Association of Glycoproteins with the Membranes. II. Isolation and Partial Characterization of "Lipophilic Fragment" from Human Erythrocyte Membrane Glycoprotein†

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ABSTRACT: The trypsin digestion of the isolated glycoprotein from human erythrocyte membranes resulted in a carbohydrate rich soluble sialoglycopeptide and an insoluble lipophilic peptide. The amino acid composition of the sialoglycopeptide showed an enrichment in hydroxy amino acids and a decrease in hydrophobic amino acids in comparison with the membrane glycoprotein, and was similar to the glycopeptide isolated from intact human erythrocytes by trypsin digestion (Winzler, R. J., Harris, E. D., Peakas, D. J., Johnson, C. A., and Weber, P. (1967), *Biochemistry 6,* 2195). The insoluble lipophilic fragment purified by Sephadex G-50 had no detectable carbohydrate and was poor in hydroxy amino acids. The hydrophobic amino acids constituted 64 mol % of the total amino acid residues. The molecular weight was 7500 and the amino terminal was leucine. These results suggested that the human erythrocyte membrane glycoprotein was an asymmetric molecule the hydrophilic part of which contains all the carbohydrates and the lipophilic part was presumably responsible for its association with the lipids of the membrane.

The presence of glycoproteins in cell membranes has been documented by many laboratories (for review see Cook, 1968; Winzler, 1970; 1972). These glycoproteins carry certain blood group antigens, transplantation antigens, and tumor specific antigens. Winzler (1969) had proposed that these glycoproteins are associated with the lipoidal trilaminer plasma membrane by means of regions rich in hydrophobic amino acids and poor in carbohydrates. This paper describes the isolation and partial characterization of the "lipophilic fragment" presumably responsible for the association of human erythrocyte glycoprotein to the membranes. The method for the isolation of the glycoprotein in the aggregated and nonaggregated forms was described in the preceding paper (Javaid and Winzler, 1974).

Materials and Methods

All reagents used were of analytical grade. Stock solution of 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride 10% in acetone, specially purified grade) was obtained from Pierce Chemical Co. Marker dansyl amino acids were obtained from Sigma Chemical Co. Dansylglycine, dansylglucosamine, dansylgalactosamine, and dansylamide were prepared according to Morse and Horecker (1966). Precoated silica gel thin-layer chromatographic plates (20 × 20 cm, glass, Batch 70313503) were from E. Merck, whereas precoated MN-Polygram Polyamid-6 plastic sheets (20 × 20 cm) were obtained from Brinkman Instruments, Inc. Ribonuclease and insulin from Sigma Chemical Co. were used as standard proteins.

Ultracentrifugation. The lipophilic fragment (1.25 mg/ml of 0.05 M phosphate buffer (pH 8.2)) was centrifuged at 32,000 rpm in a Beckman Model E analytical ultracentrifuge. Partial specific volume was calculated from the composition of the peptide as described by Schachman (1957).

NH_2-Terminal. Amino end group analysis was carried out by the dansylation procedure of Gray (1967) using 0.5–1 mg of the material dissolved in 0.5 ml of 0.5% sodium dodecyl sulfate. Dansyl amino acids were separated by thin-layer ascending chromatography and visualized under ultraviolet light in the dark.

Analytical Methods. Total neutral sugars were determined by the phenol–sulfuric acid method of Dubois *et al.* (1956). Individual neutral sugars were quantitated by gas chromatography as described by Lehnhardt and Winzler (1968). Hexosamines were determined either by the colorimetric method of Good and Bessman (1964) or by the amino acid analyzer using the borate buffer system as described by Weber and Winzler (1969). Sialic acid was determined by the thiobarbituric acid method of 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). Total proteins were determined by the colorimetric method of Lowry *et al.* (1951) using albumin as the standard.

Trypsin Digestion of the Glycoprotein. Trypsin digestion of the glycoprotein (10 mg/ml) was carried out for 24 hr at 37° in 0.05 M Tris-HCl buffer (pH 8) containing 11.5 mM $CaCl_2$ and crystalline trypsin (1:50 trypsin to substrate ratio); 1–2 drops of toluene was added to prevent bacterial growth. After 24-hr incubation the digestion was stopped by acidification of the reaction mixture to pH 4.5 with 1 N acetic acid. All the insoluble material was collected by centrifugation. The sediment or "lipophilic fragment" was then washed with 0.1 N acetic acid and the washings were combined with the first supernatant, dialyzed against distilled water, and lyophilized as the soluble sialoglycopeptide.

Results

Trypsin digestion of the aggregated glycoprotein resulted in the formation of a flocculant precipitate in 8–10 hr of diges-

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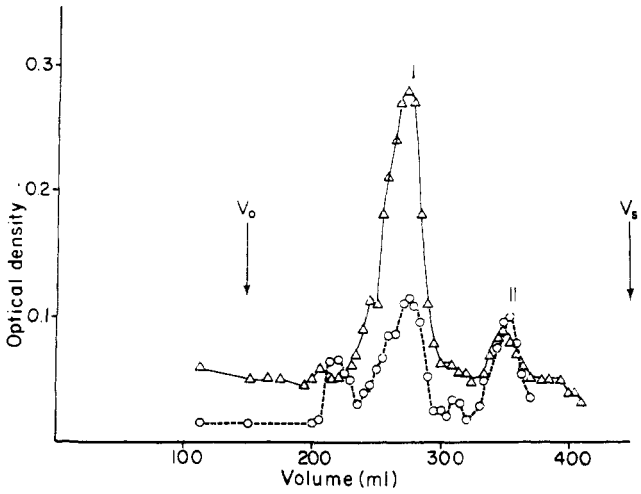

FIGURE 1: Sephadex G-100 chromatography of trypsin soluble fraction; 10 mg of the lyophilized material obtained from the trypsin digestion of the aggregated glycoproteins was loaded on the column (2.5 × 85 cm) in 3 ml of 0.1 N acetic acid and eluted with the same buffer; 5-ml fractions were collected and analyzed for proteins (OD_{280}, O- - -O) and carbohydrate (OD_{490}, Δ—Δ).

tion. After 24 hr of digestion 20–25% of the glycoprotein precipitated as the "lipophilic fragment." Recovery of the soluble fraction as nondialyzable glycopeptides was 60–65%. Increasing the trypsin to substrate ratio did not increase the yield of either fraction. Likewise, treatment with additional trypsin and incubating for further 24 hr did not affect the amount of soluble glycopeptide.

Purification of the Sialoglycopeptide. The sialoglycopeptide was chromatographed on Sephadex G-100 as indicated in Figure 1. Peak I was further purified by DEAE-cellulose chromatography (formate form) using the procedure of Winzler *et al.* (1967) to determine whether the major glycopeptide obtained from the isolated glycoprotein was similar to the major glycopeptide obtained from intact erythrocytes. It emerged as a single carbohydrate rich fraction in the volume identical with that of the trypsin fragment from intact cells.

Physicochemical Properties of the Sialoglycopeptide. More than 80% of the carbohydrates of the aggregated glycoprotein were recovered in the purified sialoglycopeptide. Proteins accounted for about 24% of the glycopeptide and total carbohydrates accounted for 76% if the glycopeptide (Table I). It possessed both M and N blood group activities but did not inhibit the agglutination of human erythrocytes by influenza virus PR_8. The amino acid composition of the glycopeptide is given in Table II. It was enriched in hydroxy amino acids whereas

TABLE I: Chemical Composition of the Soluble Sialoglycopeptide.

	g/100 g[a]
Fucose	1.5
Mannose	3.8
Galactose	18.1
N-Acetylgalactosamine	11.0
N-Acetylglucosamine	5.8
Sialic acid	37.2
Proteins	23.9

[a] Average of two determinations.

TABLE II: Amino Acid Composition of the Sialoglycopeptide from Isolated Erythrocyte Membrane Glycoprotein and Intact Erythrocytes.

	Mol %	
Amino Acid	Glycopeptide from Isolated Glycoprotein	Glycopeptide from Pooled Erythrocytes[a]
Lysine	3.6	4.2
Histidine	4.8	4.3
Arginine	2.9	2.2
Aspartic acid	7.2	9.8
Threonine	22.4	23.4
Serine	22.9	21.7
Glutamic acid	7.1	7.4
Proline	4.2	5.1
Glycine	3.8	2.6
Alanine	5.6	6.4
$^1/_2$-Cystine		
Valine	5.5	5.8
Methionine	2.1	
Isoleucine	2.7	2.9
Tyrosine	0.5	0.2
Phenylalanine		

[a] Calculated from the data of Winzler *et al.* (1967).

the amount of branched lipophilic amino acids and aromatic amino acids was reduced as compared to the glycoprotein from which it was isolated (Javaid and Winzler, 1974). Serine and threonine accounted for almost half of the total amino acids. The hydrophobic amino acids glycine, alanine, valine, isoleucine, leucine, and phenylalanine represented only 20% of the total amino acids. The amino terminal was blocked.

Sedimentation equilibrium studies of the sialoglycopeptide gave a number average molecular weight of 9344. The weight average molecular weight was found to be 10,524. These values are in excellent agreement with those of Winzler *et al.* (1967) who found a molecular weight of 10,000 for the major glycopeptide obtained from erythrocytes. On a calibrated Sephadex G-100 column the glycopeptide eluted at a volume corresponding to an apparent molecular weight of 41,000 which corresponds to a Stoke's radius of 25.5 Å. Using a molecular weight of 10,000 the frictional ratio, f/f_0, was calculated to be 3.95 indicating that the sialoglycopeptide was an asymmetric or rod-shaped molecule.

Characterization of the "Lipophilic Fragment" Obtained from Aggregated Glycoprotein. The insoluble "lipophilic fragment" isolated by the trypsin digestion of the aggregaged glycoprotein did not dissolve in any of the aqueous buffers ranging from pH 4.5 to 8.5. It was also insoluble in common lipid solvents such as acetone, methanol, butanol, and chloroform. The composition of this lipophilic fragment is given in Table III. Protein accounted for 80% of the peptide. It also contained 4–5% of chloroform–methanol (2:1, v/v) extractable material. Total carbohydrates accounted for 8–12% of the precipitated material. The most striking observation concerning the amino acid composition was the enrichment of hydrophobic amino acids and decrease in hydroxy amino acids relative to the soluble sialoglycopeptide (Table IV).

The amino end group analysis was used to ascertain the amount of heterogeneity of the lipophilic fragment obtained

TABLE III: Chemical Composition of the "Lipophilic Fragment."

	g/100 g[a]	
	I[b]	II[c]
Protein	80.4	98.4
Hexoses	4.1	0
Sialic acid	3.0	0
Hexosamines	5.2	0
Lipid	4.7	0

[a] Average of three determinations. [b] Lipophilic fragment obtained from the aggregated glycoprotein. [c] Lipophilic fragment obtained from the nonaggregated glycoprotein.

from the aggregated glycoprotein. The R_F values were determined in two different solvent systems. In chloroform-*tert*-amyl alcohol-acetic acid (140:60:1) system the insoluble peptide gave one major spot corresponding to isoleucine, leucine, or valine. There was also one minor spot. The benzene-pyridine-acetic acid (40:10:1) chromatographic system gave one major spot corresponding to leucine or valine with two minor spots. Finally the dansyl derivatives were separated in a two-dimensional chromatographic system. The plates were first developed in chlorobenzene-acetic acid (9:1), dried in air for 2 hr, and then developed in water-formic acid (200:3). The insoluble peptide gave one major spot corresponding to leucine. There was also one minor spot with an R_F close to ϵ-lysine. These results showed that there are small amounts of heterogeneity in the insoluble glycopeptide with respect to the number of peptide chains and that the major peptide had leucine as the NH_2-terminal amino acid.

TABLE IV: Amino Acid Composition of the "Lipophilic Fragment."

	Moles %[a]	
Amino Acid	I[b]	II[c]
Lysine	1.9	2.1
Histidine	3.1	2.6
Arginine	3.2	3.1
Aspartic acid	4.2	4.2
Threonine	7.1	6.8
Serine	7.6	7.2
Glutamic acid	7.4	8.2
Proline	4.5	4.5
Glycine	11.9	11.8
Alanine	7.4	7.1
$^1/_2$-Cystine		
Valine	8.4	9.1
Methionine	1.5	
Isoleucine	13.2	14.4
Leucine	12.1	11.6
Tyrosine	2.0	1.7
Phenylalanine	5.7	5.3

[a] Samples in duplicate were hydrolyzed for 24, 48, and 72 hr. The values represent the average of the two highest determinations for each amino acid. [b] Lipophilic fragment obtained from the aggregated glycoprotein. [c] Lipophilic fragment obtained from the nonaggregated glycoprotein.

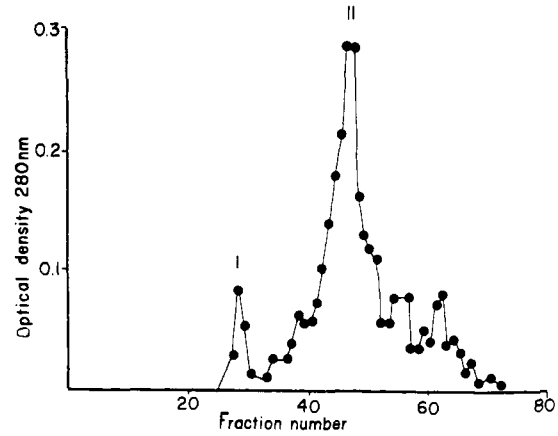

FIGURE 2: Sephadex G-50 chromatography of the "lipophilic fragment." The sample (5 ml) was loaded on the column (2.5 × 85 cm) and 7-ml fractions were collected. The fractions marked as peak II were pooled, concentrated by diaflofiltration, and rechromatographed on the same column.

Characterization of the "Lipophilic Fragment" Obtained from Nonaggregated Glycoprotein. In contrast to the aggregated glycoprotein the trypsin digestion of the nonaggregated glycoprotein did not give a precipitate until the pH of the digestion mixture was lowered to 4.5. Also, the precipitate redissolved in 0.05 M Tris-HCl (pH 8) with vigorous shaking. Figure 2 shows the elution pattern of the dissolved precipitate. Peak II was concentrated and upon rechromatography on the same column gave a symmetrical peak. The purified lipophilic fragment contained no detectable sugars. In this respect it differed from the lipophilic fragment obtained from the aggregated glycoprotein. Protein accounted for more than 98% of the material (Table III). The amino acid composition of the purified lipophilic fragment was very similar to the insoluble fragment obtained from the aggregated glycoprotein (Table IV). Glycine, isoleucine, and leucine are highest in both cases.

In the amino end determination the lipophilic fragment obtained from the nonaggregated glycoprotein gave a single dansyl amino acid spot corresponding to leucine. This also suggested that this lipophilic fragment was a homogeneous peptide as was indicated by gel filtration. The apparent molecular weight of the purified lipophilic fragment calculated by calibration of the Sephadex G-50 column was found to be 7500. The average value for the molecular weight determined by sedimentation equilibrium centrifugation was 8135. Minimum molecular weight calculated from methionine and lysine was 7500. These values were in good agreement with that determined by gel filtration.

Discussion

The trypsin digestion of the aggregated glycoprotein resulted in a soluble sialoglycopeptide and an insoluble "lipophilic fragment." More that 75% of the glycoprotein carbohydrates were present in the soluble fraction of the trypsin digest. A major glycopeptide of molecular weight 10,000 was isolated by Sephadex G-100 chromatography of the soluble fraction. The amino terminal of the sialoglycopeptide was blocked and did not react with 1-dimethylaminonaphthalene-5-sulfonyl chloride. It must, therefore, have been the amino terminal of the erythrocyte membrane glycoprotein since trypsin hydrolyzes the bond involving carboxyl group of lysine or arginine. This glycopeptide was found to be similar in properties to the glycopeptide isolated by Winzler *et al.* (1967) from the intact eryth-

rocytes by trypsin digestion. These results would suggest that the sialoglycopeptide obtained from the isolated glycoprotein represents that part of the glycoprotein which is available to trypsin attack in the outer enviroment of the intact erythrocytes.

The precipitate formed on treatment of the aggregated glycoprotein with trypsin was impoverished in its carbohydrate content and was enriched in lipophilic amino acids. Hydroxy amino acids were very low compared to the soluble glycopeptide. Further characterization of this precipitate was hampered by its limited solubility. It was heterogeneous as determined by NH_2-terminal analysis and sedimentation equilibrium studies. Recently, Lisowska and Jeanloz (1973) have also reported that the proteolytic digestion of human erythrocyte membrane glycoprotein resulted in a carbohydrate rich soluble fraction and an insoluble fraction to be heterogeneous by gel electrophoresis in the presence of detergent.

The treatment of the nonaggregated glycoprotein with trypsin, however, gave a precipitate only on subsequent lowering of the pH of the reaction mixture. Unlike the precipitate from the aggregated glycoprotein this insoluble peptide dissolved in Tris-HCl buffer. Purification of the dissolved lipophilic fragment on Sephadex G-50 gave a material which was homogeneous as determined by the amino end terminal analysis and the sedimentation equilibrium studies. This material had the amino acid composition very similar to the precipitate obtained from the trypsin digestion of the aggregated glycoprotein. However, the lipophilic fragment obtained from the aggregated glycoprotein had up to 4% neutral sugars whereas the purified lipophilic fragment obtained from nonaggregated glycoprotein had no detectable sugars. It is possible that the carbohydrate in the lipophilic fragment from aggregated glycoprotein comes from carbohydrate containing components coprecipitated with the lipophilic fragment. The fact that the NH_2-terminal amino acid in the lipophilic fragment obtained from the nonaggregated glycoprotein and the major NH_2-terminal amino acid in the lipophilic fragment from aggregated glycoprotein was leucine suggested that the same peptide bond is hydrolyzed in the two glycoprotein preparations.

From these results it was concluded that the lipophilic fragment obtained from the nonaggregated glycoprotein and the major peptide in insoluble fraction from aggregated glycoprotein are identical, and represent that part of the human erythrocyte membrane glycoprotein which presumably interacts with the lipid trilaminar portion of the membrane. These results also supported the concept that the human erythrocyte membrane glycoprotein is asymmetric with respect to the distribution of carbohydrates along the polypeptide chain. The portion of the polypeptide chain containing most of the carbohydrates is rich in hydroxy amino acids while the portion which presumably interacts with the lipids inside the membrane was rich in lipophilic amino acids. The similarities of the soluble glycopeptide from the isolated glycoprotein with that glycopeptide from the intact erythrocytes suggested that most of the carbohydrates of the glycoprotein were available in the outer environment of the cells. Since the amino terminal in the glycoprotein and the soluble glycopeptide obtained from the glycoprotein was blocked, it appears that the carboxyl terminal of the glycoprotein was blocked, it appears that the carboxyl terminal of the glycoprotein is within the membrane. These findings support the proposed model of Morawiecki (1964) later adapted by Winzler (1969) for the association of the glycoproteins to the cell membranes.

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