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THE MAJOR SIALOGLYCOPROTEIN OF THE HUMAN ERYTHROCYTE MEMBRANE

RELEASE WITH A NON-IONIC DETERGENT AND PURIFICATION

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SUMMARY

The major sialoglycoprotein of the human erythrocyte membrane has been selectively released by the non-ionic detergent Tween 20 and further purified in detergent-free buffers by hydroxyapatite chromatography and, finally, by hydrophobic interaction chromatography on pentyl-Sepharose. The purified glycoprotein shows one main zone, PAS-1, and up to three minor zones after staining both for protein and carbohydrate in polyacrylamide gel electrophoresis in the presence of dodecyl sulfate. The relative staining intensities are concentration dependent. When the purified glycoprotein has been heated to 100 °C in dodecyl sulfate, more stain appears in the most rapid zone, PAS-2, and less in the slower zones, indicating a disaggregation of oligomeric forms of this glycoprotein, including a dimer, PAS-1.

INTRODUCTION

The human erythrocyte membrane contains several glycoproteins [1]. One of these, the major sialoglycoprotein (glycophorin, the MN-glycoprotein) seems to be present in much larger amount than any of the others. It carries a large part of the sialic acid of the membrane (which amounts to 1.2 % of the membrane weight [2]). This glycoprotein is exposed on the outside of the erythrocyte [1, 3] and penetrates the membrane [1, 4], on the inside of which it might interact with the major protein components (spectrin) [5–7]. The MN blood group antigen (as well as other blood group antigens) seems to reside in the large acidic carbohydrate part of the major sialoglycoprotein [8], which gives the erythrocyte surface a highly negative charge. No specific function of this glycoprotein is known.

The major sialoglycoprotein has been purified earlier in many laboratories. It has been released from the membrane by treatment with two-phase systems of organic solvents [9, 10] or phenol [11], with 33 % pyridine [12, 13] or with lithium diiodosalicylate [14, 15]. Recently it has also been released with the non-ionic deter-

gent Triton X-100 [16, 17] and purified by adsorption chromatography on immobilized lectins in the presence of Triton X-100 [17]. We have found that the non-ionic detergent Tween 20 releases the major sialoglycoprotein with partial selectivity, which forms the basis of a mild and rapid procedure to purify this glycoprotein.

The erythrocyte membrane components solubilized with dodecyl sulfate and separated by polyacrylamide gel electrophoresis show a distribution of stain between the two dominating carbohydrate (glycoprotein) zones, PAS-1 and PAS-2, (gel zones are numbered according to Steck and coworkers [1]) that can be varied by keeping the sample solution at different temperatures before electrophoresis [18–20]. Some authors interpret this as due to a temperature-dependent equilibrium between a monomer (PAS-2) and a dimer (PAS-1) form of the major sialoglycoprotein. The purified glycoprotein is reported to aggregate reversibly even at room temperature in the presence of dodecyl sulfate [21, 22]. Other authors have, however, found some evidence that indicates that the glycoprotein in zones PAS-1 and PAS-2 might differ [23, 24] or that the zones can be heterogeneous [15].

Our results with the purified glycoprotein indicate that in dodecyl sulfate the solubilized and purified glycoprotein mainly exists as a dimer at room temperature. We also have indications that the dimerization is concentration dependent. It should be noticed that our results are obtained with glycoprotein prepared by a method different from those used earlier.

MATERIALS AND METHODS

Membranes and the membrane residue. Human erythrocyte membranes were prepared in sodium phosphate buffer (pH 7.4) according to Dodge et al. [25]. The membranes, at a total protein concentration of 4 g/l, were extracted once with 1 mM EDTA and 5 mM 2-mercaptoethanol in 5 mM glycine/NaOH (pH 9.0), which removed part of the water-soluble protein. The resulting non-solubilized material is here called the membrane residue (cf. ref. 26).

Bed material for the chromatographic experiments. Hydroxyapatite was purchased from Bio-Rad, Richmond, Calif., USA (Biogel HTP) or was prepared as described in ref. 27. Pentyl-Sepharose gel for hydrophobic interaction chromatography was prepared by coupling pentyl groups to Sepharose 4B by the glycidyl ether method, according to Hjertén et al. [28]. The pentylglycidyl ether was prepared by Dr. Jan Rosengren and Mr. Magnus Glad.

Analytical gel electrophoresis. Polyacrylamide gel electrophoresis was performed in 3 mm thick gel slabs, 90 mm long, and with 7 mm wide sample wells. The gels contained 10 mM sodium dodecyl sulfate and 50 mM glycine/NaOH (pH 9.8). The gel concentration was $T = 6\,\%$, $C = 3\,\%$ [26]. The gels were stained for protein and carbohydrate essentially according to Fairbanks et al. [29].

Chemical analyses. Protein concentrations were estimated by absorbance measurements. To estimate the yield of the glycoprotein the sialic acid concentrations in the chromatographic fractions were determined, according to Warren [30]. Amino acid analyses were performed with a Beckman 121 M automatic amino acid analyzer. The amounts of different carbohydrates in the purified glycoprotein were determined by gas-liquid chromatography according to Clamp et al. [31].

Preparation of the major sialoglycoprotein

Release from the membrane residue. The membrane residue was suspended in 60–80 ml 8 mM Tris/acetate buffer (pH 8.5), containing 2 g/l of Tween 20* (Tween 20 SD, Atlas Chemie GmbH, Essen, GFR) to a final protein concentration of 2 g/l. After 0.5 h at 0–4 °C, the mixture was centrifuged at 100 000 × *g* for 1.5 h and the supernatant was collected.

For the following chromatographic steps only detergent-free buffers were used.

Hydroxyapatite chromatography (*Fig. 2*). The above supernatant was dialyzed against 0.005 M potassium phosphate buffer (pH 6.8) and applied to a hydroxyapatite column equilibrated with the same buffer. Non-adsorbed material, which contained little glycoprotein, was washed out with the same buffer. The glycoprotein was then eluted with 0.07 M potassium phosphate buffer (pH 6.8).

Pentyl-Sepharose chromatography (*Fig. 3*). The fraction eluted with 0.07 M buffer from the hydroxyapatite chromatography was made 0.3 M in potassium phosphate buffer (pH 6.8) and applied to a pentyl-Sepharose column equilibrated with 0.3 M phosphate buffer. Non-adsorbed material was washed out with 0.3 M phosphate buffer and the adsorbed glycoprotein was eluted with 0.005 M phosphate buffer (pH 6.8).

RESULTS

Release of the major sialoglycoprotein

Solubilization of human erythrocyte membrane proteins with Tween 20 can be done more or less selectively [26]. This is true also specifically for the major sialoglycoprotein. In the present work we have used a low detergent : protein ratio and a moderate pH, thereby achieving a high selectivity. This is illustrated in sections 2 of Figs 1A and 1B, which show that mainly components 1 and 2 (spectrin), 4.1, PAS-1 and PAS-2 can be released, of which PAS-1 and possibly PAS-2 represent the major sialoglycoprotein. (Components 4.1 and PAS-1 are poorly separated in this type of dodecyl sulfate gel.)

About half of the sialic acid of the membrane, and therefore also roughly half of the major sialoglycoprotein** was released by 0.2 % Tween 20 at pH 8.5 (Table I). Less sialic acid is released at pH 7 than at pH 8.5 and, as can be expected for hydrophobic interactions, much less at the ionic strength 0.05 than at 0.005 (pH 9.8). The release of the major sialoglycoprotein by the non-ionic detergent Triton X-100 is less dependent on the ionic strength [16].

Fractionation of the glycoprotein

Hydroxyapatite chromatography. Fig. 2 shows a chromatogram of Tween 20-solubilized erythrocyte membrane proteins on hydroxyapatite. Fraction I, which

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* According to the manufacturer, Tween 20 is a polyethylene ether (with 20 oxyethylene groups in three chains) of sorbitan lauryl ester. Gas-liquid chromatographic analyses of fatty acids from Tween 20 SD also indicated considerable amounts of longer alifatic chains (Johansson, K.-E., personal communication).

** A great part of the sialic acid in the membrane is covalently bound to the major sialoglycoprotein and only a small part of the glycolipids (which also contain sialic acid) are solubilized, as judged by gel electrophoresis in dodecyl sulfate.

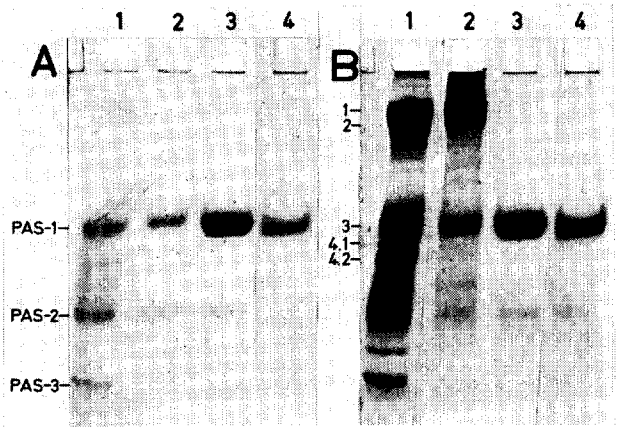

Fig. 1. Purification of the major sialoglycoprotein of the human erythrocyte membrane. Gel electrophoresis in the presence of dodecyl sulfate. Section 1: the membrane residue; gel A, 0.1 mg protein, gel B, 0.07 mg. Section 2: supernatant after solubilization with 0.2 % Tween 20, 0.02 mg. Section 3: fraction II from the hydroxyapatite chromatography (Fig. 2), 0.03 mg. Section 4: fraction II from the pentyl-Sepharose chromatography (Fig. 3), 0.02 mg. The gel was first stained with the Schiff stain (A) and then with Coomassie Brilliant Blue (B), according to ref. 27. The staining of the glycoprotein zones with Coomassie Brilliant Blue is more intense when the gel has first been stained with the Schiff stain. The glycolipids in the membrane residue have migrated out of the gel.

TABLE I

INFLUENCE OF pH AND IONIC STRENGTH UPON THE RELEASE OF SIALIC ACID FROM THE MEMBRANE RESIDUE

The membrane residue was suspended at 0–4 °C in buffer to final concentrations of 2 g protein/l and 2 g Tween 20/l and centrifuged at 100 000 × *g* for 1.5 h. The supernatant and the suspended pellet were hydrolysed with 0.075 M H_2SO_4 and their contents of sialic acid determined [30]. The sialic acid content in supernatant and pellet is expressed in percent of the total amount of sialic acid in the membrane residue. Each value in the table represents the mean of duplicate determinations from three preparations. The standard error is given.

pH	Ionic strength	Buffer	Sialic acid (%)	
			Supernatant	Pellet
7.0	0.007	0.005 M potassium phosphate	23±10	79±8
8.5	0.003	0.008 M Tris/acetic acid	48± 7	48±3
9.8	0.005	0.010 M glycine/NaOH	59± 3	35±1
9.8	0.05	0.10 M glycine/NaOH	15± 1	84±8

was not adsorbed to the column, very probably contains Tween 20 (which has some absorption at 280 nm) and lipids. It contains also small amounts (corresponding to less than 10 % of the applied sialic acid) of non-adsorbed glycoprotein. When the column was eluted with 0.07 M phosphate buffer, material containing a great part of the sialic acid was desorbed. This material (fraction II) contained components PAS-1 and PAS-2 (Figs 1A and 1B, section 3), With 0.3 M phosphate buffer spectrin was eluted (fraction III) as well as some PAS-1 and PAS-2 material and other protein components (analysis not shown).

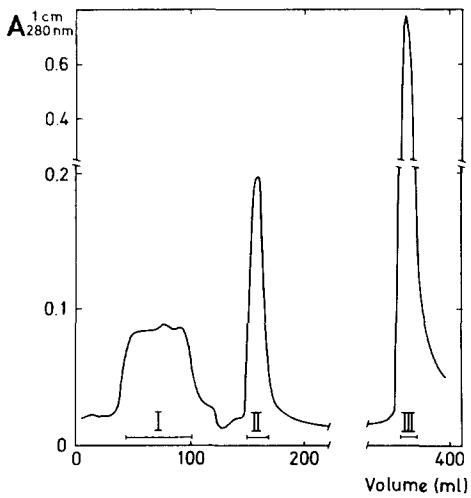

Fig. 2. Hydroxyapatite chromatography of proteins solubilized from the membrane residue with Tween 20. Sample: 70 ml of the solubilized material ($A_{280}{}^{1\,cm} = 0.4$), dialyzed aginst 0.005 M potassium phosphate buffer (pH 6.8). The column, 3 cm^2 × 25 cm, was eluted with potassium phosphate buffer (pH 6.8) of the following concentration: 0.005 M; 0.07 M; and 0.3 M. Fraction II containing the glycoprotein was applied to a pentyl-Sepharose column.

Pentyl-Sepharose chromatography. The glycoprotein in fraction II from hydroxyapatite chromatography was further purified by hydrophobic interaction chromatography on pentyl-Sepharose [28]. The material was applied to the column at moderately high ionic strength at which some components were not asdorbed onto the pentyl-Sepharose (fraction I). The glycoprotein (fraction II) was eluted at low ionic strength (Fig. 3 and Figs 1A and 1B, section 4). This fraction contained 5–20 %

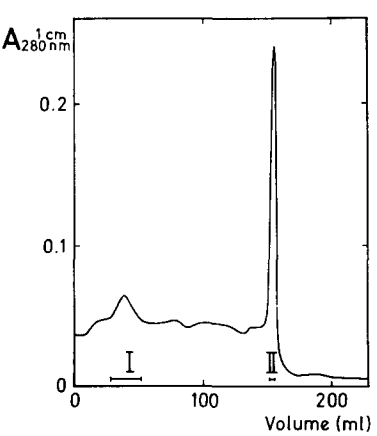

Fig. 3. Pentyl-Sepharose chromatography of fraction II from hydroxyapatite chromatography. Sample: 25 ml ($A_{280}{}^{1\,cm} = 0.1$) in 0.3 M potassium phosphate buffer (pH 6.8). The column, 1.5 cm^2 × 12 cm, was eluted with potassium phosphate buffer (pH 6.8) of the following concentration: 0.3 M; and 0.005 M. The high absorption in the beginning of the chromatogram is due to absorbing material in the 0.3 M buffer.

of the sialic acid in the membrane residue. Recently we have found that higher yields of glycoprotein can be obtained if the glycoprotein is eluted with, for example 0.001 M K_2HPO_4.

The hydrophobic interaction chromatography step can be omitted if a somewhat lower degree of purity is acceptable.

Components of the purified glycoprotein

The purified glycoprotein showed one major zone (PAS-1) and one minor zone (PAS-2) upon polyacrylamide gel electrophoresis in the presence of dodecyl sulfate (Fig. 1, section 4). In some preparations of the glycoprotein, one or two slower zones (denoted A and B in Table III) also appeared. These zones had no correspondence in the pattern for the membrane residue. All these zones stained for both protein and carbohydrate with about the same ratio between the staining intensities for the zones A, B, PAS-1 and PAS-2. In some preparations the glycoprotein was contaminated with traces of spectrin or other protein components, or with glycolipids.

Chemical analyses

Table II shows the amino acid and carbohydrate composition of the purified glycoprotein. Similar compositions have been reported for the major sialoglycoprotein prepared by other methods [8, 13, 15, 32, 33].

TABLE II

AMINO ACID AND CARBOHYDRATE COMPOSITION OF THE MAJOR SIALOGLYCOPROTEIN OF THE HUMAN ERYTHROCYTE MEMBRANE

Amino acid	Mol fraction (%)	Carbohydrate	% (w/w)
Lys	5.2		
His	4.1	Galactose	8.3
Arg	4.9	Mannose	2.0
Asx	7.4	Fucose	0.4
Thr	8.9	Glucosamine	4.8
Ser	10.4	Galactosamine	10.2
Glx	11.7	*N*-Acetylneuraminic acid	29.0
Pro	6.9		
Gly	5.5		
Ala	7.0	Carbohydrate	55
Cys	0.2		
Met	1.7		
Val	7.5		
Ile	5.1		
Leu	8.0		
Tyr	2.8		
Phe	2.8		
Trp	–		

TABLE III

DISTRIBUTION OF CARBOHYDRATE STAIN BETWEEN THE DIFFERENT ZONES IN GEL ELECTROPHORESIS OF THE PURIFIED MAJOR SIALOGLYCOPROTEIN

A solution of the purified major glycoprotein was made 0.1 M in dodecyl sulfate, 0.02 M in dithiothreitol, 0.0002 M in EDTA and 0.02 M in glycine/NaOH to pH 9.8. One half of this solution was heated to 100 °C and kept at this temperature for 5 min and then rapidly cooled to 25 °C. Another half was kept at 25 °C. Gels were prepared as described in Materials and Methods and the samples, each containing 0.05 mg, were applied and the gels run at 13 V/cm for 2 h. The gels were stained for carbohydrate and scanned at 560 nm. The distribution of stain was determined from the areas corresponding to the peaks in the scanning diagram. The given values of the uncertainties are rough estimates. Both the zones A and B seen in this experiment (but not in that of Fig. 1) had migration velocities between those of components 2 and 3.

Zone	Percent of stain	
	Normal sample	Heated sample
A	4 ± 2	~ 2
B	14 ± 3	7 ± 3
PAS-1	54 ± 3	48 ± 3
PAS-2	27 ± 3	44 ± 3

Aggregation and disaggregation of the glycoprotein

The purified glycoprotein showed a pattern in dodecyl sulfate gel electrophoresis that changed on heating the sample with dodecyl sulfate. The staining intensities of zones A and B diminished when the glycoprotein was kept at 100 °C for 5 min, while the zone PAS-2 increased in intensity (Table III). When the same experiment was performed on a preparation of the glycoprotein in which zones A and B did not appear, the staining of zone PAS-1 diminished in favor of the staining of zone PAS-2 for a sample kept at 100 °C. Two-dimensional gel electrophoresis in the presence of dodecyl sulfate in both directions gave, for a heated sample, a pattern indicating that material that had migrated as PAS-2 in the first direction (2 h) aggregated (overnight) and migrated as PAS-1 in the second direction (2 h, cf, ref. 20). Similar to the temperature effect, we have found that the amount of PAS-1 relative to the amount of PAS-2 increases with increasing total concentration of the glycoprotein.

Preliminary results with gel electrophoresis in the presence of Tween 20 indicate that the purified glycoprotein can also exist as both monomer and dimer in this detergent, In the absence of any detergent the purified glycoprotein can aggregate to larger complexes (the zones A and B in Table III) that are not easily split by dodecyl sulfate.

DISCUSSION

The non-ionic detergent Tween 20 has a much larger hydrophilic part (see note in Materials and Methods) than Triton X-100 (isooctylphenoxypolyetoxyethanol) and most other commonly used non-ionic detergents. Tween 20 is generally less capable of solubilizing membrane proteins or lipids than, for example, Triton X-100 [34]. Nevertheless, Tween 20 releases the major sialoglycoprotein of the erythrocyte membrane, which penetrates the membrane [35, 36]. Roughly half of this glycoprotein can be released by 0.2 % Tween 20 at pH 8.5 (Table I). The high capacity of Tween 20 to release the sialoglycoprotein selectively is remarkable since, under the

same conditions, less than 10 % of the phospholipids of the membrane are solubilized [26]. At present we do not know whether the solubilizing capacity of Tween 20 could be partly due to a minor fraction of the detergent with other proportions between hydrophilic and hydrophobic parts or to impurities in the detergent.

The data on the temperature-dependent distribution of carbohydrate stain between the glycoprotein components (Table III) are in close agreement with the results of Tuech and Morrison [20], who found several glycoprotein zones in dodecyl sulfate gel electrophoresis of the purified major sialoglycoprotein, and a temperature dependence for the distribution of stain (cf. also refs. 18 and 19). Similarly, Janado and coworkers [21, 22] have reported that the major sialoglycoprotein aggregates reversibly in the presence of dodecyl sulfate at 20 °C. The zone PAS-1, as well as the slower zones (A and B) obtained in some of our preparations, can be interpreted as due to aggregates of a monomer unit, PAS-2. Authors who have obtained only one zone in gel electrophoresis of their purified glycoprotein [9, 14, 17, 32, 37] may have used conditions for preparation or analysis (e.g. high protein concentration in the sample) that greatly favor one form of the protein.

Some authors have reported indications that PAS-1 and PAS-2 are entirely different glycoproteins [23, 24], which seems inconsistent with the results in Table III or in refs. 18–20. However, it is possible that the zones PAS-1 and PAS-2 can be heterogeneous owing to incomplete separation of native proteins or to artifactual aggregates [15, 23].

For the final purification we have used hydrophobic interaction chromatography, a relatively new technique, which is described in, for example ref. 28. In this technique proteins are separated on the basis of their interactions with hydrophobic ligands on a gel matrix. Generally the interactions decrease with decreasing ionic strength and the proteins can be eluted stepwise, which gives a rapid separation and a high final concentration of protein. Suitable ligands can easily be coupled to the gel matrix. For enzymes tested, no appreciable decrease in activity has been detected after adsorption and desorption [28, 38].

Since the properties of the sialoglycoprotein are somewhat elusive, it is also valuable to prepare it, as we have done, in a fundamentally new way and compare its properties with the properties reported earlier for this protein.

The purification procedure used in the present work is simple and rapid, and gives a fair yield (10–20 %) of highly purified glycoprotein. The procedure does not involve extractions with organic solvents or other procedures that are likely to change the conformation of membrane proteins. We therefore consider this procedure a good means of purifying the erythrocyte membrane sialoglycoprotein for further studies.

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