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Association of Glycoproteins with the Membranes. I. Isolation and Molecular Weight of the Monomeric Unit of the Major Glycoprotein from Human Erythrocytes†

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ABSTRACT: The major glycoprotein from human erythrocyte membranes has been isolated in the aggregated form (form I) and the nonaggregated form (form II). The two preparations of the glycoprotein were shown to be homogeneous by analytical polyacrylamide gel electrophoresis, sedimentation velocity studies, and gel filtration studies. The chemical composition and physical properties of both forms indicated that two preparations were chemically identical. However, form I was an aggregate, probably due to the presence of very small amounts of tightly associated lipids. Determination of the molecular weight by several independent methods indicated that the true molecular weight of the glycoprotein monomer was 23,000–24,000. Each glycoprotein molecule contains approximately 102 amino acid residues in the polypeptide chain with 2 molecules of fucose, 2 of mannose, 11 of galactose, 12 of *N*-acetylgalactosamine, 6 of *N*-acetylglucosamine, and 17 of *N*-acetylneuraminic acid.

The widespread occurrence of glycoproteins in cell membranes and the role ascribed to them in intercellular recognition has raised the question as to the nature of the intimate association of glycoproteins to the other constituents of the plasma membrane. According to one hypothesis the membrane glycoproteins are associated with the lipoidal trilaminar plasma membrane by means of regions poor in carbohydrate and rich in the branched chain lipophilic amino acids (Morawiecki, 1964; Winzler, 1969; Marchesi *et al.*, 1971) These studies were carried out to test the hypothesis proposed for the association of the glycoproteins to the membranes. The erythrocyte membrane glycoprotein served as a prototype for the study of cell membrane glycoproteins.

Although the erythrocyte membrane glycoproteins are the most extensively studied membrane glycoproteins, the reported molecular weight for the major glycoprotein has varied from 31,000 (Kathan *et al.*, 1961) to 12 × 10^6 (Springer, 1967). This wide variation appears to result from differences in the procedures used for preparation of the glycoprotein and the resulting different degree of aggregation and also from the methods used for the determination of the molecular weight.

This paper describes the physicochemical properties and the molecular weight of the monomeric unit of the membrane glycoprotein, using several different methods for molecular weight determination on highly purified membrane glycoprotein from human erythrocytes both in the aggregated and the nonaggregated forms.

Materials and Methods

All reagents were of analytical grade. Molecular weight markers used in gel filtration and in polyacrylamide gel electrophoresis were ribonuclease A, chymotrypsinogen A, adolase, and ovalbumin from Pharmacia Fine Chemicals Inc. calibration kit; catalase fumarase and bovine serum albumin were from Sigma Chemical Co., β-galactosidase was a gift from Dr. O. P. Bahl.

Polyacrylamide gel electrophoresis was performed in the presence of 1% sodium dodecyl sulfate and 10 mM β-mercaptoethanol as previously described (Lenard, 1970). The molecular weights were calculated by the method of Weber and Osborn (1969).

For sedimentation velocity studies the centrifugations were carried out at 52,640 rpm in a Beckman Model E analytical ultracentrifuge. A single sector Kel-F cell was used for sedimentation velocity studies in formic acid. Sedimentation equilibrium studies were carried out at 22,000 rpm and employed 3-mm liquid columns layered over an inert base of perfluorotributylamine. Diffusion coefficient was determined by low-speed centrifugation at 6000 rpm using a double sector cell.

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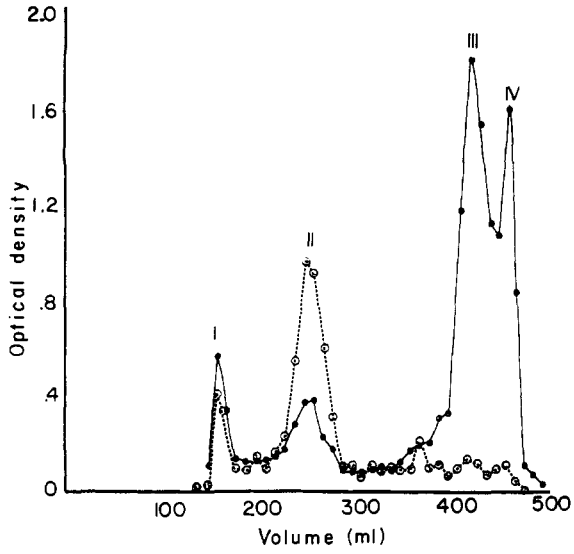

FIGURE 1: Sephadex G-100 chromatography of 67% ethanol-soluble fraction; 100 mg of the sample in 5 ml of distilled water was loaded on the column (2.5 × 90 cm); 10-ml fractions were collected and analyzed for protein and carbohydrate. (●–●) OD_{280} (protein); (O–O) OD_{490} (hexoses).

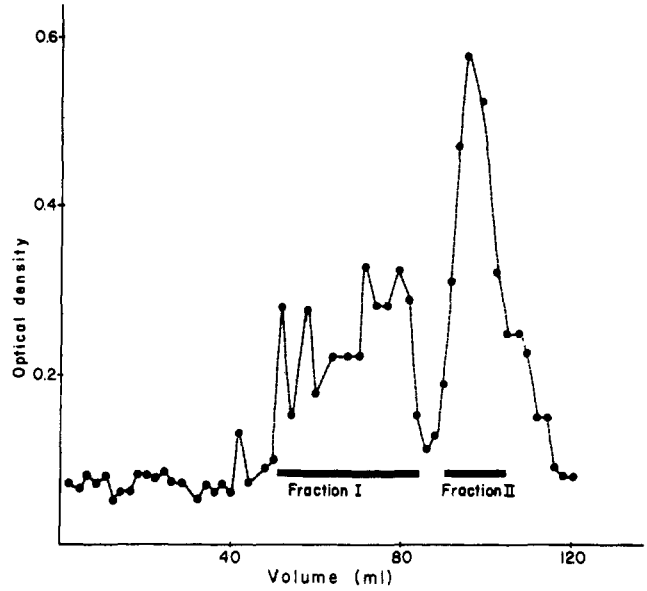

FIGURE 2: Sephadex G-200 chromatography of the aggregated glycoprotein in 6 M guanidine-HCl–4 M urea; 5 mg of the sample in 3 ml of guanidine hydrochloride–urea solution were loaded on the column (1.5 × 90 cm); 0.2-ml fractions were collected and analyzed for carbohydrates.

Partial specific volumes were calculated as described by Schachman (1957).

The osmotic pressure was determined at different concentrations by a recording membrane osmometer (Melabs Scientific Instruments). The number average molecular weight at zero concentration was obtained from the plot of π/c *vs.* concentration.

Total neutral sugars were determined by the phenol–sulfuric acid method of Dubois *et al.* (1956). Individual neutral sugars were quantitated as alditol acetates by the gas chromatography method of Lehnhardt and Winzler (1968). Hexosamines were determined either by the colorimetric method of Good and Bessman (1964) or by amino acid analyzer using the borate buffer system as described by Weber and Winzler (1969). Sialic acid was determined by thiobarbituric acid method of Warren (1959) as modified by Aminoff (1961). Amino acid composition was determined by Beckman-Spinco Model 120 B amino acid analyzer using the buffer system described by Spackman *et al.* (1958). Phosphate was determined by the molybdate method of Ames (1966).

Isolation and Purification of the Membrane Glycoprotein. Erythrocyte membranes from the outdated, pooled human blood were prepared by the procedure of Dodge *et al.* (1963). Isolation of the crude glycoprotein from the washed membranes was carried out as described by Kathan *et al.* (1961).

Aggregated Glycoprotein (Form I). Absolute ethanol to a concentration of 50% (v/v) was added to a 0.5% solution of the crude glycoprotein. After 4–6 hr the insoluble material was removed by centrifugation at 30,000*g* for 20 min. To the supernatant more ethanol was added to bring the final ethanol concentration to 67% (v/v). Sodium acetate (1 mg/ml) was added to facilitate the precipitation. After standing overnight the precipitate was collected by centrifugation, dissolved in 0.9% saline, and pH adjusted to 8.5 with dilute NaOH. Equal volume of 90% phenol was then added, and the mixture was heated at 65° for 10 min, cooled to room temperature, and centrifuged. The aqueous layer was dialyzed against water and lyophilized; 20-mg aliquots were then dissolved in 3 ml of 0.05 M pyridine adjusted to pH 4.5 with acetic acid and chromatographed on a phosphocellulose column (Cellex P, Bio-Rad, pyridine form 25 × 1.5 cm). The void volume fractions were pooled, lyophilized, and chromatographed on Sephadex G-200 in 0.1 M acetic acid. The void volume peak was lyophilized to give the purified aggregated glycoprotein (form I).

Nonaggregated Glycoprotein (Form II). During the purification of the glycoprotein as described above, the 67% ethanol-soluble fractions were collected and pooled. The volume of the pooled fractions (1500 ml) was reduced to 50 ml by evaporation at 30° under reduced pressure. The cloudy concentrate was dialyzed against distilled water and lyophilized. The lyophilized material (0.5 g) dissolved readily in 67% ethanol, but gave cloudy suspensions in aqueous buffers. This was extracted at room temperature, once with 50 ml of 95% ethanol, then twice with 50 ml of chloroform–methanol (2:1 v/v). The residue after lipid extraction was readily soluble in water and was chromatographed on a Sephadex G-100 column (Figure 1). The peaks were pooled and lyophilized. Peak I contained the aggregated glycoprotein as well as some other proteins and peak II, the nonaggregated form of the glycoprotein.

Nonaggregated glycoprotein could also be prepared from the aggregated form by gel filtration in the presence of 6 M guanidine-HCl and 4 M urea (Figure 2). Fraction II was pooled and dialyzed against distilled water and concentrated by ultrafiltration using Amicon cell. Rechromatography on Sephadex G-100 gave a single peak corresponding to peak II of Figure 1. This fraction also had the same electrophoretic mobility in polyacrylamide gel electrophoresis as the nonaggregated glycoprotein.

Results

The crude glycoprotein from human erythrocyte membranes was isolated in yields of 9–10% of the dry weight of erythrocyte membranes. From this material the purified stromal glycoprotein obtained as the aggregated form amounted to about 3% of the dry weight of the membrane. Recovery of neutral sugars and of sialic acid was 17 and 57%, respectively. The isolated nonaggregated glycoprotein accounted for 0.3% of the erythrocyte membrane glycoprotein and contained 1% of the membrane neutral sugars and 6% of the membrane sialic acid.

TABLE I: Chemical Composition of Erythrocyte Membrane Glycoprotein.

	g/100 g[a]	
	Aggregated	Nonaggregated
Neutral sugars	13.4	14.9
Acetyl hexosamines	17.3	17.7
Sialic acid	22.8	24.5
Proteins	45.6	42.9
Phosphate	0.4	<0.05

[a] These values are averages of three different determinations.

Chemical Composition of the Glycoprotein Preparations. The composition of the aggregated and nonaggregated glycoproteins shown in Table I indicates that both have the same amount of neutral sugars, amino sugars, and protein. Tables II and III show that the content of individual neutral sugars and of amino acids is also very similar in the two preparations of the glycoprotein. The amino acid composition is in good agreement with the values obtained by other investigators.

Physical Properties and Molecular Weight Determination. The molecular weight calculated for the six amino acids present in lowest concentration indicated a minimal molecular weight of about 23,000 for both the aggregated and the nonaggregated glycoprotein preparations.

Gel Electrophoresis. The aggregated glycoprotein did not migrate into the polyacrylamide gel during electrophoresis unless a detergent was added. In the presence of sodium dodecyl sulfate, however, the glycoprotein migrated into 7.5% of gel as a single band with an apparent molecular weight of 110,000. When the gel concentration was increased to 12% the apparent molecular weight decreased to 95,000.

The nonaggregated glycoprotein preparation migrated into 7.5% polyacrylamide gels in the absence or presence of 1% sodium dodecyl sulfate giving a single band corresponding to an apparent molecular weight of 55,000.

Sedimentation and Diffusion Studies. Schlieren photographs of the sedimentation velocities of the aggregated and nonaggregated forms of the glycoprotein revealed single symmetrical boundaries with both preparations (Figure 3). The $s_{20,w}$ values for aggregated and nonaggregated forms were 6.9 S and 1.6 S, respectively. In 90% formic acid, however, the aggregated glycoprotein sedimented with an $s_{20,w}$ of 1.6 S.

The $D_{20,w}$ value (5.55×10^{-7} cm^2 sec^{-1}) was similar to that reported by Kathan *et al.* (1961) and Morawiecki (1964).

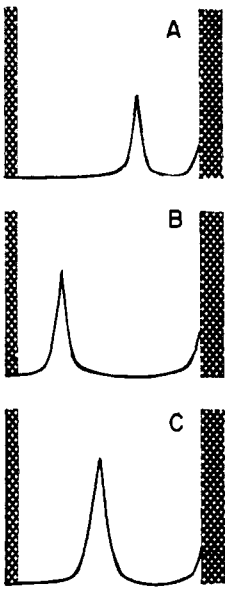

FIGURE 3: Sedimentation velocity patterns of human erythrocyte membrane glycoprotein: (A) aggregated glycoprotein in 0.05 M PO_4 (pH 8.2) (2 mg/ml); (B) aggregated glycoprotein in 90% formic acid (2 mg/ml). (C) nonaggregated glycoprotein in 0.05 M PO_4 (pH 8.2) (2 mg/ml).

The partial specific volume, calculated from the amino acid and carbohydrate composition, was 0.656. The molecular weight for the nonaggregated glycoprotein calculated from sedimentation velocity, diffusion coefficient, and partial specific volume was 20,700.

Sedimentation Equilibrium Method. The molecular weight of nonaggregated glycoprotein in 0.05 M PO_4 (pH 8.2) was determined to be 20,600. The molecular weight of the aggregated glycoprotein in 90% formic acid was found to be 23,600.

Gel Filtration. The aggregated glycoprotein was eluted with the void volume of Sephadex G-200 columns. However, the nonaggregated glycoprotein was eluted in the included volume

TABLE II: Neutral Sugar Composition of the Erythrocyte Membrane Glycoprotein.

	Aggregated		Nonaggregated	
Sugar	%	Molar Ratio[a]	%	Molar Ratio[a]
Fucose	1.05	1.0	0.91	1.0
Mannose	1.74	1.6	1.78	1.8
Galactose	8.68	8.2	8.84	9.0
Glucose	0.60	0.6	0.79	0.6
Total	12.1		12.3	

[a] Molar ratio = moles of sugar per mg/moles of fucose per mg.

TABLE III: Amino Acid Composition of Human Erythrocyte Membrane Glycoprotein.

	Aggregated		Nonaggregated	
Amino Acid	g/100 g	Moles %	g/100 g	Moles %
Lysine	2.56	4.7	2.43	3.6
Histidine	1.69	3.9	1.88	2.7
Arginine	2.89	5.6	3.10	4.3
Aspartic acid	4.54	5.6	4.68	8.2
Threonine	4.86	10.3	5.32	10.7
Serine	4.11	10.5	4.43	10.1
Glutamic acid	7.19	9.5	7.61	11.8
Proline	3.23	6.6	3.37	6.7
Glycine	2.15	6.3	2.35	6.1
Alanine	3.28	6.9	3.14	7.4
$^1/_2$-Cystine				
Valine	3.85	8.2	4.01	8.2
Methionine	1.01	1.6	1.15	1.9
Isoleucine	2.48	7.1	2.68	4.9
Leucine	4.86	8.22	5.00	8.6
Tyrosine	2.20	2.6	2.17	2.4
Phenylalanine	1.79	2.4	1.93	2.6

of Sephadex G-100 columns (Figure 1). The nonaggregated glycoprotein had an apparent molecular weight of 55,000 as calculated from a plot of K_{av} *vs.* the log of molecular weight of reference proteins. The higher molecular weight value for the nonaggregated glycoprotein obtained by gel filtration was attributable to the asymmetry of the molecule (Andrews, 1964). The Stoke's radius was calculated to be 30 Å using the method described by Reichert *et al.* (1969).

Osmotic Pressure Studies. The number average molecular weight of the nonaggregated glycoprotein determined by osmotic pressure studies was 24,800.

Discussion

The combined procedure of Kathan *et al.* (1961) and Springer *et al.* (1966) involving partitioning between phenol and water was further modified to isolate highly purified aggregated and nonaggregated forms of the glycoprotein from human erythrocyte membranes. The purity of the two preparations was established by polyacrylamide gel electrophoresis and sedimentation velocity studies.

Studies on the chemistry of the aggregated glycoprotein preparation and the nonaggregated glycoprotein preparation showed that the two were similar in composition. However, the aggregated glycoprotein preparation contained small amounts of phosphate whereas the nonaggregated form had no detectable phosphate. The nature of the phosphate is not known, although a preliminary ^{31}P nuclear magnetic resonance study indicated that there might be small amounts of phosphatidylcholine present in the glycoprotein preparation. The presence of lipids in the aggregated glycoprotein is also suggested by Lisowska (1968) who has studied the nature of fatty acids in glycoprotein.

The aggregated form could be converted to the nonaggregated form by chromatography in the presence of 6 M guanidine-HCl and 4 M urea on Sephadex G-200. However, attempts to convert the nonaggregated form to the aggregated form were unsuccessful.

The apparent molecular weight of the aggregated glycoprotein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was 95,000. Nonaggregated glycoprotein preparation, on the other hand, migrated with an apparent molecular weight of 55,000. It appears that the difference in the apparent molecular weight by polyacrylamide gel electrophoresis may represent a difference in the state of aggregation. The aggregated form in sodium dodecyl sulfate is still a dimer of the nonaggregated form.

The state of aggregation of high molecular weight glycoprotein preparations from human erythrocyte membranes has been found to be quite variable. Springer (1967) has reported molecular weights ranging from 1.6 to 12 × 10^6 for different preparations of the erythrocyte membrane glycoprotein. Morawiecki (1964) had shown that in the presence of detergents the glycoprotein had a molecular weight of 30,000. Segrest *et al.* (1971) observed the anomalous behavior of glycoproteins in polyacrylamide gel electrophoresis. Using different polyacrylamide gel concentrations they concluded that the apparent monomeric molecular weight of the glycoprotein was 55,000. However, in the present studies the molecular weight of the monomeric unit of the erythrocyte membrane glycoprotein determined by independent methods was found to be 23,000. The excellent agreement in the molecular weight values by different methods was also evidence for the homogeneity of the glycoprotein preparations. The $s_{20,w}$ 1.63 S determined in these studies was close to $s_{20,w}$ 1.57 S reported for the monomeric unit by Morawiecki (1964) in the presence of detergent. The higher molecular weight (31,000) calculated by Morawiecki (1964) based on sedimentation and diffusion studies was due to the fact that he estimated the value for partial specific volume to be 0.774 for the glycoprotein, whereas we calculated the partial specific volume of 0.656 from the glycoprotein composition. The higher apparent molecular weight for the nonaggregated glycoprotein by Sephadex gel filtration is due to the fact that the human erythrocyte membrane glycoprotein was more like a rod than globular as indicated by the frictional ratio of 1.74 calculated from the Stoke's radius.

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