Carbohydrate Structures of Haptoglobin in Sera of Healthy People and a Patient with Congenital Disorder of Glycosylation

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Haptoglobin, Glycosylation, Congenital Disorder of Glycosylation

Haptoglobin is one of acute phase glycoproteins often used as markers in glycopathology studies. In this work the oligosaccharide structures of haptoglobin from 'healthy' subjects have been studied in detail, taking into consideration the possible dependence of glycosylation on the phenotype. About 75% of charged haptoglobin glycans were of biantennary complex structure, and some of them lacked one terminal sialic acid molecule. Triantennary structures made up almost 25% of the charged glycans pool, and highly branched tetrasialylated oligosaccharides did not exceed 1%. The main difference between haptoglobin derived from the sample of pooled 44 sera and from the 2–2 phenotype individual concerned the relative content of trisialylated oligosaccharide with one 2–3 linked sialic acid residue. The oligosaccharide profile of haptoglobin derived from serum of a patient suffering from congenital disorder of glycosylation was compared to 'healthy' controls. It was shown, that four main glycans are identical in patient and 'normal' haptoglobins. Some alterations were found in the relative content of mono-, bi-, and trisialylated glycans as well as in the appearance of some tracely abundant oligosaccharides in haptoglobin of the patient with congenital disorder of glycosylation.

Introduction

Biological importance of glycosylation, as well as the significance of its alterations following the pathological events are intensively studied and discussed over last years (Dwek, 1998; Wormald and Dwek, 1999; Qasba, 2000; Kim and Varki, 1997; Ørntoft and Vestergaard, 1999; Durand and Seta, 2000). Recent interest is focused to examine the role of glycan moiety in the functional activity of glycoconjugates as folding-mediating or recognition molecules (Qasba, 2000; Rudd *et al.*, 1999; Watson *et al.*, 1999). On the other hand in some diseases, such as cancer, rheumatoid arthritis and inflamation, altered branching, fucosylation and sialylation of glycans was reported (Kim and Varki, 1997; Ørntoft and Vestergaard, 1999; Durand and Seta, 2000; Watson *et al.*, 1999; Van Dijk *et al.*, 1998; Havenaar *et al.*, 1998). Although in such cases modifications of oligosaccharides are considered as secondary markers for the disease, these changes could be potent diagnostic parameters, and they also can play a significant role in the progression of the disease (Ørntoft and Vestergaard, 1999; Krause and Turner, 1999; De Graaf *et al.*, 1993). Altered glycan structures of acute phase proteins are supposed to mediate the interaction of selectins with sialyl-Lewis X epitopes, acting as the immunomodulators (De Graaf *et al.*, 1993).

Inherited disorders in the metabolic pathways of oligosaccharides biosynthesis and degradation is another field of interests, and, among them, the group of congenital disorders of glycosylation (Aebi *et al.*, 2000), formerly called carbohydrate-deficient glycoprotein (or Jaeken's) syndromes. These disorders result from the deficiency in a single enzyme of the glycosylation pathway. Some defects were identified in the initial steps of N-glycosylation, namely in the synthesis of GDP-mannose or lipid-linked oligosaccharide precursor, and clasified as CDG type I, subtypes Ia – Ie (Aebi *et al.*, 2000; Freeze, 1998; Carchon *et al.*, 1999). Only one

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defect was identified in the processing of oligosaccharides attached to the polypeptide chain and called CDG type IIa (Schachter and Jaeken, 1999). In all patients abnormal glycosylation of serum proteins, mainly transferrin, was found (Freeze, 1998; Carchon *et al.*, 1999; Schachter and Jaeken, 1999). As the result of particular enzyme deficiencies glycoproteins lack some glycan units in CDG type I or one of the glycan antennae in CDG type II. The structure of oligosaccharides attached to polypeptide chain was found to be normal for serum transferrin in CDG type I (Iourin *et al.*, 1996). In all CDG I patients substantial glycosylation of glycoproteins still persists, suggesting that some kind of 'bypassing' of the defect is possible (Freeze, 1998). It was also reported, that the frequency of different underglycosylated forms of glycoproteins differ among patients, and, probably, particular serum glycoproteins are not affected to the same degree even in the same individual (Stibler *et al.*, 1998). For all these reasons studying glycosylation of glycoproteins other than transferrin should give new information on the biochemistry of CDG.

The glycosylation of haptoglobin in physiological and pathological conditions has been studied for the last years (Kątnik *et al.*, 1994; Kątnik and Jadach, 1996; Turner *et al.*, 1995; Brinkman-van der Linden *et al.*, 1998; Kątnik *et al.*, 1996). Anyway, data on the oligosaccharide structure of healthy human haptoglobin are limited to the 1–1 phenotype of this glycoprotein (Nilsson *et al.*, 1981). This phenotype is the most rare one in Polish population.There is also no information if there are any phenotype-dependent variations in oligosaccharide structures. On the other hand, biological and clinical importance of haptoglobin polymorphism was confirmed (Langlois and Delanghe, 1996). Previously, we have described (Ferens-Sieczkowska *et al.*, 1999) the lack of some glycan chains in haptoglobin isolated from the serum of the only diagnosed so far in Poland patient with congenital disorder of glycosylation. Althoug the genetic defect was not confirmed, the pattern of serum Hp indicated for type I of the disease.

In the current study we report on the structure of sugar chains of haptoglobin isolated from healthy human sera and from the serum of the CDG patient.

Materials and Methods

Serum samples

Control haptoglobins were isolated from the sera of healthy volunteers. Sample designated 'control-pooled Hp' was isolated from the pooled sera of 44 healthy subjects (23–57 years, average 39, median 38); 24 women (23–57 years, average 39.5, median 37) and 21 men (23–50 years, average 39, median 38). Haptoglobin levels in studied sera were comprised within the values accepted as 'normal' (0.8–1.6 g/l). Haptoglobin phenotypes were determined in all subjects. Pooled serum contained 25 samples of Hp 2–1 phenotype (12 women and 13 men), 15 samples of Hp 2–2 phenotype (9 women and 6 men) and 4 samples of Hp 1–1 phenotype (3 women and 1 man). The relative content of phenotypes was as follows: 57% of 2–1 phenotype, 34% of 2–2 phenotype and 9% of 1–1 phenotype.

The sample designated 'control Hp 2–2′ was isolated from serum of one healthy woman (43 years), whose Hp phenotype was identical with that of CDG patient.

Two blood samples obtained from the girl suffering from the congenital disorder of glycosylation (Midro *et al.*, 1996) were kindly provided by Prof. A. Midro (Białystok Medical Academy). The patient was 14 years old at the moment of the first blood collection, and this haptoglobin sample was designated 'CDG-$Hp_{(1)}$'. The second sample was taken one year later and designated 'CDG-$Hp_{(2)}$'. Hp phenotype was determined as 2–2 (Ferens-Sieczkowska *et al.*, 1999).

Haptoglobin isolation

Haptoglobin was isolated from serum samples using affinity chromatography on Sepharose-bound monoclonal anti-Hp antibodies (clones Mo 2.36.71.41 and Mo 7.60.65.55) as described earlier (Kątnik and Jadach, 1993). The purity of preparations was examined by SDS-PAGE. Only the CDG-$Hp_{(1)}$ sample contained an impurity, a small amount of haemoglobin (Hb) bound in Hp-Hb complex.

Determination of Hp phenotype

Hp phenotypes were determined electrophoretically. Electrophoresis was performed in 6% polyacrylamide gel according to Whitaker (1967). Af-

ter electrophoresis the gel was soaked with 0.1% horse haemoglobin, and after washing out the excess of haemoglobin the peroxidase activity of Hb-Hp complex was visualized with diaminobenzidine and H_2O_2 as substrates. Alternatively, in the samples with lower Hp levels, serum proteins were transferred onto nitrocellulose membrane and Hp subunits were detected with goat anti-Hp antibody and anti-goat IgG-HRP conjugate (Sigma).

Isolation of glycans

Oligosaccharides were released from the tryptic digests of haptoglobin by endoglycosidase treatment. Lyophilized protein samples (100–500 μg) were dissolved in 20 μl of 8 M urea in 0.4 M ammonium bicarbonate pH 7.9, reduced by adding 5 μl of 45 mM dithiothreitol, incubated for 15 min at 50 °C and then carboxymethylated by adding 5 μl 100 mM iodoacetic acid for 15 min at room temperature. The mixture was diluted to obtain 1 M urea concentration. Proteins were digested by sequencing grade, modified trypsin (Boehringer Mannheim) for 12 hours at 37 °C with a trypsin/protein ratio of 1:20. The resulting peptide pool was purified on Eurosil Bioselect 300 C-18 (5 μm) column (250 × 4 mm, Knauer). The trypsin digest mixture was injected onto the column and washed with 2% acetonitrile in water, containing 0.06% trifluoracetic acid (TFA). Peptides monitored at 215 nm were eluted from the column by 60% acetonitrile, containing 0.052% TFA, then dissolved in 50 μl of 50 mM phosphate buffer, pH 7.8, containing 0.2% SDS. The mixture was boiled for 5 min, and this step was followed by adding a Complete Protease Inhibitor Coctail (Boehringer Mannheim). Then, peptide–N-glycosidase F from *Flavobacterium meningosepticum* (PNGase, Boehringer Mannheim) was added to obtain a final concentration of 20 U/ml and the mixture was incubated at 37 °C for 40 h. Glycans were separated from peptides on the C-18 column. The void-volume fractions containing sugars were collected and dried. Samples containing free oligosaccharides were then purified on a GlycoSep H column (100 × 3 mm, Oxford GlycoSciences). The column was washed with 3% acetonitrile in water, containing 0.05% TFA and eluted with 30% of acetonitrile, containing 0.05% TFA. Oligosaccharide containing fractions were monitored at 200 nm, collected and dried.

Fluorescence labelling of glycans

The reducing ends of the oligosaccharides were fluorescently labelled with 2-aminobenzamide (2-AB) (Bigge *et al.*, 1995) by the use of a 2-AB Signal Labelling Kit (Oxford GlycoSciences) and stored at −20 °C.

Chromatographic separations of labelled glycans

Oligosaccharide fractionation was performed by HPLC on a Knauer apparatus with a Shimadzu RF-551 fluorescence detector with detection at λ_{max} excitation=330 nm and λ_{max} emission= 420 nm. The 2-AB glycan derivatives were separated on an anion exchange GlycoSep C column (100 × 3 mm, Oxford GlycoSciences). Then, fractions containing neutral, monosialylated, disialylated and trisialylated structures were separated on a normal-phase amide column (GlycoSep N, 250 × 4.6 mm, Oxford GlycoSciences). Gradient conditions were used with a low salt solvent system (LSSS) as described by Guile *et al.* (1996). The total run time was 110 min and the column was maintained at 30 °C. The partially hydrolyzed dextran (Oxford GlycoSciences), labelled with 2-AB was used as an external standard and expressed in glucose units (GU). Fractions containing oligosaccharides were collected, dried, redissolved in a minimal volume (up to 6 μl) of water and injected onto a GlycoSep H column. Glycans were eluted with a 10–30% acetonitrile gradient containing 0.1% TFA, following manufacturer's protocol (GlycoSep™ Oxford GlycoSystem instructions, 1995). Fractions containing oligosaccharides were desalted and dried.

Exoglycosidase sequencing

Table I presents the specificity and concentration of exoglycosidases (Oxford GlycoSciences, except of α-mannosidase from *X. manihoti,* which was purchased from New England Biolabs) used in sequential digestions. Before glycosidase treatment, purified and fluorescently labelled glycans (0.1–2 nmol of each) were dissolved in 100 mM sodium citrate-phosphate buffer, pH 4.5, containing 100 mM NaCl and 0.2 mM $ZnCl_2$, then the first ex-

Table I. Specificity of exoglycosidases used for glycan sequencing.

Enzyme	Concentration *	Specificity
β-Galactosidase from *Streptococcus pneumoniae*	80 mU/ml	Gal(β1–4)
β-N-Acetylhexosaminidase from *Canavalia ensiformis*	10 U/ml	GlcNAc(β1–2,3,4,6)
β-N-Acetylhexosaminidase from *S.pneumoniae*	10 mU/ml	GlcNAc(β1–2)
α-Mannosidase from *Xanthomonas manihoti*	20 mU/ml	Man(α 1–2,3)
α-Mannosidase from *Canavalia ensiformis*	10 U/ml	Man(α1–2,3,6)
β-Mannosidase from *Helix pomatia*	2.5 U/ml	Man(β 1–4)
Sialidase from Newcastle disease virus (NDV)	1 U/ml	Neu (α2–3)
Sialidase from *Arthrobacter ureafaciens*	1 U/ml	Neu (α2–3,6,8)

* Concentration of the enzymes was expressed with activity units defined by the manufacturer as the amount of enzyme required to hydrolyse one μmol of appropriate substrate per min at the test conditions defined.

oglycosidase was added to obtain a final concentration as described in Table I and a total volume of 30–40 μl. After 24–72 h of incubation at 37 °C the reaction was stopped by filtration through a 5 kDa membrane (Ultrafree MC, Millipore), and the membrane was rinsed with 100 μl of water. The first and second filtrates were combined. A small aliquot from the filtrate (usually about 1/6 of total volume) was dried on a rotary evaporator, redissolved in 10 μl of 70% acetonitrile in water (v/v) and injected onto a GlycoSep N column. The glucose unit (GU) values of glycans were determined by comparing their retention times with the external standard (GlycoSep™ Oxford GlycoSystem instructions, 1995). The rest of the filtrate containing about 5/6 of the total digested glycan was dried, dissolved in water and the next enzyme was added to obtain the required concentration of the exoglycosidase in 30–40 μl of total volume.

Results and Discussion

Four preparations of haptoglobin, derived from pooled 'healthy' human sera, 'healthy' control individual of 2–2 phenotype and two samples from CDG patient were digested with trypsin prior to the endoglycosidase treatment, to increase the efficiency of oligosaccharide release. Direct endoglycosidase digestion of native haptoglobin resulted in incomplete release of glycans, as under mild reducing conditions the accessibility of glycans of highly polimeric Hp 2–1 and Hp 2–2 molecules for the enzyme was limited. Complete pool of released glycans was labelled with 2-AB and purified from peptide fragments on GlycoSep H column using HPLC system.

The content of neutral and charged structures in Hp glycans

2-AB-labelled haptoglobin glycans were separated using ion exchange chromatography on the GlycoSep C column. As shown in Fig. 1, separated peaks were obtained, corresponding to neutral and charged glycans, containing from one to four sialic acid molecules per oligosaccharide chain. The percentage content of particular neutral and charged structures was calculated from the area under appropriate peaks.

We have found high difference in neutral oligosaccharide content among examined haptoglobin preparations. In control Hp from pooled sera the amount of neutral glycans was low, calculated as 2.6%. The content of neutral structures was higher in 'healthy' individual Hp (control Hp 2–2) and reached 13.5%. A high abundance of neutral carbohydrates was found in the first haptoglobin sample from the CDG patient, CDG-$Hp_{(1)}$, as the uncharged structures comprised almost 90% of 2-AB labelled derivatives. The content of asialoglycans found in CDG-$Hp_{(2)}$ isolated from the blood sample taken one year later, was significantly lower and similar to those in control Hp 2–2.

Because of such differences in the uncharged structures amount it seemed to be convenient to calculate the relative content of particular sialylated structures as the percentage of total charged haptoglobin glycans. These results are shown in Table II. In all four examined Hp preparations glycans containing two sialic acid residues were the dominant charged fraction. In both 'healthy control' preparations this fraction contained about 60% of charged glycans. The difference in the

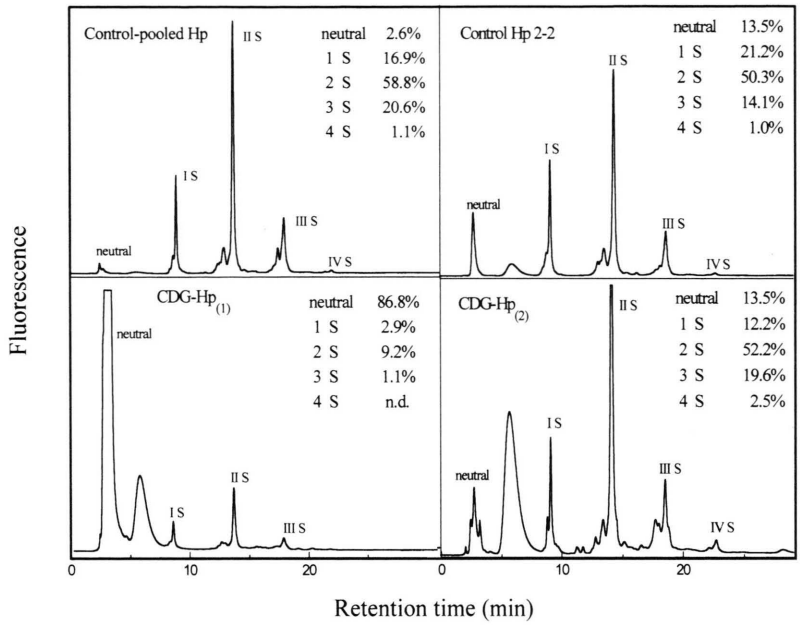

Fig. 1. GlycoSep C separations of haptoglobin oligosaccharides.
Oligosaccharides released by PNGase F treatment from four haptoglobin preparations derived from 'healthy' subjects (control-pooled Hp and Hp 2–2) and from congenital disorder of glycosylation patient (CDG-$Hp_{(1)}$ and CDG-$Hp_{(2)}$) were submitted to GlycoSep C column.

Table II. Relative content of glycans differing in the amount of sialic acid residues in the samples of haptoglobin.

Hp-sample	Control-pooled Hp	Control Hp2–2	CDG-$Hp_{(1)}$	CDG-$Hp_{(2)}$
1S-glycans	17.3%	24.4%	21.9%	14.2%
2S-glycans	60.3%	58.2%	70.1%	60.3%
3S-glycans	21.3%	16.3%	8.0%	22.7%
4S-glycans	1.1%	1.1%	0	2.8%

content of mono- and trisialylated chains reached several percent. As much as about 75% of oligosaccharide chains of normal haptoglobin were of biantennary structure, triantennary glycans were less abundant (slightly less than 25%). Nilsson *et al.* (1981) reported on glycan structures of 1–1 Hp and claimed that the content of bi- and triantennary structures was equal. The discrepancy of our results towards that study could suggest possible dependence of the glycosylation on Hp phenotype. In our Hp pool the 1–1 phenotype constituted only 9% of all serum samples. This amount reflected the occurence of this phenotype in the population, but its influence on the values obtained in our study was less significant.

The content of highly branched tetrasialylated structures in both healthy controls did not exceed 1%.

The content of particular charged chains in CDG patient haptoglobins has shown some difference towards controls. In the first sample, CDG-$Hp_{(1)}$, the content of bisialylated glycans was elevated to 70% and the content of trisialylated oligosaccharides was significantly decreased. The tetrasialylated form was undetectable. In the second Hp preparation obtained from the blood sample taken after one year, CDG-$Hp_{(2)}$, the content of particular charged oligosaccharides resembled the normal pattern; only the slight increase in tetrasialylated forms was found.

The pattern of sialylated glycans of healthy controls and CDG patient haptoglobins

Fractions of mono-, bi- and trisialylated glycans of all haptoglobin preparations collected after GlycoSep C column were further analysed on the GlycoSep N column.

The chromatographic profiles of sialylated structures were similar when both control hapto-

globin preparations were compared as well as when CDG-Hp samples were compared towards controls. Among monosialylated oligosaccharides of control-pooled Hp, 8.22 GU glycan was dominant comprising 81.5% of this fraction. The 9.43 GU glycan constituted 72.3% of bisialylated glycans of the same haptoglobin preparation. Among trisialylated glycans of pooled Hp two structures were comparably abundant; the 11.1 GU glycan constituted 47.4% and the 11.56 GU glycan 38.7%. The only difference found in control Hp 2–2 sample was the decreased content of 11.56 GU glycan towards the 11.1 GU one.

The dominant glycans of both CDG-Hp samples showed the same GU values as the control Hp oligosaccharides and their percentage content also did not differ from the control samples. The relative amounts of trisialylated glycans in CDG-Hp samples resembled the control Hp 2–2 rather than control pooled-Hp, so this difference could be due to Hp phenotype rather than to the disease. Anyway, in the chromatographic profiles of charged CDG-Hp glycans slight alterations could be observed. These concern the increase in one of monosialylated fractions in CDG-$Hp_{(2)}$ sample, and the increased heterogeneity and the appearance of the small additional peak in trisialylated glycans of low retention time, also in both CDG-Hp samples. In transferrin glycan sequences no significant difference was described in CDG I patients (Iourin *et al.*, 1996). Our observations indicate, that also in haptoglobin the main abnormality on the glycosylation pathway lies in incomplete occupation of glycosylation sites.

Sequencing of dominant haptoglobin glycans

Sequences of four dominant glycans, constituting 75% oligosaccharides of haptoglobin obtained from pooled sera of healthy people, were determined. Calculated GU values (Guille *et al.*, 1996; GlycoSep™ Oxford Glycosystem instructions, 1995) were used for predicting the structure and designing the strategy for sequential digestion. Proposed structures of sequenced oligosaccharides and their relative content in the total pool of control haptoglobin glycans are presented in Table III.

8.22 GU glycan. According to its GU value this glycan was supposed to be the biantennary with only one arm sialylated. The purpose of the sequential analysis was to establish which arm was sialylated and what was the type of sialic acid link-

Table III. Proposed structures of dominant haptoglobin glycans.

Glycan	GU	Proposed structure	Relative content*
I	8.22	Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↗	14%
II	9.43	Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Manβ1→4GlcNAcβ1→4GlcNAc 3 Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↗	44%
III	11.10	Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Neu2→3Galβ1→4GlcNAcβ1 ↘ 4 Manβ1→4GlcNAcβ1→4GlcNAc 3 Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↗	10%
III′	11.59	Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↘ 6 Neu2→6Galβ1→4GlcNAcβ1 ↘ 4 Manβ1→4GlcNAcβ1→4GlcNAc 3 Neu2→6Galβ1→4GlcNAcβ1→2Manα1 ↗	8%

* calculated as a percent of total charged glycans; abbreviations: Man – mannose, GlcNAc – N-acetylglucosamine, Gal – galactose, Neu – sialic acid.

age. The digestion was started from the sialic acid lacking arm. The treatment with galactosidase β 1–4 resulted in 0.86 GU decrease of hydrodynamic volume of the reaction product, which corresponded to the loss of one galactose residue. Next enzyme, hexosaminidase β 1–2, caused the release of one N-acetylglucosamine residue giving 7.05 GU product. This oligosaccharide was resistant to specific α 1–3-mannosidase indicating, that α 1–3 linked mannose was substituted with the succeeding sugar and not accessible for the enzyme digestion. The 7.05 GU glycan was also resistant for α 2–3 sialidase. This proved that the sialic acid was bound to the hydroxyl group of sixth carbon of galactose. Sequential digestion of the glycan with unspecific α 2–3,6 sialidase followed by the treatment with β-galactosidase and unspecific hexosaminidase led to a 4.29 GU product, obtained by the release of sialic acid, galactose and N-acetylglucosamine. This value was consistent with reported by Guile *et al.* (1996) for 'trimannosyl core' of N-glycans. Further digestion with unspecific mannosidase 1–2,3,6 led to a release of two mannose residues; the obtained 2.45 GU glycan was identical in its retention time with mannochitobiosyl core glycan.

9.43 GU glycan. The GU value for this glycan was close to the values reported for biantennary structures sialylated at both arms. The glycan was resistant to α 2–3 specific sialidase digestion, indicating that both sialic acid residues are bound by α 2–6 linkage. Sequential digestion with unspecific sialidase, galactosidase β1–4, hexosaminidase β 1–2,6 and mannosidase α1–2,3,6 resulted in a release of two appropriate monosaccharide residues at each step. The final product (2.43 GU) represented mannochitobiose trisaccharide.

11.1 GU glycan. The 11.1 GU glycan was the only one susceptible for digestion with α 2–3 specific sialidase. This treatment gave a 0.48 GU decrease of hydrodynamic volume and suggested the loss of one out of three sialic acid residues. The product after galactosidase treatment was resistant to β 1–2 hexosaminidase. This indicated that α 2–3 sialic acid was linked to the third antenna, on which N-acetylglucosamine was bound to mannose by α 1–4 or α 1–6 linkage, and not to any outer α 1–2 bound arm. The digestion of the 8.76 GU oligosaccharide obtained as described above with unspecific sialidase released two sialic acid residues (decrease of 2.2 GU); further treatment with β 1–4 galactosidase resulted in two galactose residues release. The next enzyme, hexosaminidase α 1–2 caused the release of two N-acetylglucosamine residues, suggesting that the third undigested N-acetylglucosamine residue was β 1–4 bound. Glycans with N-acetylglucosamine bound to mannose with α 1–6 linkage are resistant to the latter enzyme. In such a case the release of only one N-acetylglucosamine residue should be expected. The former suggestion was further confirmed by the resistance of obtained 4.77 GU glycan for the digestion with specific α 1–2,3 mannosidase. At the next step of sequencing, unspecific hexosaminidase released the remaining N-acetylglucosamine residue giving as the result the final product of 4.25 GU, identical with the 'trimannosyl core' of N-glycans.

11.59 GU glycan. Predicted from the theoretical values structure should be triantennary, completely sialylated. Resistance for sialidase 2–3 digestion indicated that all three sialic acid molecules were α 2–6 linked. Unspecific α 2–3,6 sialidase treatment resulted in a decrease of hydrodynamic volume corresponding to 2.81 GU, which indicated for the release of three sialic acid residues. Galactosidase released three galactose molecules from the obtained glycan. The reaction product was then digested with hexosaminidase specific for the β 1–2 linkage. The enzyme released two N-acetylglucosamine residues like in the case of described above glycan 11.1 GU. Similarly, obtained glycan was resistant to α 1–3 specific mannosidase. Finally, the treatment with unspecific hexosaminidase and mannosidase mixture resulted in the 2.43 GU product, corresponding to mannochitobiose.

The values obtained in our experiments were in good agreement with values calculated for these structures by general rules reported by Oxford Glycosystem (1995) and also with the values reported by Guile (1996). The reported and/or calculated values for the same structures were 8.28, 9.44, 11.2 and 11.65 GU, respectively. It should be underlined, that none of these four dominant oligosaccharides contained fucose. In our previous studies we have found that haptoglobins isolated from both healthy and CDG sera did not react with fucose specific lectin from *Alleuria aurantia*. Expression of fucosylated oligosaccharides of hap-

toglobin increases in cancer and inflamation (Turner *et al.*, 1995; Kątnik *et al.*, 1996; Brinkman-van der Linden *et al.*, 1998), albeit even in disease it is significantly lower than in other acute phase protein, α_1-acid glycoprotein (Brinkman-van der Linden *et al.*, 1998).

Analysis of neutral compounds

Neutral glycans obtained from all four haptoglobin preparations were collected after glycoSep C chromatography and analysed further on the GlycoSep N column. In all examined haptoglobin preparations the fractions of uncharged oligosaccharides were heterogenous (Fig. 2). The patterns obtained for particular Hp samples varied from each other. The retention times of glycans corresponded to 1 to 10 glucose units, as it was marked

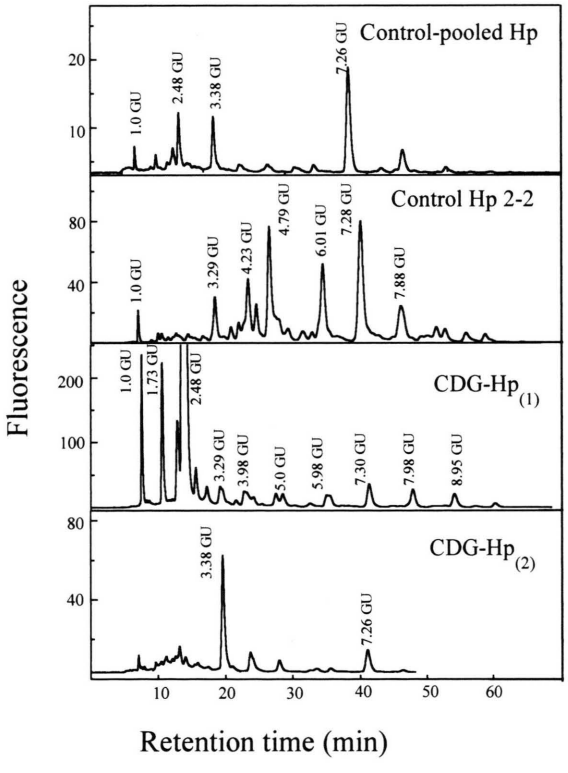

Fig. 2. GlycoSep N separations of haptoglobin neutral glycans.
Neutral oligosaccharides of four haptoglobin preparations derived from 'healthy' subjects (control-pooled Hp and Hp2–2) and from congenital disorder of glycosylation patient (CDG-$Hp_{(1)}$ and CDG-$Hp_{(2)}$) were collected after separation on GlycoSep C column and submitted to GlycoSep N chromatography.

in Fig. 2. Structures of lower mass (1–3.38 GU) predominated in both samples of CDG-Hp. Glycan of 7.26 GU, prevailing in control-pooled Hp, was detected also in the other samples, although with relatively decreased abundance. As the samples of CDG-Hp glycans were collected in trace amounts, their further analysis was impossible. The only neutral glycan collected after GlycoSep N chromatography in the amount high enough for sequential analysis was the 7.26 GU oligosaccharide of control-pooled Hp. This glycan was examined by exoglycosidase digestion. The glycan appeared to be resistant to the treatment with galactosidase, hexosaminidase and mannosidase, as the retention times after enzyme treatment were not shifted. Therefore we were not able to determine the structure with enzymatic digestion followed by chromatographic separation and we can only speculate on the nature and origin of this and other low retention time carbohydrate derivatives. It is possible that the main, neutral glycan contained modified sugars which were resistant to exoglycosidases used in our experiments. There should be also considered that some of uncharged compounds of CDG-$Hp_{(1)}$ could be the result of partial glycation of haemoglobin. The haemoglobin contamination of CDG-$Hp_{(1)}$ resulted from the presence of tight Hb-Hp complex in the patient plasma sample, which could not be dissociated under isolation conditions (Ferens-Sieczkowska *et al.*, 1999).

In our previous studies we have found, that haptoglobin of the CDG patient partially lacks oligosaccharide chains (Ferens-Sieczkowska *et al.*, 1999). About 50% of its β subunits has all four glycosylation sites occupied, about 30% lacks one out of four and 20% lacks two out of four glycans. Our present study indicates, that the structure of oligosaccharide chains attached to the β subunit is very similar to that observed in healthy human Hp. Slight difference was found in the relative content of mono, bi, tri, and tetrasialylated structures in Hp obtained from 14-year-old CDG patient, however, the sample taken one year later shown the oligosaccharide content similar when compared to the control Hp samples. So far, the information on the glycosylation patterns in the time course of the disease is limited. The only report comparing glycosylation of several glycoproteins in almost 50 patients revealed, that the level of glycoproteins, sig-

nificantly decreased in early childhood, became normal later. Patients differed in respect to the level of glycosylation defects, and defective glycoforms were more frequent in younger patients (Stibler *et al.*, 1998). Thus it is possible, that the change of glycans profile reported in this work could be explained by general stabilisation of the CDG symptoms with age.

Concluding, we have characterised glycosylation of haptoglobin obtained from healthy human sera, and defined the relative content and the sequence of dominating glycans. We have also shown, that alterations in glycan composition, concerning the relative amount of α 2–3 bound terminal sialic acid, could be dependent on the phenotype of this glycoprotein. It has been pointed out as well, that the structure of dominant oligosaccharides of haptoglobin isolated from the serum of CDG type I patient does not differ from the control structures and the differences in the content and profiles of oligosaccharides concern the relatively less abundant fractions.

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