Carbohydrate Research, 92 (1981) 328–332
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Heterogeneity in the carbohydrate moiety of band-3 glycoprotein of human erythrocyte membranes*

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(Received July 15th, 1980; accepted for publication, October 20th, 1980)

The band-3 glycoprotein is one of the preponderant intrinsic proteins of human erythrocyte membrane[1]. This glycoprotein was recently studied in several laboratories[2] since it had been found that it plays an important role in many biological functions, such as anion transport[3] and blood-group I activity[4]. Heterogeneity of band-3 glycoprotein with respect to the degree of glycosylation has been suggested[5–7], as it migrates as a diffuse band on sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis, and may be resolved into a few bands by crossed immuno-affino-electrophoresis in the presence of various lectins[7]. We have previously shown that oligosaccharides prepared from this glycoprotein have a unique structure containing the repeating disaccharide unit 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-β-D-glucopyranosyl, with a variable number of this repeating unit being attached to a core pentasaccharide composed of three D-mannose and two 2-acetamido-2-deoxy-D-glucose residues[8]. It was still unclear, however, whether the diffusion of the band was caused specifically by the heterogeneity of the sugar component. Therefore, we fractionated band-3 species that showed various electrophoretic mobilities, and analyzed the respective chemical composition and size of the sugar chains.

When purified band-3 glycoprotein was subjected to poly(acrylamide) gel electrophoresis in sodium dodecyl sulfate, a characteristically broad band was observed (Fig. 1, A). This band was divided into three zones according to mobility. These were separately removed by cutting the gel and extracting it with a buffer containing 1% of sodium dodecyl sulfate, followed by a dialysis against ethanol and water. Re-investigation of these fractions by electrophoresis showed diffuse bands. the relative mobilities of which corresponded to the original positions in the gel (Fig. 1; B, C, and D). This result, indicating that each band-3 fraction has its own specific mobility, is in good agreement with that reported by Yu and Steck[5].

*This work was supported by research grants from the Ministry of Education, Science, and Culture of Japan, and the Toray Science Foundation.
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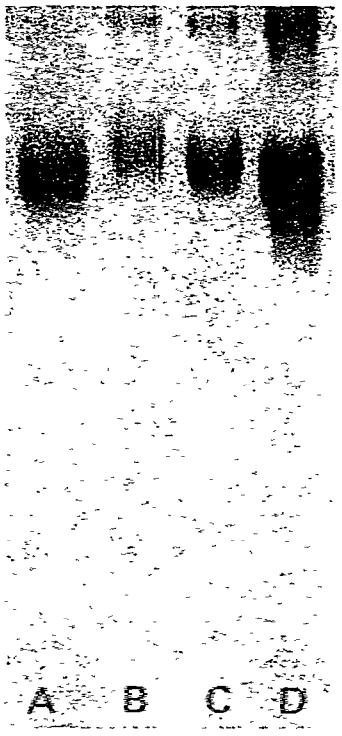

Fig. 1. Heterogeneity in the electrophoretic profile of band-3 glycoprotein. Purified band-3 glycoprotein was subjected to sodium dodecyl sulfate electrophoresis (A), and its region was cut into three segments according to mobility. The three fractions were re-examined by electrophoresis (B, C, and D).

The results of the chemical analysis of the three band-3 fractions, obtained by extraction from the gel, are shown in Table I. The amino acid compositions of the three fractions were found to be identical within experimental error. On the other hand, the carbohydrate analysis revealed that the fraction that had the lower mobility in the gel contains the larger amount of carbohydrate. Furthermore, this fraction had a higher content of D-galactose and 2-acetamido-2-deoxy-D-glucose than the other fractions, although the D-mannose content of the three fractions was found to be similar. These results suggest that the trailing zone of the band-3 glycoprotein, as seen in sodium dodecyl sulfate electrophoresis, contains oligosaccharides of a relatively higher molecular-weight than the other portion of the band-3 glycoprotein, which contain a large number of the repeating disaccharide unit consisting of D-galactose and 2-acetamido-2-deoxy-D-glucose. This observation was confirmed by the determination of the molecular sizes of the oligosaccharides obtained from the three fractions by hydrazinolysis. Fig. 2 shows the elution patterns, from a Sephadex G-50 column, of the tritium-labeled oligosaccharides, released by hydrazinolysis from the glycoproteins. The oligosaccharide from the fraction that had the lowest mobility was eluted somewhat faster, indicating that this oligosaccharide had

TABLE I

CHEMICAL ANALYSIS OF THREE FRACTIONS OF BAND-3 GLYCOPROTEIN[a]

Component	*Band-3 fractions having*		
	Lowest mobility[a] (mmol/100 g)	*Intermediate mobility[b] (mmol/100 g)*	*Highest mobility[c] (mmol/100 g)*
Aspartic acid	49.0	48.3	48.0
Threonine	33.7	33.9	34.2
Serine	52.7	51.0	48.4
Glutamic acid	84.4	80.0	82.8
Proline	48.4	49.8	52.3
Glycine	62.7	64.3	63.7
Alanine	47.8	52.1	52.9
Half-cystine	2.3	2.4	2.3
Valine	32.1	36.6	38.6
Methionine	17.0	12.8	14.8
Isoleucine	18.7	21.8	28.3
Leucine	82.4	87.3	81.4
Tyrosine	12.3	12.3	12.0
Phenylalanine	33.4	37.1	38.5
Lysine	22.2	22.5	23.5
Histidine	13.7	14.4	13.5
Arginine	32.9	33.7	34.5
Fucose	3.2	2.8	2.1
Mannose	3.7	4.0	3.6
Galactose	16.0	9.8	8.0
2-Acetamido-2-deoxyglucose	17.7	11.8	8.5
2-Acetamido-2-deoxygalactose	1.5	0.9	0.5
Sugar content (%, w/w)	8.3	5.7	4.4

[a]B in Fig. 1. [b]C in Fig. 1. [c]D in Fig. 1.

a higher molecular-weight. These results demonstrate that the characteristic diffusion of band-3 glycoprotein, as seen in sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis, may be attributed to the heterogeneity of the molecular size of the sugar chains.

EXPERIMENTAL

Analytical procedures. — Poly(acrylamide) gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Fairbanks *et al.*[1], except that a 7.5% poly(acrylamide) slab-gel was used to distinguish band-3 glycoprotein from the major sialoglycoprotein. Amino acids were determined with a Hitachi 835 amino acid analyzer, after hydrolysis in 6M hydrochloric acid for 24 h at 110°. Neutral sugars were determined by g.l.c. in columns of 0.05% ECNSS-M (0.3 × 100 cm) and 3% OV-225 (0.3 × 200 cm), after conversion to the respective alditol acetates

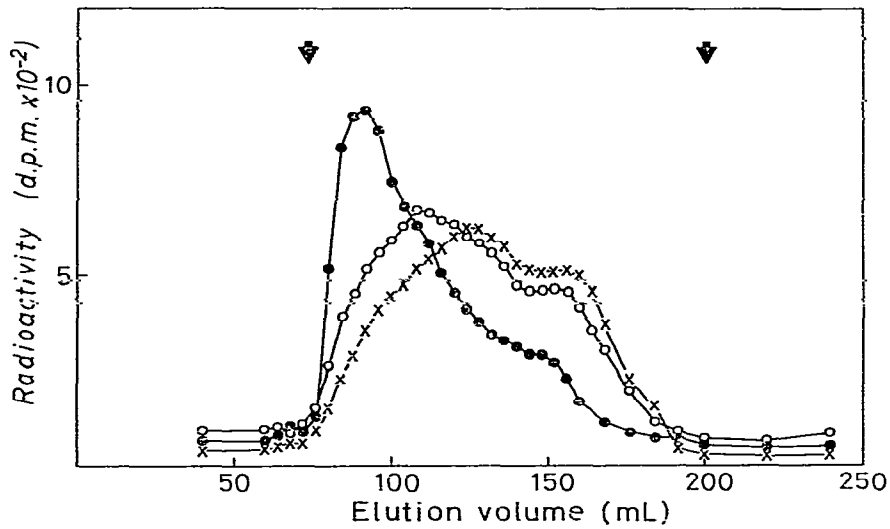

Fig. 2. Gel chromatography of tritium-labeled oligosaccharides released by hydrazinolysis from the glycoprotein fraction in a column (1.5 × 100 cm) of Sephadex G-50. Elution was achieved with 0.5M sodium chloride at a flow rate of 5 mL/h. The arrows indicate the elution positions of Blue Dextran and D-glucose. (●) Oligosaccharide from the fraction of lowest mobility; (○) oligosaccharide from the fraction of intermediate mobility; and (×) oligosaccharide from the fraction of highest mobility.

according to the method of Spiro[9], the hydrolysis being performed with 0.5M sulfuric acid for 6 h at 100°, and with 3M hydrochloric acid for 3 h at 100°. 2-Amino-2-deoxyhexoses were also determined with an amino acid analyzer, according to the method of Spiro[9]. The hydrolysis for this assay was performed with 4M hydrochloric acid for 4 h at 100°.

Isolation of band-3 glycoprotein. — Band-3 glycoprotein was purified from blood-group type A, human erythrocytes by selective solubilization and gel chromatography in a Sepharose 6B column, in the presence of sodium dodecyl sulfate, as described previously[8]. The purified, whole band-3 glycoprotein was subjected to sodium dodecyl sulfate–poly(acrylamide) gel-electrophoresis, and its region of migration was detected by staining with Coomasie Brilliant Blue. The band-3 region of the gel was cut into three segments which had different mobilities. Each gel slice was homogenized, and the glycoprotein extracted with 10mM Tris · HCl (pH 8.0) containing 1% of sodium dodecyl sulfate, mM ethylenediaminetetraacetic acid, and 40mM 2-mercaptoethanol for 16 h at 37°. After centrifugation at 30 000*g* for 60 min, the supernatant solution was dialyzed against absolute ethanol and then water, and lyophilized.

Isolation of oligosaccharides from band-3 glycoprotein. — The oligosaccharide chains from band-3 glycoprotein were split off by hydrazinolysis, according to the method described previously[10]. The dried sample was heated for 4 h at 105° in a sealed, evacuated tube with freshly distilled, anhydrous hydrazine (500 μL) containing hydrazine sulfate (1 mg). After evaporation, and addition and evaporation of

toluene *in vacuo*, the residue was dissolved in a small amount of 4.5M sodium acetate and N-acetylated according to the method of Spiro[9]. The *N*-acetylated oligosaccharides were isolated from the reaction mixture by gel chromatography in a column (0.6 × 45 cm) of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) at room temperature.

Isotope labeling of oligosaccharides. — Tritium labeling of oligosaccharides was performed according to Takasaki and Kobata[11]. *N*-Acetylated oligosaccharides were reduced with sodium borotritide (2 μmol, 500 μCi) (New England Nuclear, Boston, MA 02118) in 10mM sodium hydroxide (200 μL) for 4 h at 25°. Unlabeled sodium borohydride (2 mg) was then added, and the reaction was allowed to continue for another 2 h at 25°. The reaction was stopped by passing the reaction mixture through Dowex 50WX-8 (H^+). The remaining boric acid was removed by repeated additions and evaporations of methanol. To remove any likely radioactive contaminants from sodium borotritide, descending paper-chromatography was performed[12] for 16 h on Whatman No. 1 paper in 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water. The area of the radioactive sample was monitored with a radiochromatogram scanner (Packard Model 7220). The radioactive peak that remained at the origin was eluted with water.

Gel chromatography. — Tritium-labeled oligosaccharides were chromatographed in a column (1.5 × 100 cm) of Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution was achieved at room temperature with 0.5M sodium chloride at a flow rate of 5 mL/h. The radioactivity in each tube was determined by a liquid-scintillation spectrometer (Aloka LSC-700, Tokyo, Japan).

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