Sulphation of proteins secreted by a human hepatoma-derived cell line

Sulphation of *N*-linked oligosaccharides on α_2 HS-glycoprotein

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Several human glycoproteins, including α_1 -antitrypsin, α_1 -acid glycoprotein, transferrin, caeruloplasmin and α_2 HS-glycoprotein, synthesized by the hepatoma-derived cell line HepG2 were observed to contain covalently linked sulphate. These proteins were estimated to contain about 0.1 mol of sulphate/mol of protein. The most abundant of the sulphated glycoproteins, α_2 HS-glycoprotein, was analysed in detail. All of the sulphate on this protein was attached to *N*-linked oligosaccharides which contained sialic acid and resisted release by endoglycosidase H. Several independent analytical approaches established that approx. 10% of the molecules of α_2 HS-glycoprotein contained sulphate. Our results suggest that a number of human plasma proteins contain small amounts of sulphate linked to oligosaccharides.

INTRODUCTION

Sulphation of oligosaccharides generally has been considered to be a rare modification of glycoproteins (Parsons & Pierce, 1980; Heifetz *et al.*, 1980). The increasing number of examples of glycoproteins reported to contain sulphate, however, suggests that it is a widespread post-translational modification. A broad phylogenetic spectrum of organisms, ranging from simple halobacteria (Lechner *et al.*, 1985) and slime moulds (Freeze *et al.*, 1983) to mammals (Hortin *et al.*, 1981; Heifetz *et al.*, 1982; Green *et al.*, 1984; and references therein) contain sulphated glycoproteins. Sulphation of glycoproteins has been noted in diverse tissues such as brain (Simpson *et al.*, 1976; Klinger *et al.*, 1985), liver (Bertrand *et al.*, 1983), thyroid (Edge & Spiro, 1984) and pituitary (Green *et al.*, 1984). The catalogue of proteins reported to contain sulphated oligosaccharide is growing rapidly, just as, similarly, a considerable number of proteins are being recognized to contain sulphate linked to tyrosine residues (Huttner, 1982, 1984). A comprehensive listing of examples is too lengthy to be included here.

Although numerous studies have reported the sulphation of particular glycoproteins, most studies have been limited to the identification of products which are biosynthetically labelled with [35 S]sulphate and to the determination of whether incorporated sulphate is linked to tyrosine or to oligosaccharides. The physiological significance and many basic aspects about the sulphation of proteins are not understood. It is not known what effect sulphation has on the stability and biological activity of glycoproteins, how the specificity of sulphation is determined, or what percentage of glycoproteins are so modified. There is also a shortage of data on detailed structural determination of sulphated oligosaccharides and on the stoichiometry of sulphation. Most studies have not ascertained whether the sulphated forms of glycoproteins represent major or minor components.

This basic information is essential for understanding the functional and structural role of the sulphation of glycoproteins.

The present study sought to identify new examples of glycoproteins which contain sulphate and to examine two basic issues about the sulphation of glycoproteins, i.e. the prevalence of sulphation and its stoichiometry. We examined the sulphation of proteins by the human hepatoma-derived cell line HepG2 because these cells are known to synthesize actively most of the proteins made by normal hepatocytes (Knowles *et al.*, 1980), the cell type synthesizing the greatest number of well-characterized glycoproteins. Unexpectedly, we observed that a considerable number of proteins secreted by these cells contained sulphate. Previously, the only reported example of a protein sulphated by HepG2 cells is the fourth component of complement (C4), which has sulphate linked to tyrosine (Karp, 1983). The only other human proteins reported to contain sulphate are lutropin (Parsons & Pierce, 1980) and unidentified glycoproteins secreted by vascular endothelium (Heifetz *et al.*, 1982), which have sulphate bound to *N*-linked oligosaccharides. The present report describes the sulphation of several proteins secreted by HepG2 cells, and the sulphation of the most abundant of these glycoproteins, α_2 HS-glycoprotein (α_2 HS), is analysed in detail.

EXPERIMENTAL

Materials

HepG2 cells were obtained from Dr. Barbara Knowles of the Wistar Institute (Philadelphia, PA, U.S.A.). Purified α_2 HS was bought from Calbiochem/Behring. Carrier-free $\text{Na}_2^{35}\text{SO}_4$ was purchased from ICN, and [^3H]leucine, [^3H]tyrosine and [^3H]valine were from Amersham. Antisera against the following human proteins were purchased from the indicated sources:

α_2 HS, haemopexin, eighth component of complement, β_2 -glycoprotein I, coagulation factor IX, zinc α_2 -glycoprotein, α_2 -antiplasmin, C1s esterase inhibitor, antithrombin III, apolipoprotein B and α_2 -histidine-rich glycoprotein from Calbiochem/Behring; albumin, fibrinogen, C1s, second, third, fourth, fifth and ninth components of complement, coagulation factor XII, factor B and prothrombin from Miles Scientific; caeruloplasmin, α_1 -acid glycoprotein, α_1 -antitrypsin, transferrin, plasminogen, fibronectin and haptoglobin from Sigma; C1r and α_2 HS from Atlantic Antibodies (Scarborough, ME, U.S.A.); α_1 -antichymotrypsin from Dako Corp. (Santa Barbara, CA, U.S.A.). Protein A-Sepharose CL was obtained from Pharmacia. Chymotrypsin and trypsin (treated with Tos-Phe-CH₂Cl) were from Worthington. Endoglycosidase F was purchased from New England Nuclear or prepared as previously described (Elder & Alexander, 1982).

Cell culture

HepG2 cells were maintained in Earle's medium without antibiotics, supplemented with 10% (v/v) foetal-calf serum and 4 mM-glutamine. Cells were incubated in serum-free medium with no unlabelled sulphate for incorporation of [³⁵S]sulphate, with 0.3 mM-glucose for incorporation of [³H]mannose, and with 5 μ M of unlabelled amino acid when labelling with ³H-labelled amino acids.

Isolation and analysis of labelled proteins

Radiolabelled products in medium were isolated by immunoprecipitation with monospecific antibodies bound to protein A-Sepharose. Each immunoprecipitation used 25 μ l of serum unless indicated otherwise in Figure legends. For immunoprecipitations, culture medium was adjusted to 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mg of bovine serum albumin/ml, 1 mM-benzamidine, 5 mM-EDTA, 1 mM-*p*-chloromercuribenzenesulphonic acid and 50 mM-Tris, pH 8.0. Medium was then mixed with bound antibody for 1.5–2 h at room temperature. Immunoprecipitates were washed extensively and transferred to a different tube to remove unbound products. Products were analysed by electrophoresis in polyacrylamide gels containing SDS (Gordon *et al.*, 1984), or for cationic-detergent electrophoresis (Mocz & Balint, 1984) 0.2% cetyltrimethylammonium bromide was added to the top-chamber buffer. Sample buffer for electrophoresis contained 20 g of detergent/l, 100 ml of glycerol/l, 50 ml of 2-mercaptoethanol/l, and Bromophenol Blue or Crystal Violet as tracker dye. Samples were heated for 3 min at 100 °C before electrophoresis. Labelled products in gels were detected by fluorography with sodium salicylate as the fluor and Kodak XAR film. Sequential Edman degradation was performed with a Beckman 890C sequenator using a 0.33 M-Quadrol program (Gordon *et al.*, 1984).

Reverse-phase h.p.l.c.

Proteinase digestions were performed in 100 mM-NH₄HCO₃, pH 8.0, with 0.02% NaN₃. Peptides were then injected directly or after concentration by freeze-drying on to a Waters C18 μ Bondapak column equilibrated with 0.05% trifluoroacetic acid. Elution was isocratic for 5 min, followed by a linear gradient of acetonitrile increasing in concentration by 0.5%/min. Flow rate was 1 ml/min, and 1 min fractions were

collected for scintillation counting in cocktail 3a70 (Research Products International, Mount Prospect, IL, U.S.A.).

Analysis of oligosaccharides

N-Linked oligosaccharides were released from peptides by endoglycosidase F as described by Elder & Alexander (1982). Anion-exchange h.p.l.c. of oligosaccharides was performed on a Varian MicroPak AX-10 column as described by Baenziger & Natowicz (1981) and Green *et al.* (1984). Elution was with 2.5 mM-KH₂PO₄ at pH 4.0 for 10 min, followed by a linear gradient to 250 mM-KH₂PO₄ over 20 min. Oligosaccharides were desialylated by heating for 15 min at 100 °C in 2 M-acetic acid, followed by treatment with neuraminidase from *Clostridium perfringens* in 100 mM-cacodylate buffer, pH 6.0, for 16 h at 37 °C. Affinity of oligosaccharides for concanavalin A was assayed by loading them on a column (1 cm $\times$ 7 cm) of concanavalin A-Sepharose (Pharmacia) in 150 mM-NaCl/1 mM-CaCl₂/1 mM-MgCl₂/0.02% NaN₃/10 mM-Tris/HCl, pH 8.0, at room temperature. Bound oligosaccharides were eluted at 65 °C with the same buffer containing 0.1 M- α -methyl mannoside.

RESULTS

Sulphation of multiple proteins secreted by HepG2 cells

Culture medium of HepG2 cells incubated with [³⁵S]sulphate was assayed for the presence of sulphated proteins by immunoprecipitation with polyvalent antisera specific for individual human plasma proteins. Polyacrylamide-gel electrophoresis of immunoprecipitates and detection of labelled products by autofluorography indicated that several proteins contained sulphate. Fig. 1 shows an example of sulphate-labelled transferrin, caeruloplasmin, α_1 -antitrypsin and α_1 -acid glycoprotein isolated by specific immunoprecipitation. Faint background bands in each lane are due to slight contamination of these immunoprecipitates with several peptides derived from C4, which was the protein most heavily labelled by sulphate. We also detected sulphate-labelled bands corresponding to α_2 HS (shown below), prothrombin, fibrinogen, and the second and fifth components of complement (not shown). Not all products of HepG2 cells incorporated detectable quantities of sulphate, however. No sulphate was detected attached to the third component of complement, apolipoprotein AI or albumin, which are major secretory products of HepG2 cells. For other proteins examined sulphation was not detected, but it was not clear whether this resulted from absence of sulphate on the products or from secretion of insufficient amounts of protein to detect sulphation.

Among 31 proteins precipitated with specific antisera (listed under 'Materials'), C4 incorporated the greatest amount of sulphate, α_2 HS incorporated about one-third as much sulphate, and other proteins incorporated lesser amounts. The quantities of [³H]leucine and [³⁵S]sulphate precipitated by antisera specific for C4, α_2 HS, albumin and α_1 -acid glycoprotein are compared in Table 1. Several times more ³⁵S was incorporated into C4 and α_2 HS than into other products. However, comparison of the absolute quantities of sulphate label precipitated by each antiserum can be misleading, because HepG2 cells synthesize different quantities of each protein and because the efficiency of immunoprecipitation with different antisera may vary. The quantity of [³H]leucine

Fig. 1. Polyacrylamide-gel electrophoresis of sulphate-labelled products

One 75 cm² flask of HepG2 cells at confluency was incubated for 15 h in medium containing 5 mCi of [³⁵S]sulphate. Culture medium was divided into aliquots, which were precipitated with antisera against transferrin (lane 1), caeruloplasmin (lane 2), α_1 -acid glycoprotein (lane 3), or α_1 -antitrypsin (lane 4). One-third of each immunoprecipitate was loaded on a 10%-polyacrylamide gel. Labelled products were detected by fluorography with a 1-day exposure. The positions of M_r markers are indicated.

molecule can be calculated from the ratio of ³⁵S to ³H. This comparison shows that the amount of sulphate incorporated per molecule of C4 was manyfold greater than for the other proteins. Also, it is possible to estimate the absolute number of sulphate groups per molecule, since we have ascertained that C4 contains an average of approx. 3 sulphate groups per molecule (Hortin *et al.*, 1986). These calculations suggest that sulphate is attached to a small fraction of the molecules of the proteins other than C4. In particular, 9% of the molecules of α_2 HS are estimated to contain sulphate. Substantial amounts of sulphate label were incorporated into α_2 HS, despite the low degree of sulphation of this protein, because it was one of the major secretory products of HepG2 cells; 10–20 times more moles of α_2 HS were synthesized than of C4. The small amount of sulphate associated with the albumin immunoprecipitate, 0.007 mol/mol of protein, is presumed to represent non-specific binding, since no sulphate-labelled product corresponding to albumin was detected by electrophoresis. Among the proteins that we examined, stoichiometric sulphation was apparent only for C4, although quantification of ratios is increasingly imprecise for proteins such as α_1 -acid glycoprotein, which were synthesized in smaller quantities by HepG2 cells. Similar comparison of the ratios of label in sulphate and amino acids in several other proteins yielded the following estimates of mol of sulphate/mol of protein: prothrombin 0.1, caeruloplasmin 0.4, second component of complement 0.04 and fifth component of complement 0.1. Our further studies focused on detailed characterization of the sulphation of α_2 HS as the most abundant representative of the class of non-stoichiometrically sulphated proteins.

Characterization of α_2 HS

HepG2 cells incubated with [³⁵S]sulphate or [³H]leucine secreted a single product precipitated by antiserum specific for α_2 HS, and the product had an M_r of approx. 55000 by electrophoresis in the presence of SDS under reducing conditions (Fig. 2, lanes 1 and 2). This is consistent with previous studies of α_2 HS by Gejyo & Schmid (1981) and Matsushima *et al.* (1982), who obtained slightly higher M_r values by electrophoresis than the value of 49000 determined by analytical centrifugation (Schmid & Burgi, 1961). There are conflicting data regarding whether α_2 HS isolated from

in each product can be used to normalize for these factors, and, for proteins with a known number of leucine residues, the relative number of sulphate groups per

Table 1. Quantification of sulphation of proteins

One 75 cm² flask of HepG2 cells was incubated for 154 h with 3 mCi of [³⁵S]sulphate and 300 μ Ci of [³H]leucine in 10 ml of sulphate-free medium. The culture medium was divided into five aliquots, from which products were immunoprecipitated by antibodies bound to Protein A–Sepharose. Background values were from immunoprecipitation with non-immune rabbit serum. Each value represents duplicate counts of 20% of the total immunoprecipitate. The numbers of leucine residues in each protein are reported values from compositional or sequence analyses (Gigli *et al.*, 1977; Dayhoff, 1978). The average number of sulphate groups per molecule of C4 was assumed to be 3, based on the content of tyrosine sulphate sulphate in this protein (Hortin *et al.*, 1986).

Protein	[³⁵ S]Sulphate (d.p.m.)	[³ H]Leucine (d.p.m.)	[³⁵ S]/[³ H]	Leucine content (mol/mol)	Sulphate content (mol/mol)
C4	37000	67200	0.55	187	3
α_2 HS	12200	141000	0.087	34	0.09
Albumin	1400	359000	0.0039	61	0.007
α_1 -Acid glycoprotein	1580	10400	0.15	13	0.06
Background	700	2590			

plasma consists of a single peptide chain or of two disulphide-linked peptide chains of different size (Lebreton *et al.*, 1979; Gejyo & Schmid, 1981; Matsushima *et al.*, 1982; Gejyo *et al.*, 1983). α_2 HS secreted by HepG2 cells consists of a single chain, since only a single band was detected for α_2 HS labelled with [3 H]leucine (lane 2) or [3 H]valine (results not shown). These amino acids have been detected in both peptides of two-chain forms of α_2 HS (Gejyo & Schmid, 1981; Gejyo *et al.*, 1983). α_2 HS also had an apparent M_r of approx. 55000 when analysed without reduction (result not shown). Sulphate-labelled C4 is shown (Fig. 2, lane 3) to illustrate that precipitation of sulphate label by antibody to α_2 HS does not result from precipitation of C4. Specificity of immunoprecipitation was also shown by competitive inhibition of immunoprecipitation of sulphate-labelled α_2 HS by purified α_2 HS isolated from plasma. Almost no sulphate-labelled α_2 HS was precipitated in the presence of 50 μ g of purified α_2 HS (lane 7) as compared with the control (lane 6). Fig. 2 also shows the total profile of [3 H]leucine- and [35 S]sulphate-labelled products secreted by HepG2 cells

Fig. 2. Electrophoresis of α_2 HS, C4 and total secretory products, from HepG2 cells

HepG2 cells were labelled for 14 h with 1 mCi of [35 S]sulphate or 400 μ Ci of [3 H]leucine in 10 ml of medium. Labelled products secreted into the medium were isolated by immunoprecipitation with 25 μ l of antiserum to α_2 HS (lanes 1 and 2) or to C4 (lane 3). For analysis of the total profile of labelled products, medium was mixed with concentrated sample buffer. Samples in lanes 1–5 represent 8%, 4%, 8%, 4% and 4% respectively of the total product from one 75 cm² flask of cells. Fluorography was for 2 days. In lanes 6 and 7 sulphate-labelled α_2 HS was precipitated with a limiting quantity of antiserum (2 μ l) in the absence (lane 6) or presence (lane 7) of 50 μ g of purified α_2 HS. The gel was exposed for 4 days.

(lanes 4 and 5). Immunoprecipitable α_2 HS accounted for as much as 15% of total acid-precipitable [3 H]tyrosine in culture medium. A band co-migrating with α_2 HS is prominent in the total profile of leucine-labelled protein. The most intensely labelled band is albumin. Individual protein bands among the total sulphate-labelled products (lane 5) were obscured by labelled glycosaminoglycans, which accounted for most of the acid-precipitable sulphate label. More than 60% of the total acid-precipitable sulphate in medium was released by chondroitinase ABC, whereas less than 1% of the total was immunoprecipitated with antibody to α_2 HS.

Examination of the total profile of sulphate-labelled proteins was possible by electrophoresis in the cationic detergent cetylpyridinium bromide. This system separates proteins on the basis of size (Mocz & Balint, 1984), similarly to electrophoresis in SDS. We observed that glycosaminoglycans did not enter the separating gel in this system, permitting analysis of sulphated proteins free of interference from glycosaminoglycans (Fig. 3). Cationic detergents are precipitating agents for glycosaminoglycans; this may account for the inability of glycosaminoglycans to migrate into these gels. Three major sulphate-labelled proteins, of M_r 55000, 75000 and 95000, were observed in the total profile of proteins in the medium. α_2 HS (M_r 55000; lane 1) migrated similarly to the lower band of the total profile (although the band in the total profile is broader and may contain other sulphate-labelled proteins of similar size). The product with M_r 75000 has not been identified. The α -chain of C4 (M_r 95000; lane 2) co-migrated with the third band in the total profile. Notably, there was no sulphate-labelled band at the position of albumin (shown in lane 3 labelled with [3 H]leucine), although it was the most abundant secretory protein.

The identity of the product precipitated by α_2 HS-specific antiserum was further established by sequential Edman degradation of the [3 H]valine-labelled product isolated by immunoprecipitation. Valine residues were detected at positions 2, 3, 7, 14, 15 and 25 (Fig. 4). This is, with one exception, identical with the *N*-terminal sequence of α_2 HS isolated from plasma by Matsushima *et al.* (1982), which has valine at positions 2, 3, 7, 14, 15 and 27. Thus the sequence analysis not only confirms the identity of the product secreted by HepG2 cells, but also indicates that the secreted form has the same *N*-terminus as the mature form which circulates in plasma.

Localization of sulphate attached to α_2 HS

The number and types of sites that were sulphated in α_2 HS were examined by analysis of tryptic peptides. α_2 HS labelled with [35 S]sulphate plus [3 H]mannose or [3 H]tyrosine was isolated and was digested with trypsin. Resulting peptides were separated by reverse-phase h.p.l.c. (Fig. 5). Two sulphate-labelled peaks were resolved, which were co-eluted with two mannose-labelled peaks. (In the top panel there is too little ^{35}S label for the small second peak to be apparent; see the middle panel.) Thus sulphate was linked to the same tryptic peptides as asparagine-linked oligosaccharides. Previously reported data on the sugars attached to α_2 HS are consistent with the presence of two *N*-linked oligosaccharides per molecule (Gejyo & Schmid, 1981), and one *O*-linked oligosaccharide, which does not contain mannose (Gejyo *et al.*, 1983). There was some discrimination among the sites to which sulphate was added, since 80% of the

Fig. 3. Polyacrylamide-gel electrophoresis with cationic detergent

Products of HepG2 cells, i.e. α_2 HS (lane 1), C4 (lane 2), albumin (lane 3) and total sulphate-labelled medium (lane 4), were analysed by electrophoresis in a 9% polyacrylamide gel with the cationic detergent cetyltrimethylammonium bromide added to samples and to the top-chamber buffer. α_2 HS and albumin were isolated by immunoprecipitation from medium labelled with [3 H]leucine and [35 S]sulphate. C4 was isolated from medium labelled with [35 S]sulphate. For the total profile of [35 S]sulphate-labelled products, medium was diluted with sample buffer. The positions of M_r markers are indicated. With this system the cathode is at the bottom of the gel.

sulphate was eluted with the first glycosylated peptide and only 20% with the second glycosylated peptide. Sulphated peptides were well separated from tyrosine-containing peptides, providing evidence against linkage to tyrosine residues. Linkage of sulphate directly to oligosaccharides was suggested by reverse-phase h.p.l.c. of tryptic peptides treated with endoglycosidase F, which cleaves oligosaccharides linked to asparagine (Elder & Alexander, 1982). After treatment with endoglycosidase F, sulphate label was eluted near the non-retained volume as expected for free oligosaccharide chains, whereas elution of tyrosine-labelled peptides was unaltered with respect to acetonitrile concentration (The elution gradient is steeper in the lower panel of Fig. 5).

The resistance to acid hydrolysis of sulphate bound to α_2 HS provided further evidence for linkage of sulphate to oligosaccharide rather than to tyrosine. Paper

Fig. 4. Sequence analysis of valine-labelled α_2 HS

One 75 cm² flask of HepG2 cells was incubated 4 h with [3 H]valine (1 mCi in 10 ml). α_2 HS was isolated by immunoprecipitation. A portion of the immunoprecipitate with 1 000 000 d.p.m. was subjected to sequential Edman degradation as described in the Experimental section. The quantity of [3 H]valine released for each cycle is shown.

electrophoresis showed that only 5% of the sulphate bound to α_2 HS was released by heating for 10 min in 0.1 M-HCl. These conditions released 80% of the sulphate from C4 (Fig. 6), which contains tyrosine sulphate (Karp, 1983). Generally, sulphate bound to oligosaccharides is more stable in acid than is tyrosine sulphate (Rees, 1963; Huttner, 1984).

Analysis of sulphated oligosaccharides from α_2 HS

Sulphated oligosaccharides of α_2 HS were characterized by using endoglycosidase digestion, lectin binding and anion-exchange h.p.l.c. Endoglycosidase H, which cleaves high-mannose and hybrid types of *N*-linked oligosaccharides (Tarentino & Maley, 1974), did not release any sulphate from tryptic peptides (results not shown). Endoglycosidase F released all sulphate label from tryptic peptides, and 56% of the released sulphate-labelled oligosaccharides was retained by concanavalin A-Sepharose and eluted with α -methyl mannoside. These results suggest that at least half of the sulphate was linked to dibranched complex *N*-linked oligosaccharides, with the remainder attached to other forms of complex *N*-linked oligosaccharides. Anion-exchange h.p.l.c. of sulphate-labelled oligosaccharides released by endoglycosidase F yielded two peaks (Fig. 7a). The major peak was eluted with a retention time similar to that of a dibranched oligosaccharide from human lutropin known to contain one sulphate and one sialic acid moiety (Green *et al.*, 1985). The elution positions of marker oligosaccharides containing one or two sulphate groups and no sialic acid are indicated by arrows. A minor peak, which is more highly charged, is eluted later than the markers and may correspond to oligosaccharides containing one sulphate and two sialic acid moieties, since none of the oligosaccharides appear to have more than one sulphate group (see below).

To ascertain what proportion of the oligosaccharides on α_2 HS contained sulphate, oligosaccharides labelled with [3 H]mannose and [35 S]sulphate were analysed by anion-exchange h.p.l.c. The oligosaccharides were pre-

Fig. 5. H.p.l.c. of tryptic peptides from α_2 HS

HepG2 cells were incubated for 15 h with 500 μ Ci of [3 H]mannose and 200 μ Ci of [35 S]sulphate or for 20 h with [3 H]tyrosine and [35 S]sulphate in 10 ml of medium. α_2 HS was isolated by immunoprecipitation. Immunoprecipitates were electrophoresed in a 10%-polyacrylamide gel, and α_2 HS was eluted from the gel by incubation overnight with 4 ml of trypsin (10 μ g/ml). For the top two panels tryptic peptides were analysed by reverse-phase h.p.l.c. as described in the Experimental section, with a gradient of increasing acetonitrile of 0.5%/min; 1 min fractions were collected and samples counted for radioactivity. For the bottom panel, a portion of the tryptic peptides labelled with [3 H]tyrosine and [35 S]sulphate was freeze-dried, dissolved in 200 μ l of 0.1 M-sodium phosphate (pH 6.1)/20 mM-EDTA/1% 2-mercaptoethanol with 0.5 unit of endoglycosidase F (Endo F), and incubated for 3 h at 37 °C. This sample was analysed as described above, except that the gradient of acetonitrile was 1%/min.

pared by digestion of purified tryptic peptides with endoglycosidase F as before. To ensure accurate quantification of 3 H in this experiment, the amounts of [35 S]sulphate added to cultures were decreased so that [3 H]mannose was the predominant label. H.p.l.c. resolved four peaks (A–D) of [3 H]mannose (Fig. 7b; only the 3 H channel is shown), with the [35 S]sulphate peaks being eluted at the positions of shoulders of peaks C and D (indicated by arrows). Thus only a small fraction (5–10%) of the [3 H]mannose co-migrated with the peaks of [35 S]sulphate. Removal of sialic acid with neuraminidase

Fig. 6. Comparison of acid-lability of sulphate on α_2 HS and C4

α_2 HS and C4 labelled with [35 S]sulphate were isolated by immunoprecipitation. Labelled proteins were heated to 100 °C for 10 min in 0.1 M-HCl and then freeze-dried. Sulphate released from protein as detected by paper electrophoresis for 40 min at 200 V on 3MM paper with a buffer of 1% acetic acid adjusted to pH 5.0 with triethylamine. The paper was cut into 1 cm strips and counted for radioactivity. The top panel shows [35 S]sulphate run as a marker, and brackets indicate the position of tyrosine sulphate. Middle and bottom panels show analyses of C4 and α_2 HS respectively.

and heating in acetic acid converted 90% of mannose-labelled oligosaccharides into a neutral form (Fig. 7c). After removal of sialic acid, 5–10% of the mannose-labelled oligosaccharides and all of the [35 S]sulphate were eluted at the same position as a known monosulphated oligosaccharide (arrow). This experiment indicates that sulphated oligosaccharides of α_2 HS contain only a single sulphate group. Also, since α_2 HS appears to contain two N-linked oligosaccharides, an estimate that 5% of the total oligosaccharides contain sulphate is consistent with 10% of the molecules of α_2 HS containing sulphate.

DISCUSSION

α_2 HS was characterized in detail as a representative example of a number of secretory glycoproteins non-stoichiometrically sulphated by the human hepatoma-derived cell line HepG2. All of the sulphate on α_2 HS was found to reside on N-linked oligosaccharides, which were resistant to endoglycosidase H and which contained sialic acid, and only a single sulphate was attached to any oligosaccharide. Only about 10% of the molecules of α_2 HS were sulphated. Similar estimates of the stoichiometry of sulphation were obtained by three independent methods: comparison of the ratio of [35 S]sulphate to [3 H]leucine with the ratio in C4, analysis of the quantity of [3 H]mannose-labelled oligosaccharides co-eluted with peaks of [35 S]sulphate on anion-exchange h.p.l.c., and determination of the amount of [3 H]mannose-labelled oligosaccharide remaining charged and being co-eluted

Fig. 7. Anion-exchange h.p.l.c. of asparagine-linked oligosaccharides

(a) $[^{35}\text{S}]$ Sulphate-labelled oligosaccharides (3000 c.p.m.) were released from $\alpha_2\text{HS}$ by endoglycosidase F as in Fig. 5 and were analysed by anion-exchange h.p.l.c. as described in the Experimental section. The elution positions of monosulphated (S-1), disulphated (S-2) and monosulphated/monosialylated (S-N) N -linked oligosaccharides from human lutropin are indicated. (b) and (c) Tryptic peptides of $\alpha_2\text{HS}$ labelled with $[^3\text{H}]$ mannose and small amounts of $[^{35}\text{S}]$ sulphate were purified by reverse-phase h.p.l.c. and digested with endoglycosidase F. Samples containing 5000 c.p.m. of ^3H and 700 c.p.m. of ^{35}S were analysed by anion-exchange h.p.l.c. before (b) and after (c) removal of sialic acid residues by acid hydrolysis and neuraminidase treatment. The elution profile for ^3H is plotted, with the positions of ^{35}S peaks indicated by arrows. In (c) the ^{35}S peak was eluted at the same position as a monosulphated (S-1) oligosaccharide from human lutropin (arrow).

proteins by HepG2 cells and verifies the estimates in Table 1. Few previous studies have determined the stoichiometry of the sulphation of oligosaccharides. Reported values of the percentage sulphation of oligosaccharides on different glycoproteins are: bovine lutropin, nearly 100% (Parsons & Pierce, 1980); lysosomal enzymes from slime mould, 75% (Freeze *et al.*, 1983); two thyroid membrane proteins, 20% (Edge & Spiro, 1984); HN and F glycoproteins of paramyxovirus SV5, 10% (Prehm *et al.*, 1979); and ovalbumin, 3% (Yamashita *et al.*, 1983). It appears that the extent of sulphation of oligosaccharides is widely variable, and that, in a number of cases, only a small fraction of the molecules of a protein is sulphated.

In order to characterize the sulphation of $\alpha_2\text{HS}$ in the present study, it was necessary to examine basic aspects of the biosynthesis of $\alpha_2\text{HS}$ by HepG2 cells. Newly synthesized $\alpha_2\text{HS}$ has not been analysed previously, although synthesis of an analogous protein in rabbits, bone α -glycoprotein, has been studied (Triffitt *et al.*, 1976), and secretion of $\alpha_2\text{HS}$ by HepG2 cells has been detected (Knowles *et al.*, 1980). We found that $\alpha_2\text{HS}$ was one of the major secretory products of HepG2 cells, and that it was secreted as a single-chain protein with the same N -terminus as $\alpha_2\text{HS}$ isolated from plasma (Matsushima *et al.*, 1982). Previous structural characterizations of this abundant plasma glycoprotein (normal plasma concentration 600 mg/l; Lebreton *et al.*, 1979) of unknown function have disagreed with respect to whether $\alpha_2\text{HS}$ is a single- or two-chain molecule (Lebreton *et al.*, 1979; Gejyo & Schmid, 1981; Matsushima *et al.*, 1982; Gejyo *et al.*, 1983). Our results indicate that it is secreted as a single-chain molecule, but do not rule out the possibility that it may be cleaved during circulation in blood. Previous studies have noted that $\alpha_2\text{HS}$ isolated from plasma exhibits isoelectric heterogeneity (Matsushima *et al.*, 1982). Most, but not all, of the heterogeneity was eliminated by removal of sialic acids. Our results suggest that sulphation may contribute to the remaining isoelectric heterogeneity.

Our results suggest that a number of major human plasma proteins are sulphated on a small proportion of their molecules. Huttner's laboratory has observed similarly that a number of rat plasma proteins contain sulphate (Hille *et al.*, 1984), and concluded that most of the sulphate was linked to tyrosine residues. We have not yet analysed the sites of sulphation of the proteins other than C4 and $\alpha_2\text{HS}$. This has been difficult, owing to the smaller amounts of these products secreted by HepG2 cells. All of the sulphate is released from α_1 -acid glycoprotein by endoglycosidase F, suggesting that the sulphate is bound to oligosaccharides, but similar experiments with transferrin and caeruloplasmin have been inconclusive. Although we have not identified the site of sulphation of these proteins, similarity of the stoichiometry of their sulphation suggests that, like $\alpha_2\text{HS}$, they are sulphated on their oligosaccharides. The oligosaccharide structures of several glycoproteins sulphated by HepG2 cells, such as α_1 -antitrypsin and caeruloplasmin, have been determined (Mega *et al.*, 1980; Yamashita *et al.*, 1981) without any indication that the oligosaccharides are sulphated. However, sulphation of a small percentage of the molecules of a glycoprotein would not have been detected during most investigations of oligosaccharide structure, because, until recently, the charge of anionic oligosaccharides was assumed to result

with the peak of $[^{35}\text{S}]$ sulphate during h.p.l.c. after exhaustive removal of sialic acids. Accurate determination of the stoichiometry of sulphation of $\alpha_2\text{HS}$ provides a standard for comparison with the sulphation of other

completely from sialylation. As an example, the structure of the oligosaccharides attached to ovalbumin was thoroughly investigated without noting the presence of any sulphated oligosaccharides (references in Yamashita *et al.*, 1983). These were detected only recently when Kobata's laboratory systematically analysed the 5% of the oligosaccharides that were not neutral (Yamashita *et al.*, 1983). One-third of the anionic oligosaccharides contained sialic acid, and two-thirds contained sulphate. Our results suggest that low-level sulphation of glycoproteins, similarly to that of ovalbumin and α_2 HS, may be a common process. Many proteins not at present recognized to contain sulphate possibly contain small quantities of sulphate.

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