neuraminidase (2). Thus, the regulation of sulfation may be complex and reflect relatively subtle differences among the hormones and their respective oligosaccharide structures. We have utilized reduced and alkylated bLH, heterogeneous mixtures of endoglycosidase-released oligosaccharides, and purified oligosaccharide species to define the structural requirements for sulfation in the cell-free system.

MATERIALS AND METHODS

Synthesis of Adenosine 3′-phosphate 5′-phospho[^{35}S]sulfate (*P*Ado*P*[^{35}S]*S*). A mixture (5 ml) containing 5 mCi of [^{35}S]sulfate (ICN; 1 Ci = 37 GBq), 1 ml of ribosome-free supernatant (S-100) derived from Krebs ascites tumor cells (9), 3 mM ATP, 3 mM $MgCl_2$, 4 mM NaF, and 50 mM Tris Cl (pH 8.0) was incubated at 37°C for 8–10 hr. *P*Ado*P*[^{35}S]*S* was purified by ascending chromatography on Whatman 3MM paper in ethanol/1 M ammonium acetate, pH 7.5 (7:3, vol/vol) for 20 hr and stored in 5 mM Tris Cl (pH 8.0) at −70°C. Typical yields of 1.5 mCi were obtained.

Cell-Free Sulfation. Cell-free sulfation was carried out as described (3) with either 10 μCi of *P*Ado*P*[^{35}S]*S* or unlabeled *P*Ado*PS* (Sigma) and crude membrane preparations (9). Sulfated oligosaccharides were released from glycoproteins by digestion with endo-β-*N*-acetylglucosaminidase F (endo F). Reaction mixtures with *P*Ado*P*[^{35}S]*S* were passed through a MicroPak AX-10 (Varian) HPLC column in a solution containing 270 mM KH_2PO_4 and 10% methanol. At a flow rate of 1 ml/min, mono- and disulfated oligosaccharides were eluted at 3–7 min, whereas sulfate and *P*Ado*PS* were eluted at 7–10 and 14–20 min, respectively.

Sulfate Removal by Methanolysis. Dried samples were resuspended in 0.5 M HCl/anhydrous methanol, incubated at room temperature for 5–10 hr, and dried under a stream of N_2 or under reduced pressure. Residual HCl was removed either

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Abbreviations: LH, lutropin (luteinizing hormone); bLH and hLH, bovine and human LH, respectively; hCG, human chorionic gonadotropin; FSH, follitropin (follicle-stimulating hormone); *P*Ado*PS*, adenosine 3′-phosphate 5′-phosphosulfate; endo F and H, endo-β-*N*-acetylglucosaminidase F and H; S-0, neutral oligosaccharide(s); S-1, monosulfated oligosaccharide(s); S-2, disulfated oligosaocharide(s); S-1/Man_n, monosulfated oligosaccharide(s) containing *n* mannose residues; Sia, sialic acid.

by repeated evaporation from methanol or by gel filtration with Bio-Gel P-2. These conditions cleave sulfate-sugar linkages with >90% efficiency, as determined by analysis of [^{35}S]sulfate-labeled oligosaccharides.

Enzyme Digestions. Oligosaccharides were released from the protein by overnight digestion with endo F or endo H as described (3) or with *N*-glycanase (Genzyme, Boston, MA) in 0.2% Nonidet P-40/1 mM EDTA/75 mM sodium phosphate, pH 8.6. Exoglycosidase digestions were as described (10).

NaB^{3}H$_4$ Labeling of Oligosaccharides from Bovine LH. Purified bLH (11) was exhaustively digested with endo H or *N*-glycanase and labeled by reduction with 5 mCi of NaB^{3}H$_4$ (ICN) in 0.3 ml of sodium borate (pH 9.8) at 30°C for 3 hr as described (12). The oligosaccharides were purified by preparative anion-exchange HPLC.

Separation by HPLC. Anion-exchange HPLC of oligosaccharides was performed on a MicroPak AX-10 (Varian) column (13); elution was achieved with a linear gradient of 2.5 mM KH_2PO_4 to 125 mM KH_2PO_4 (pH 4.0) over 25 min. Oligosaccharide size was determined by ion-suppression amine absorption HPLC with a Micropak AX-5 (Varian) column as described (12).

RESULTS

Cell-Free Sulfation with *P*Ado*P*[^{35}S]*S*. We previously demonstrated that cell-free sulfation of asparagine-linked oligosaccharides and amino acids on glycoprotein hormones requires the presence of sulfotransferases from crude membrane fractions, the sulfate donor *P*Ado*PS*, and a proper substrate (3). Sulfation of exogenous bLH increases linearly with time for up to 24 hr and is dependent on the concentrations of pituitary membranes, hormone, and *P*Ado*P*[^{35}S]*S*. The sulfated oligosaccharides obtained in the cell-free system are identical to those labeled metabolically in pituitary explants. Cell-free sulfation is also highly substrate-specific, since nonsulfated glycoproteins do not act as acceptors of [^{35}S]sulfate. Sulfation of bLH is not affected by heat treatment (100°C for 5 min) or by reduction and alkylation, suggesting that the native conformation of the protein is not critical for sulfation. The signal for sulfation, therefore, could reside in the primary amino acid sequence, the oligosaccharide, or a combination of both. To establish whether peptide is required, sulfation of oligosaccharides on intact bLH was compared to sulfation of endo F-released bLH oligosaccharides.

Incubation of bLH in the cell-free system followed by digestion with endo F resulted in release of oligosaccharides that are resolved as monosulfated (S-1) and disulfated (S-2) species on anion-exchange HPLC (Fig. 1*a*). Incubation of endo F-released bLH oligosaccharides in the cell-free system yielded the same distribution of sulfated products (Fig. 1*b*). The amount of [^{35}S]sulfate incorporated was the same for intact bLH and endo F-digested bLH. Incubations containing neither bLH nor endo F-released bLH oligosaccharides yielded virtually no detectable radioactivity after removal of *P*Ado*P*[^{35}S]*S* and free [^{35}S]sulfate. Intact bLH is not eluted from the HPLC column under these conditions. These results indicate that the information required for sulfation of bLH is predominantly, if not exclusively, in the oligosaccharides and not in the peptide.

Generation of NaB^{3}H$_4$-Reduced bLH Oligosaccharides. To further examine the structural requirements for sulfation, we prepared individual ^{3}H-labeled oligosaccharides of defined structure and known concentration. Oligosaccharides released from bLH by digestion with endo H were labeled by reduction with NaB^{3}H$_4$. The monosulfated (S-1) species (Fig. 2*a*) consisted of two populations of oligosaccharides, S-1/Man$_5$ and S-1/Man$_4$, when examined by ion-suppression amine absorption HPLC (Fig. 2*b*). The structures of these

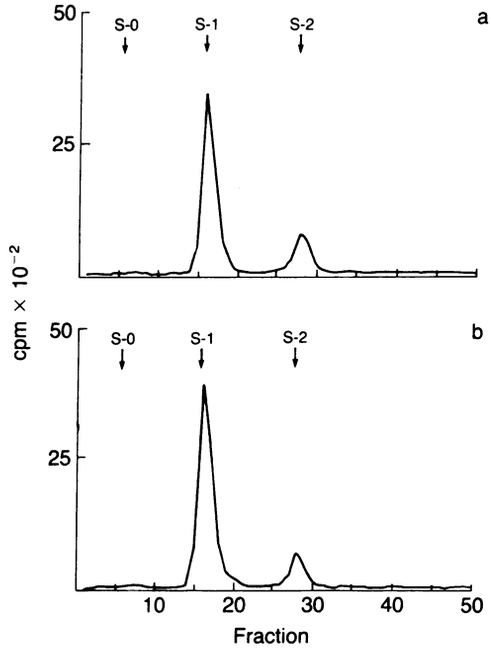

FIG. 1. Cell-free sulfation of bLH oligosaccharides using *P*Ado*P*[^{35}S]*S*. An equivalent of 100 μg each of desulfated bLH (*a*) and endo F-digested, desulfated bLH (*b*) were incubated in the cell-free sulfation system with 10 μCi of *P*Ado*P*[^{35}S]*S* and pituitary membranes (400 μg) for 24 hr. The sulfated protein sample (*a*) then was treated with endo F to release oligosaccharides. Excess *P*Ado*P*-[^{35}S]*S* and [^{35}S]sulfate were removed, yielding ≈10^5 cpm of [^{35}S]sulfated oligosaccharides from each incubation. An aliquot of each (10^3 cpm) was analyzed by anion-exchange HPLC. Elution positions of endo F-released non- (S-0), mono- (S-1), and disulfated (S-2) oligosaccharides from bLH are indicated.

oligosaccharides have been determined by exoglycosidase sensitivity, methylation analysis, periodate oxidation, and lectin binding and are shown in Fig. 3.

S-2 oligosaccharides, which are resistant to release with endo H, were liberated by digestion with *N*-glycanase or endo F. Since digestion with endo F does not yield a reducing terminus, endo F-released oligosaccharides could only be obtained from metabolically labeled bLH. S-2 oligosaccharides were, however, labeled by reduction with NaB^{3}H$_4$ following release by *N*-glycanase. Sulfate can be removed from bLH oligosaccharides by methanolysis, as illustrated for S-1/Man$_5$ and S-1/Man$_4$ oligosaccharides (Fig. 4 *a* and *b*).

Cell-Free Sulfation with Unlabeled *P*Ado*PS*. The ^{3}H-labeled, desulfated S-1/Man$_5$ and S-1/Man$_4$ oligosaccharides were used as substrates in the cell-free system to further study the structural requirements for oligosaccharide sulfation. In the presence of unlabeled *P*Ado*PS* and sulfotransferase(s) from pituitary membranes, a significant portion of desulfated, ^{3}H-labeled S-1 oligosaccharides was sulfated in 24 hr (Fig. 4*c*). The extent of sulfation can be quantitated by comparing the relative areas of the S-0 and S-1 peaks. Under these conditions, sulfate was added to 20% of the desulfated S-1 oligosaccharides. No evidence of S-2 oligosaccharides is seen, consistent with the presence of only one sulfation site (GalNAc) on S-1/Man$_5$ and S-1/Man$_4$ oligosaccharides. Ion-suppression HPLC analysis confirmed that cell-free sulfate addition to the desulfated S-1 oligosaccharides produces the original structures shown in Fig. 3.

By use of this type of cell-free assay, the relative amount of sulfate incorporated into desulfated, neutral oligosaccharides can be quantitated. The extent of sulfation is expressed as the percent of desulfated S-1 (neutral) converted to S-1 (anionic). Sulfation of desulfated S-1/Man$_5$ and S-

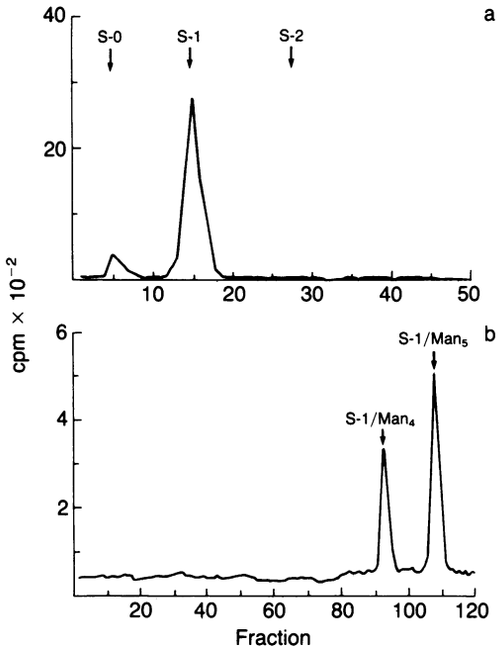

FIG. 2. Characterization of endo H-released, NaB^3H_4-reduced oligosaccharides from bLH. Endo H-released oligosaccharides from bLH were reduced with NaB^3H_4, and an aliquot of the final product (10^3 cpm) was analyzed by anion-exchange HPLC (*a*). Elution positions of bLH oligosaccharides are indicated as in Fig. 1. Following purification by anion-exchange HPLC, S-1 oligosaccharides (3000 cpm) were examined by ion-suppression amine absorption HPLC (*b*). Peaks corresponding to oligosaccharides with 4 (S-1/Man_4) and 5 (S-1/Man_5) mannose residues are indicated.

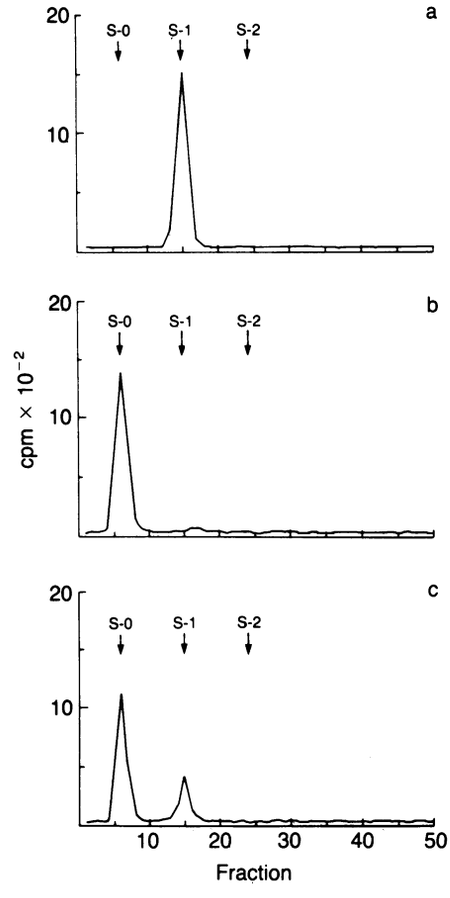

FIG. 4. Cell-free sulfation using unlabeled *PAdoPS*. Endo H-released, NaB^3H_4-reduced S-1 oligosaccharides (5000 cpm) from bLH were analyzed by anion-exchange HPLC before (*a*) and after (*b*) removal of sulfate by methanolysis. Desulfated S-1 oligosaccharides (5000 cpm) were also incubated for 24 hr in the cell-free sulfation system containing 2 mM unlabeled *PAdoPS* and 400 μg of pituitary membranes and then analyzed by anion-exchange HPLC (*c*). Positions of oligosaccharides from bLH are indicated as in Fig. 1.

1/Man_4 is directly proportional to the amount of pituitary membranes (Fig. 5*a*), the *PAdoPS* concentration up to 2 mM (Fig. 5*c*), and the incubation time up to 40 hr (Fig. 5*b*). The fraction of desulfated oligosaccharides that was susceptible to sulfation varied. Some preparations of desulfated substrates have yielded as high as 50–70% sulfation; however, the majority were in the range of 30–40%. This variation appears to reflect damage to reduced oligosaccharides during desulfation, since metabolically labeled, unreduced oligosaccharides consistently yielded 50–70% sulfation. Susceptibility to damage during desulfation may be due to deacetylation of the *N*-acetylglucosaminitol.

Substrate Specificity of Pituitary Sulfotransferase(s). Results of comparative studies using radiolabeled oligosaccharides and monosaccharides in the cell-free sulfation system are summarized in Table 1. Saturating conditions [400 μg of membranes (protein), 2.0 mM *PAdoPS*, and 48-hr incubation] were used to assess which substrates could be sulfated under optimal conditions. Equal amounts of radioactive oligosaccharide substrate were used, which corresponded to a final concentration of ≈40 μM. Desulfated S-1/Man_5 and S-1/Man_4 oligosaccharides of bLH (structures 1 and 2) were sulfated to an equal extent under saturating conditions (30–40% sulfated). Exhaustive digestion of these oligosaccharides with α-mannosidase yielded desulfated S-1/Man_3 and S-1/Man_2 (structures 3 and 4), both of which are equally susceptible to sulfation as desulfated S-1/Man_5 and S-1/Man_4. Thus a structure as small as the pentasaccharide GalNAc(β1-4)GlcNAc(β1-2)Man(α1-3)Man(β1-4)GlcNAc-ol can act as a substrate. Desulfated S-2 (structure 5) was sulfated to the same extent at the desulfated S-1 oligosaccharides. Unlike the endo H-released, desulfated S-1 species, addition of two sulfates was observed on 5–10% of the sulfated oligosaccharides obtained with desulfated S-2. This is consistent with the presence of two terminal GalNAc residues on S-2 and only one on the S-1/Man_5 and S-1/Man_4 oligosaccharides. Structures 1–5 were also sulfated equally when the reaction was terminated while sulfate incorporation was still increasing linearly (200–400 μg of membranes, 2.0 mM *PAdoPS*, and 24-hr incubation). Structural features of the oligosaccharide, other than the presence of β1,4-linked GalNAc, do not seem to influence the efficiency or extent of sulfation in the cell-free system.

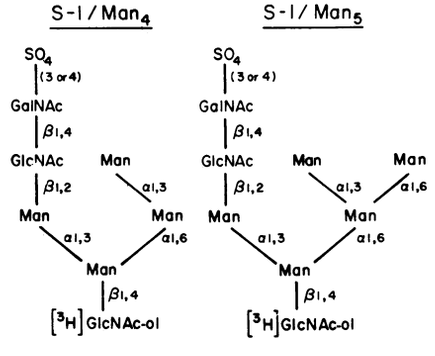

FIG. 3. Proposed structures for endo H-released, NaB^3H_4-reduced S-1/Man_4 and S-1/Man_5 oligosaccharides from bLH. GlcNAc-ol, *N*-acetylglucosaminitol.

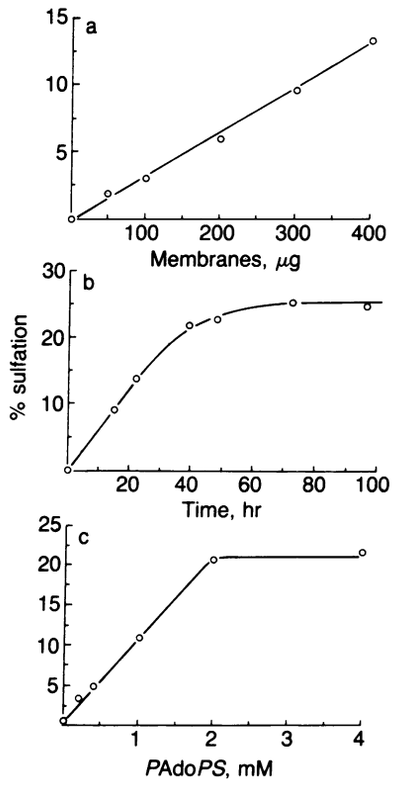

FIG. 5. Optimization of cell-free sulfation using unlabeled *P*AdoPS. Endo H-released, NaB^3H_4-reduced S-1 oligosaccharides from bLH were desulfated by methanolysis and used as sulfate acceptors in the cell-free system. Each incubation mixture (5000 cpm) was analyzed by anion-exchange HPLC, and the percent of oligosaccharides sulfated was determined by peak integration (see Fig. 4*c*). (*a*) *P*AdoPS (2 mM) and various amounts of bovine pituitary membranes were incubated for 20 hr. (*b*) *P*AdoPS (2 mM) and 300 μg (protein) of bovine pituitary membranes were incubated for various times. (*c*) Various concentrations of *P*AdoPS and 400 μg of bovine pituitary membranes were incubated for 24 hr.

Oligosaccharides devoid of GalNAc were not sulfated in the cell-free system when examined at the same concentration as bLH oligosaccharides. Sulfation of asialo (structure 6), agalacto (structure 7), and ahexosamino (structure 8) dibranched complex oligosaccharides was not observed, even under saturating conditions. Likewise, radiolabeled GalNAc, GlcNAc, and galactose were not sulfated.

To further investigate specificity, we tested larger quantities of unlabeled substrates for sulfation with *P*AdoP[^{35}S]S. The monosaccharides shown in Table 1 were tested at concentrations up to 40 mM in the cell-free assay, but no detectable sulfated products were observed. Smith degradation (6, 14) of bLH yielded some disaccharide with the structure SO_4-GalNAc-GlcNAc-X, where X represents a portion of the underlying mannose residue that was destroyed by periodate oxidation. Following desulfation, this structure was sulfated by sulfotransferase(s) in pituitary, but not placental, membranes (data not shown). However, the amount of [^{35}S]sulfate incorporated was only 25% of that found with parallel incubations of an equivalent amount of bLH or endo F-digested bLH. These results suggest that the disaccharide GalNAc(β1-4)GlcNAc can be specifically sulfated. Sulfation of the disaccharide may be less efficient than that of the larger oligosaccharides. Since the amount of sulfated disaccharide that was generated by Smith degradation of bLH could not be ascertained, quantitative comparison to other structures was not possible.

Table 1. Oligosaccharide substrate requirements of pituitary sulfotransferase(s)

No.	Substrate* Structure	Sulfation†
1	Man | Man-Man | GalNAc-GlcNAc-Man-Man-GlcNAc-ol	+
2	Man-Man | GalNAc-GlcNAc-Man-Man-GlcNAc-ol	+
3	Man | GalNAc-GlcNAc-Man-Man-GlcNAc-ol	+
4	GalNAc-GlcNAc-Man-Man-GlcNAc-ol	+
5	GalNAc-GlcNAc-Man | GalNAc-GlcNAc-Man-Man-GlcNAc-ol	+
6	Gal-GlcNAc-Man | Gal-GlcNAc-Man-Man-GlcNAc-ol	−
7	GlcNAc-Man | GlcNAc-Man-Man-GlcNAc-ol	−
8	Man | Man-Man-GlcNAc-ol	−
9	GalNAc, GlcNAc, or Gal	−

GlcNAc-ol, *N*-acetylglucosaminitol.

*For structures 5–8, both reduced (shown) and unreduced oligosaccharides were incubated in sulfation reaction mixtures. For structures 1, 2, and 5, both endoglycosidase- (shown) and *N*-glycanase-released oligosaccharides (containing an additional core GlcNAc) were incubated.

†−, No sulfated product detected.

Tissue Specificity of Sulfation. The sulfotransferase(s) responsible for sulfation of bLH oligosaccharides is probably pituitary-specific, since cell-free addition of [^{35}S]sulfate to bLH is mediated by membranes from pituitary but not placenta (2, 3). Intact bLH is, however, weakly sulfated by liver membranes (3). Using saturating conditions for cell-free addition of unlabeled sulfate, membrane fractions from bovine pituitary, bovine placenta, and rat liver were tested for their ability to sulfate desulfated S-1/Man_5 and S-1/Man_4. Whereas the pituitary membranes sulfated these oligosaccharides, placenta and liver membranes were devoid of detectable activity over a wide range of concentrations (Fig. 6). The oligosaccharides whose structures are shown in Table 1 were each incubated with placental membranes and analyzed for sulfate incorporation. In contrast to pituitary membranes, the placental membranes did not sulfate any of these structures, verifying that sulfation of the partially degraded, GalNAc-containing oligosaccharides (structures 3 and 4) is also tissue-specific.

DISCUSSION

The asparagine-linked oligosaccharides on the α and β subunits of bLH contain sulfate and GalNAc but no sialic acid or galactose. We recently characterized the structures of the sulfated oligosaccharides on bLH and found that they invariably contained one or two branches with the sequence SO_4-GalNAc(β1-4)GlcNAc(β1-2)Manα in place of the more common sequence Sia(α2-6 or 3)Gal(β1-4)GlcNAc(β1-2)Manα. hCG, which is synthesized by placental trophoblasts, contains the latter sequence in its asparagine-linked

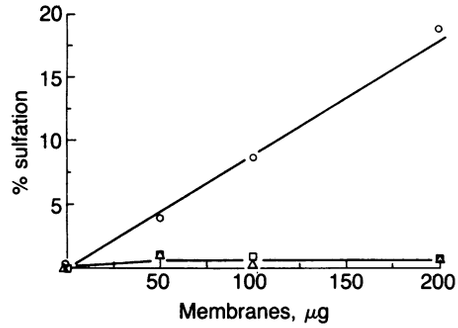

FIG. 6. Tissue specificity of oligosaccharide sulfation. Endo H-released, NaB^3H_4-reduced S-1 oligosaccharides from bLH were desulfated and incubated for 48 hr in the cell-free sulfation system with 2 mM *PAdoPS* and various amounts of bovine pituitary (○), bovine placenta (△), or rat liver (□) membranes. Each incubation mixture (5000 cpm) was analyzed by anion-exchange HPLC, and the percent of oligosaccharides sulfated quantitated (see Fig. 4*c*).

oligosaccharides, which are devoid of sulfate and GalNAc. Exogenously added bLH, but not hCG, is sulfated in the cell-free system by pituitary membranes, but neither protein is sulfated by placental membranes (2, 3). These and other data have led us to conclude that sulfation is both substrate- and tissue-specific.

Two observations suggested that the sulfotransferase(s) recognized features of the oligosaccharide rather than the peptide. (*i*) Reduction and alkylation of bLH did not destroy its ability to act as a sulfate acceptor (3). (*ii*) The GalNAc(β1-4)GlcNAc(β1-2)Manα sequence, which thus far has only been found in pituitary glycoprotein hormones, is sufficiently distinct from other oligosaccharides that it may provide the signal for sulfation. The purpose of this study was to establish the minimal structural requirements for specific sulfation of bLH oligosaccharides. Our data show that sulfation of the asparagine-linked oligosaccharides of bLH is not dependent on peptide but does require the sequence GalNAc(β1-4)GlcNAc(β1-2)Manα. GalNAc alone or oligosaccharides devoid of GalNAc are not sulfated in the cell-free system.

Sulfation of pituitary hormone oligosaccharides is distinctly different from phosphorylation of high-mannose oligosaccharides on lysosomal enzymes. Phosphorylation of these structures by the UDP-GlcNAc phosphotransferase is dependent on the recognition of the protein (15, 16). Any perturbation of the native conformation of lysosomal enzymes inhibits phosphorylation. Deglycosylated lysosomal enzymes can act as competitive inhibitors of phosphorylation, further demonstrating that recognition by the UDP-GlcNAc phosphotransferase is directed specifically at the peptide and not the oligosaccharides.

Tissue specificity is displayed for oligosaccharides released from bLH, which are sulfated by pituitary membranes but not those from placenta or liver. The lack of an appropriate placental sulfotransferase(s) and the absence of GalNAc in hCG asparagine-linked oligosaccharides suggest that the placenta is incapable of synthesizing asparagine-linked oligosaccharides containing sulfate or GalNAc. Regulation of the synthesis of asparagine-linked oligosaccharides may, however, be more complex in the pituitary. We have compared the human and bovine hormones as sulfate acceptors in the cell-free system and found that the ability to be sulfated correlates with the presence of GalNAc (2). hFSHhas little GalNAc as isolated from pituitary and is not sulfated unless digested with neuraminidase. hLH is sulfated in the cell-free system, yielding products that are similar, but not identical, to bLH. Thus the oligosaccharides on LH and FSH appear to differ significantly even though these hormones may be synthesized in the same cell and share α subunits with identical amino acid sequences.

The sulfated oligosaccharides present on bLH differ from dibranched complex oligosaccharides that are found on many glycoproteins in that they possess the peripheral sequence SO_4-GalNAc(β1-4)GlcNAc rather than Sia-Gal(β1-4)GlcNAc. The sequence found on bLH is likely to be present on other sulfated glycoprotein hormones, including bFSH, hLH, and bovine and human thyrotropins (2). In contrast to bLH which contains no sialic acid or galactose, bFSH, hLH, and human thyrotropin contain asparagine-linked oligosaccharides with only Sia-Gal and oligosaccharides with both Sia-Gal and SO_4-GalNAc sequences (2). In the case of hFSH, only oligosaccharides with the terminal sequence Sia-Gal are found. This indicates that a pivotal step in the synthesis of sulfated, as opposed to sialylated, oligosaccharides is the addition of GalNAc rather than galactose. The differences in the susceptibility to sulfation, in conjunction with the specificity of the sulfotransferase(s), suggest that addition of β1,4-linked GalNAc is a hormone-specific, peptide-dependent event that occurs in the pituitary and provides the signal, as well as the site, for sulfate addition.

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