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Characterization of sheep hemopexin glycovariants

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The hemopexin phenotype HpxB1 isolated from sheep serum, yields three major bands when subjected to starch gel and/or polyacrylamide gel electrophoresis which are here designated as subcomponents HpxB1-I, HpxB1-II and HpxB1-III. Electrospray mass spectrometric analysis of samples of the isolated subcomponents prepared by ion exchange chromatography showed that each was composed of three glycoproteins and that the major difference between the subcomponents was due to their constituent glycoproteins possessing different numbers of sialic acid residues. Combined analysis of the ESI-MS data and of the overall carbohydrate compositional data obtained by colorimetric procedures, leads to the composition of the glycan of each glycoprotein, and a combined methylation and 400 MHz H-NMR analysis of the alkaline cleaved glycans identified them as being of only the biantennary *N*-acetyllactosamine type. Taking into account the molecular mass, the carbohydrate content and structure it may be concluded that each of the constituent glycoproteins contain five *N*-glycosidically linked glycans.

Keywords: sheep hemopexin, glycoform, ^{1}H-NMR spectroscopy, glycan structure

Abbreviations: HpxB1, hemopexin phenotype B1; Man, mannose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; GlcNAc-ol, *N*-acetylglucosaminitol.

Introduction

Hemopexin is a serum β-glycoprotein which binds to heme in an equimolar ratio and which is involved in the transport of heme to the liver, where the heme-hemopexin complex is catabolized [1]. Hemopexin has been isolated from blood plasma of mammals, birds and fishes, its carbohydrate content ranging from 15% to 21% [1]. The number and the structure of glycans appears to be species specific. In fact, human hemopexin contains six glycans, five of which are N-glycosidically linked to asparagine and one O-glycosidically linked to amino terminal threonine [2, 3]. Human hemopexin is the only one in which O-glycosidically linked glycan has so far been found. N-glycosidically linked glycans appear to be a mixture of bi- and triantennary structures [3]. Rat hemopexin has four glycans, which are of the monosialyl, disialyl and trisialyl biantennary and triantennary types [4, 5]. Rabbit hemopexin has been suggested to have four biantennary glycans [6]. Chicken hemopexin appears to have three N-linked glycans of the bi-, tri- and possibly tetra-antennary types [7]. For hemopexins of other studied species, i.e. mouse [8], sheep [9], bovine [10] and pig [11] no data on glycan structures have been published.

Preliminary studies have shown that human and sheep hemopexins possess a similar amino acid composition and molecular mass but differ in their carbohydrate content [9, 12]. Moreover, using starch gel electrophoresis, three phenotypes (A, B1 and B2) of sheep hemopexin have been characterized [9]. Purified hemopexin phenotype A (Hpx A) was demonstrated to be heterogeneous, since four zones were characterized by starch electrophoresis and six to seven components were separated on a DEAE-Sephadex column [9, 13].

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In the present study, we have purified the hemopexin phenotype B1 (HpxB1) subcomponents, determined their glycan structures and have compared them with those found in human hemopexin. Further we have attempted to elucidate the role played by the glycans in the heterogeneity of HpxB1.

Materials and methods

Materials

CM-Sephadex C-50 and DEAE-Sephadex A-50 were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden), Bio-Gel P-2 and Bio-Gel P-6 from Bio-Rad Laboratories (Richmond, CA, USA). Fractogel TSK HW-40 S was obtained from Merck (Darmstadt, Germany). D_2O (99.95 atom % D_2) was from Commissariat à l'Energie Atomique (Saclay, France).

Isolation of hemopexin

Blood was collected from adult sheep (Hpx phenotype B1) of the Merino breed. After clotting, the serum was stored at −15 °C. The rivanol-ammonium sulphate procedure [14] was applied to precipitate the serum proteins. Transferrin and hemopexin present in the supernatant were fractionated on a DEAE-Sephadex A-50 column. Briefly, the crude hemopexin preparation was absorbed on a DEAE-Sephadex column (3.6 cm × 50 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. Hemopexin was eluted with a linear gradient from 0.05 M to 0.12 M NaCl in the buffer. The isolated hemopexin components were further purified on a CM-Sephadex C-50 column (2.6 cm × 14.5 cm) equilibrated with a 0.1 M sodium acetate-acetic acid buffer, pH 5.0. About 200 mg of each hemopexin component was applied. The hemopexin subcomponents were eluted with a linear gradient from a 0.1 M sodium acetate buffer, pH 5.0, to 0.2 M sodium acetate buffer, pH 6.5.

Electrospray ionization mass spectrometry (ESI-MS)

The molecular masses of the three HpxB1 subcomponents were determined by electrospray mass spectrometry as follows: the glycoprotein samples were first de-salted by being exchanged into 50 mM NH_4HCO_3 and then lyophilized several times, the residue being redissolved in H_2O after each lyophilization. Electrospray mass spectra were measured with a VG Quattro-BQ quadrupole mass spectrometer equipped with an electrospray interface and data handling system. Aliquots (10 μl) of the sample solution, (approximately 10 pmol μl^{-1}) prepared in a solvent mixture of 50:50 (v/v) acetonitrile:water (0.2% formic acid), were injected directly into the electrospray ion source via a loop injector (Rheodyne 5717) at a solvent flow rate of 5 $\mu l\,min^{-1}$. The mass spectrometer was routinely scanned over the m/z range 600–2500 at 10 s per scan and a total of approximately 70 scans were summed over a 12 min period to acquire the final data. The spectrometer mass scale was calibrated from the spectrum obtained by separate introductions of 10 μl aliquots of a solution of trypsinogen (Sigma T-1143; M_r = 23980.9 Da) scanned at 10 s per scan over a 2–3 min period.

Electrophoretic methods

Either starch gel electrophoresis with a Tris-citric acid-lithium hydroxide-boric acid buffer [15] or polyacrylamide gel electrophoresis [16] were used for analyses of serum hemopexin and isolated components. The haematin solution was added to the serum samples, before electrophoresis, and the gels were stained with the benzidine solution.

Release of O-glycosidically linked glycans

The sheep Hpx-B1 was treated with 0.1 M sodium hydroxide, 1 M potassium borohydride for 48 h at 37 °C [17]. The β-elimination was stopped by the addition of concentrated acetic acid until a pH = 4.5 was reached. The solution was dried under vacuum, redissolved in methanol and dried again. This operation was repeated three times. The glycans were purified by gel-permeation on a Bio-Gel P-6 column (1.4 cm × 80 cm) eluted with 0.5% acetic acid.

Release of N-glycosidically linked glycans

The glycans N-glycosidically-linked were released by using strong alkaline conditions. Briefly, the cleavage was performed by treating 30 mg of Hpx-B1 subcomponents with 1 ml of a solution containing 1 M potassium borohydride, 1 M sodium hydroxide at 100 °C for 6 h [18]. The reaction was stopped by the addition of concentrated acetic acid until a pH = 4.5 was reached. The solution was applied to a Bio-Gel P-2 column (1.2 cm × 70 cm) and eluted with distilled water. The oligosaccharide-alditols were *N*-reacetylated [19] and purified by gel permeation on a Fractogel TSK HW-40 S column (1 cm × 30 cm) with a Shimadzu LC 5A Liquid Chromatograph equipped with a LKB Uvicord S 2138 detector and a SP 6040 differential refractometer. Elution was carried out with 0.5% acetic acid at a flow-rate of 1 $ml\,min^{-1}$.

Carbohydrate composition

Percentage compositions of neutral sugars, hexosamines and sialic acids were determined by colorimetric microprocedures [20]. The monosaccharide molar ratios present in the glycans of the HpxB1 subcomponents and in the oligosaccharide-alditols released from these subcomponents were determined after methanolysis [21] and gas-liquid chromatography of the trimethyl-sialylated methyl-glycosides on a capillary CP SIL 5CB column (25 m × 0.25 mm) [22].

Methylation analysis

The oligosaccharide-alditols were permethylated after methanolysis, the partially methylated methyl-glycosides were peracetylated [23, 24]. The peracetylated methyl derivatives were then separated by gas-liquid chromatography [25] and also identified by GLC-MS using a mass spectrometer RIBERMAG R 10.10 (Riber, Rueil-Malmaison, France) coupled to the data system Sydar 121.

^{1}H-NMR spectroscopy

For ^{1}H-NMR spectroscopic analysis, the oligosaccharide-alditols were repeatedly dissolved in D_2O at room temperature and at pD = 7 with intermediate freeze drying [26]. The deuterium-exchanged oligosaccharide-alditols were submitted to ^{1}H-NMR spectroscopy performed at 400 MHz on a Bruker AM 400-Wb spectrometer operating in the pulsed-Fourier transform mode and equipped with a Bruker Aspect 3000 computer at a probe temperature of 300 K (Centre Commun de Mesures, Université des Sciences et Technologies de Lille). Chemicals shifts (δ) are given relative to sodium 4,4-di-methyl-4-sila-pentane-1-sulphonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm) with an accuracy of 0.002 ppm.

Results

Fractionation of hemopexin and purification of the subcomponents

The scheme of fractionation on a DEAE-Sephadex column of a homozygous phenotype HpxB1 was similar to that previously described for Hpx A [9]. Three main subcomponents (I, II and III) of Hpx-B1 were isolated using conditions described in Materials and methods. Polyacrylamide gel electrophoresis of these main subcomponents indicated that they were well separated from transferrin but were still heterogeneous (Fig. 1). Further purification of the three individual HpxB1 subcomponents accomplished on CM-Sephadex C-50 increases their homogeneity without however, completely eliminating all traces of the other subcomponents.

Molecular masses of the HpxB1 subcomponents

Electrospray mass spectrometric analysis performed on the three HpxB1 subcomponents showed that in fact each of them was composed of three glycoprotein species, thus confirming the heterogeneity found by the PAGE analysis. In particular, every subcomponent was characterized by the presence of a major (M1) and two less abundant (M2 and M3) glycoproteins (Table 1). The molecular mass of the major glycoprotein (M1) of HpxB1-III (58621.2 Da) differs by +287.7 Da from that of the major glycoprotein found in HpxB1-II (58333.5 Da) which in turn differs by +295.7 Da from the major glycoprotein present in HpxB1-I (58037.8 Da). Similar molecular mass differences are also found for the other two minor glycoprotein species (see Table 1), leading to an overall mean mass difference value of 291.7 Da. These quantitative results suggest that the measured mass difference between each of the substituent glycoproteins forming the three HpxB1 subcomponents is due to a difference of one *N*-acetylneuraminic acid residue (M = 291.2 Da).

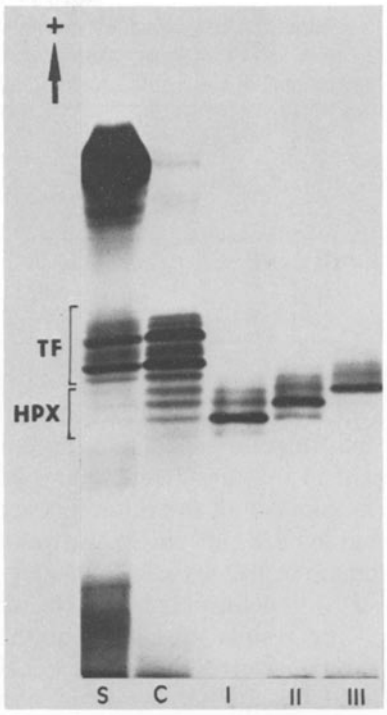

Figure 1. Polyacrylamide gel electrophoresis of the three main subcomponents of sheep hemopexin, phenotype HpxB1, obtained by fractionation of a crude hemopexin preparation on a DEAE-Sephadex A-50 column; S, serum; C, crude hemopexin preparation; I, II, III, isolated hemopexin subcomponents; TF, transferrin; Hpx, hemopexin. Under these staining conditions hemopexin zones in the serum are very weak (for serum benzidine staining is normally used).

Carbohydrate composition of the HpxB1 subcomponents

Data pertaining to the total carbohydrate content of HpxB1 subcomponents are shown in Table 2. The results

Table 1. Molecular masses (Da) of the three glycoproteins (M1, M2 and M3) comprising the separated HpxB1 subcomponents as determined by electrospray ionization mass spectrometry and of the calculated total glycan residues.

Subcomponents	*Glycoproteins*			*Glycans*
	M1	*M2*	*M3*	
HpxB1-I	58037.8	57928.4	57853.5	10447.5
HpxB1-II	58333.5	58225.2	58146.5	10738.8
HpxB1-III	58621.2	58516.3	58438.0	11030.0

Table 2. Percentage of molar carbohydrate compositions of sheep Hpx subcomponents. The number of monosaccharide residues per mol of protein was calculated on the basis of the molecular mass of the major constituent (M1) of each of the HpxB1 subcomponents.

Subcomponents	*Centesimal carbohydrate composition*				*Number of residues per mol of Hpx*		
	Total hexoses	*GlcNAc*	*NeuAc*	*Total*	*Total hexoses*	*GlcNAc*	*NeuAc*
HpxB1-I	6.9	6.8	3.9	17.6	24.6	19.5	7.8
HpxB1-II	7.0	7.0	4.4	18.4	25.0	20.0	8.9
HpxB1-III	6.9	7.0	4.9	18.8	24.8	20.0	9.8

indicate that the weight percentage of different monosaccharides present in the three components of HpxB1 are very close. The number of the different monosaccharide residues present in each subcomponent was calculated on the basis of the molecular mass of the major glycoprotein constituent M_1 as determined by electrospray mass spectrometry. The results revealed that the number of *N*-acetylneuraminic acid residues increases with the concentration of NaCl in the corresponding elution buffers. These results suggest that the number and the structure of the glycans located on the polypeptide chain of the three subcomponents are most probably similar and that one of the important differences revealed by analysis of the monosaccharides of the HpxB1 subcomponents concerned their degree of sialylation. Using the total number of monosaccharide residues:25 hexoses, 20 GlcNAc and 8, 9 or 10 NeuAc were found per mol for each HpxB subcomponent as given in Table 2. The corresponding glycan residue masses were calculated and are listed in column 5 of Table 1.

Release of O-glycosidically linked glycans

Purification on a Bio-gel column of the β-eliminated products from component I of HpxB1 leads to the isolation of only one fraction possessing a molar carbohydrate composition similar to that of the native protein (results not shown). No free glycan was isolated, and it was therefore concluded that sheep HpxB1 most probably does not contain O-glycosidically linked glycans.

Release of oligosaccharide-alditols

The oligosaccharide-alditols released by strong alkaline-reductive treatment from the three subcomponents HpxB1-I, HpxB1-II and HpxB1-III were obtained in a good yield (70%) by gel filtration on a Bio-Gel P-2 column, and fully re-*N*-acetylated. The ratios of the different monosaccharides of these oligosaccharide-alditols were similar to those found in the intact protein (results not shown). The number of GlcNAc residues was however decreased since one residue of GlcNAc was transformed into GlcNAc itol during reductive alkaline treatment of the protein.

Methylation analysis of the released oligosaccharide-alditols

The relative proportions of the permethylated derivatives of monosaccharides taken from the mass fragmentometry scans and combined by using the data of the molar carbohydrate compositions are given in Table 3. The presence of one residue of 2,4-di-*O*-methyl-Man for 1.9 or 1.6 residues of 3,4,6-tri-*O*-methyl-Man in the oligosaccharide-alditols isolated from HpxB1-I, HpxB1-II and HpxB1-III respectively suggests the presence of a trimannosidic core. The presence of 2,3,4,6-tetra-*O*-methyl-Gal in the oligosaccharide-alditols from HpxB1-I and HpxB1-II, shows that some Gal residues are not fully sialylated in both glycans. The presence of 2,3,4-tri-*O*-methyl-Gal in the three subcomponents of HpxB1 favours a substitution of a galactose residue at C6. The presence of 2,4,6-tri-*O*-methyl-Gal in HpxB1-III indicates that in this subcomponent, the Gal residue is also substituted at the C-3 position. The occurrence of methyl derivatives of the GlcNAc-itol in the oligosaccharide-alditols indicates that one GlcNAc residue is located at the reducing end position of the cleaved oligosaccharides. In summary, the results obtained by methylation analysis

Table 3. Molar ratios of monosaccharide methyl ethers in methanolysates of permethylated oligosaccharide-alditols released by reductive alkaline cleavage from the three subcomponents of HpxB1. Molar proportions were calculated on the basis of 1 mol of (2,4)Me_2 Man per mol of glycan.

Monosaccharide methyl ethers	*Molar ratios of monosaccharide methyl ethers*		
	HpxB1-I	*HpxB1-II*	*HpxB1-III*
(2,4)Me_2 Man	1.0	1.0	1.0
(4,7,8,9)Me_4 NeuAcMe	1.2	1.8	1.7
(2,3,4)Me_3 Gal	1.2	1.8	1.1
(2,4,6)Me_3 Gal	0.0	0.0	0.6
(2,3,4,6)Me_4 Gal	0.5	0.2	0.0
(3,4,6)Me_3 Man	1.9	1.6	1.6
(3,6)Me_2 GlcNAcMe	2.8	2.7	2.5
(1,3,5,6)Me_4GlcNAcMe-ol	0.4	0.2	0.2

suggest that the glycans from sheep HpxB1 subcomponents possess a classical *N*-acetyllactosamine biantennary structure and differ by their degree of sialylation and the nature of the *N*-acetylneuraminic acid linkage.

400 MHz ^{1}H-NMR spectroscopic analysis of the released oligosaccharide-alditols

The 400 MHz ^{1}H-NMR spectra of the glycan fractions isolated from the three subcomponents HpxB1 confirm that all the oligosaccharide-alditols which were analysed possess a biantennary *N*-acetyllactosamine structure. The typical spectral features, except those of the *N*,*N*′-diacetylchitobiositol unit, match the structural-reporter groups of the glycopeptide structure reported for the human serum transferrin [26, 28]. Furthermore, small signals were present at $\delta = 1.800$ (NeuAc H-3ax) and $\delta = 2.758$ (NeuAc H-3eq), indicating the presence of NeuAc in (α2,3) linkage in HpxB1-III. Based on the accompanying low-intensity signals at $\delta = 5.118$ (Man-4 H-1), $\delta = 4.926$ (Man-4′ H-1), $\delta = 2.047$ (GlcNAc-5 NAc) and $\delta = 2.040$ (GlcNAc-5′ NAc), this latter NeuAc residue was found to be located both on the Man (α1,3) and Man (α1,6) antennae of the major disialylated biantennary structure. The different possible structures of glycans from HpxB1 are shown in Fig. 2.

Discussion

The hemopexins of most species studied are heterogeneous and the heterogeneity in individual species has not yet been fully explained. Preliminary experiments performed on sheep hemopexin phenotype A have shown that a variation in glycan structure due to the number of sialic acid residues present may be the main cause of this heterogeneity [9]. In this study we have isolated, by ion exchange chromatography, three subcomponents of HpxB1 which were subsequently found by PAGE and by ESI-MS to be heterogeneous. Analysis of the mass spectral data showed that each of the three separated subcomponents was in fact composed of three different glycoproteins. However, consideration of the carbohydrate analytical data together with the molecular masses led to the conclusion that the three subcomponents of HpxB1 consists of only three protein species which overall differ in glycosylation by one or two *N*-acetylneuraminic acid residues.

In contrast to human Hpx, sheep Hpx does not contain an O-glycosidically linked glycan. In the glycan found in human Hpx, *N*-acetyl galactosamine is linked to the amino-terminal threonine [2]. Absence of this O-glycosidically linked glycan, is explained by the fact that the N-terminal sequence of sheep hemopexin (Leu-Pro-Pro) [9] differs from that characterized in human (Thr-Pro-Leu-Pro-Pro) [2] by the absence of the two first amino acids.

In addition to the O-glycosidically linked glycan, human Hpx contains five N-glycosidically linked glycans located at the five sites of Asn-X-Thr/Ser attachment found on the polypeptide chain [2]. The aminoacid sequence of the sheep Hpx polypeptide chain has not yet been determined, it is, therefore, difficult to assess the location of the glycans on the sheep Hpx polypeptide chain. However, the number of glycans found in sheep Hpx may be deduced from the results obtained in the present data. In particular, on the basis of the number of neutral monosaccharides found in a biantennary *N*-acetyllactosaminic type glycan and on the basis of the total number of neutral monosaccharides deduced from the mass determination and carbohydrate composition, it may be suggested that the three subcomponents of sheep HpxB1 contain five N-glycosidically linked glycans. This number is therefore identical to those found in human hemopexin [2, 3].

The structure of the sheep HpxB1 glycans is of only the biantennary *N*-acetyllactosaminic type. Only one glycan out of the five glycans found in HpxB1-II is monosialylated. In HpxB1-I, the lack of two NeuAc residues suggest that two glycans might be monosialylated or that one glycan might be asialylated. The location of these monosialylated or asialylated glycans on the polypeptide and the peculiar biological roles of these partially desialylation glycans are not yet known. In particular, it may be interesting to determine if the different hemopexin glycovariants possess the same haeme binding capacity and if the different hemopexin-haeme complexes bind to a hepatocyte receptor with the same affinity.

Genetic polymorphism of Hpx has been described in human [12], rabbit [29], pig [30], goat and sheep [9]. Studies performed previously have presented evidence that sheep Hpx phenotypes are genetically controlled by three alleles Hpx^A, Hpx^{B1} and Hpx^{B2} of a single autosomal locus [9, 13]. Preliminary experiments have shown that HpxA and HpxB2, like HpxB1, are also separated by starch gel and polyacrylamide gel electrophoresis into three main subcomponents, I, II and III, and on the basis of neuraminidase treatment, it was determined that these subcomponents possess 8, 9 and 10 residues of NeuAc,

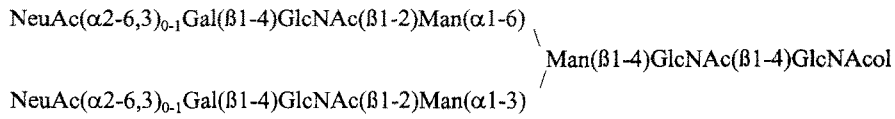

Figure 2. Proposed structures of the glycans from the subcomponents I, II and III of sheep HpxB1.

respectively. Complete desialylation of each of these phenotypes reduces their heterogeneity, since only one band was detected by gel electrophoresis [9]. Differences in the electrophoretic mobility of the three asialo-Hpx phenotypes (A, B1 and B2) indicated that variations in glycan structure other than neuraminic acid should play a role in the polymorphism of these proteins [9].

Preliminary results concerning glycan structure analysis of HpxA subcomponents indicated that the three subcomponents contain, like those of HpxB1, a biantennary *N*-acetyllactosamine type glycan differing by the number of NeuAc residues.

The determination of the complete aminoacid sequence of the polypeptide chain of any one of the phenotypes of sheep Hpx appears to be necessary to elucidate the polymorphism of these proteins.

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