

The Molecular Weight of the Major Glycoprotein from the Human Erythrocyte Membrane

(anomalous binding/sodium dodecyl sulfate)

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ABSTRACT The molecular weight of the major glycoprotein from the human erythrocyte membrane is 29,000, of which 55% is carbohydrate and 45% is protein. The binding of sodium dodecyl sulfate to this glycoprotein is anomalous when compared to water soluble proteins and leads to migration rates in sodium dodecyl sulfate-polyacrylamide gels that cannot be interpreted in terms of molecular weight. Anomalous sodium dodecyl sulfate binding may be a general characteristic of many intrinsic membrane proteins even if they are not glycoproteins, and such proteins are likely to have mobilities in sodium dodecyl sulfate-gel electrophoresis that do not correspond to the mobilities of water soluble proteins of identical molecular weight.

The major glycoprotein from human erythrocyte membranes has been the subject of a large number of investigations since its description by Kathan *et al.* (1) and by Winzler (2). It has been implicated as a trans-membrane protein by a variety of techniques (see refs. 3 and 4 for recent reviews) and partial sequences of the polypeptide moiety have been reported (5-7).

A wide variety of estimates of the molecular weight of the glycoprotein have appeared in the literature. Springer *et al.* (8) reported aggregate molecular weights in aqueous buffer of about 500,000-600,000. Attempts to determine the monomer molecular weight in the presence of detergent (9-11) on sodium dodecyl sulfate (NaDodSO₄)-polyacrylamide gels have shown that the migration rate depends on the acrylamide concentration, and extrapolation techniques have been used to estimate a molecular weight of 55,000. Banker and Cotman (11) have clearly stated the errors inherent in such procedures, and cautioned against placing too much credence in their own value for the glycoprotein molecular weight of 53,000. Several authors (12-14) have reported sedimentation equilibrium data for the glycoprotein in the presence of detergent, but in all cases the investigators did not measure or correct for detergent binding, thus invalidating the result.

We have recently published a detailed discussion of the rigorous determination of molecular weights of proteins in the presence of detergents (15). If the binding to the protein (covalent or noncovalent) of all ligands present and the partial specific volumes are known, sedimentation equilibrium then gives the correct molecular weight for the protein in a multi-component system. We have used this method to determine the molecular weight of the major glycoprotein from the human erythrocyte membrane.

EXPERIMENTAL PROCEDURES

[*S]NaDodSO₄ was purchased from Amersham Searle (specific activity 35 mCi/g) and was diluted with unlabeled Na-

DodSO₄ from Schwarz/Mann. [¹⁴C]NaDodSO₄ was synthesized by Dr. William Stone in this laboratory. Radioactivity was determined in a Beckman LS-100 scintillation counter using Bray's solution (16). NaDodSO₄ concentrations were also assayed by the methylene blue extraction procedure which we have described in detail elsewhere (17).

Protein concentrations were routinely measured by the method of Lowry *et al.* (18) using bovine serum albumin as a standard. Calibration of this procedure was accomplished by quantitative amino-acid analysis, and all Lowry determinations were increased by 18% which was the difference between the true protein weight and the Lowry value for the glycoprotein.

Amino acid and amino sugar determinations were done on a Beckman Automatic Amino Acid Analyzer. Internal standards of *L*-2-amino guanido propionic acid for the short column and norleucine for the long column were included in all analyses. Amino sugars were determined by 10-, 18-, and 24-hr hydrolyses. Amino acids were determined by 24-, 48-, and 96-hr hydrolyses.

Hexose was determined by the orcinol method of Winzler (19) and the anthrone method (20) using galactose as a standard. Sialic acid was measured by the method of Svennerholm (21) using *N*-acetyl neuraminic acid as a standard.

Human erythrocyte ghosts were prepared as described previously (22). Spectrin was removed by the procedure of Trayer and Reynolds (22). The spectrin depleted ghosts were suspended in 10⁻³ M NaN₃, 10⁻² M Tris·HCl (pH 7.4) at a concentration of 2 mg of membrane protein per ml, and the glycoprotein extracted by the procedure of Hamaguchi and Cleve (10).

The aqueous phase containing the glycoprotein was concentrated to about 4 mg/ml and sufficient NaDodSO₄ was added to bring the total NaDodSO₄ concentration to 10%. This solution was heated for 10-20 min at 80° and eluted from a Sepharose 4B column previously equilibrated with NaDodSO₄. This step in the purification procedure separates the major glycoprotein from two minor glycoprotein components and from glycolipid. Pooled column fractions were dialyzed exhaustively against 5 × 10⁻³ M phosphate buffer, pH 7.2, 10⁻³ M NaN₃ until all NaDodSO₄ had been removed (about 4-5 days).

NaDodSO₄ binding was determined by equilibrium dialysis, column chromatography, and sedimentation equilibrium (23).

Molecular weights were determined in a Beckman model E ultracentrifuge equipped with a photoelectric scanner.

Partial specific volumes for the carbohydrates were taken from Gibbons (24), for the amino acids from Cohn and Edsall

Abbreviation: NaDodSO₄, sodium dodecyl sulfate.

TABLE 1. Chemical composition of glycoprotein

	g/g Polypeptide	Weight %
Polypeptide*	1.0	45.1
Sialic Acid	0.56 ± 0.03	25.2 ± 1.5
Hexose	0.27 ± 0.02	12.2 ± 1.0
Hexoseamine	0.36 ± 0.01	16.2 ± 0.6
<i>N</i> -acetylgalactoseamine	0.26 ± 0.01	11.7 ± 0.2
<i>N</i> -acetylglucoseamine	0.095 ± 0.004	4.3 ± 0.2
Fucose†	0.029	1.3

* Quantitative amino-acid analysis.

† Fukuda and Osawa (12).

(25), and for sodium dodecyl sulfate from Tanford *et al.* (15). Partial specific volume of the glycoprotein was also measured directly in a Paar Densimeter.

RESULTS

Table 1 shows the chemical composition of the major glycoprotein from the human erythrocyte membrane, and Table 2 presents the amino-acid composition data. These results are in substantial agreement with analytical data obtained by other laboratories for this glycoprotein (2, 12, 26).

The final column of Table 2 gives the minimal molecular weight of the polypeptide chain based on the presence of two methionine residues per chain, which in turn is derived from the reported presence of three independent fragments after hydrolysis with cyanogen bromide (7). It should be noted that

TABLE 2. Amino-acid composition of glycoprotein

Amino acid	Moles*	Moles
	Mole alanine	7 moles alanine
Lys	0.74 ± 0.08	5.2 (5)
His	0.73 ± 0.06	5.1 (5)
Arg	0.89 ± 0.05	6.2 (6)
Asp	1.24 ± 0.07	8.7 (9)
Thr†	2.14 ± 0.14	15.0 (15)
Ser‡	2.83 ± 0.18	19.8 (20)
Glu	2.16 ± 0.09	15.1 (15)
Pro	1.44 ± 0.07	10.1 (10)
Gly	0.89 ± 0.10	6.2 (6)
Ala	1.00	7.0 (7)
Val	1.43 ± 0.14	10.0 (10)
Met	0.24 ± 0.01	1.7 (2)
Ile	1.01 ± 0.19	7.1 (7)
Leu	0.98 ± 0.08	6.9 (7)
Tyr	0.56 ± 0.04	3.9 (4)
Phe	0.28 ± 0.02	2.0 (2)
Trp§	0.04 ± 0.01	0 (0)
Total		130
Molecular Weight		14,093

* Average of three 24-hr hydrolyses from one preparation and duplicates of 24-, 48-, and 96-hr hydrolyses from a second preparation.

† Corrected for 3% decrease/24-hr hydrolysis.

‡ Corrected for 10% decrease/24-hr hydrolysis.

§ Determined by the method of Edelhoch (1967) (29). The protein is highly aggregated in 6 M guanidine hydrochloride and the small amount of absorbance measured is undoubtedly due to difficulties in correcting for light scattering.

a minimal molecular weight of 7000 would be obtained if only a single methionine were assumed to be present, but several amino acids would then be present in nonintegral molar ratios.

The glycoprotein aggregates in aqueous buffer and in 6 M guanidine hydrochloride. However, it is dissociated into a homogeneous species when amphiphilic ligands such as anionic detergents are bound. Fig. 1 shows the binding isotherms for sodium dodecyl sulfate to the glycoprotein at two different ionic strengths. It is significant that very little detergent is bound below the critical micelle concentration and further that the saturation levels of binding are much larger than those observed with water soluble proteins. The complete binding data with a variety of detergents will be presented and discussed in detail in another communication. For the present purposes, we are concerned with the amount of NaDodSO₄ bound at a given equilibrium concentration in order to obtain the correct molecular weight for the glycoprotein.

We have previously described in detail the rigorous procedure for obtaining molecular weights in the presence of bound detergent (15). In sedimentation equilibrium the following equation applies

$$M = \frac{2RT}{\omega^2} \left(\frac{d \ln c}{dr^2} \right) \frac{1}{(1 - \phi' \rho)}$$

where $d \ln c/dr^2$ is the quantity obtained directly from the experiment at a rotor speed ω , ρ is the solvent density and ϕ' the effective partial specific volume. It is an excellent approximation (15) to divide $(1 - \phi' \rho)$ into independent contributions from the glycoprotein (subscript GP) and from the bound detergent. Where $\delta_{\text{NaDodSO}_4}$ is the amount of detergent bound per gram of protein,

$$(1 - \phi' \rho) = (1 - \bar{v}_{\text{GP}} \rho) + \delta_{\text{NaDodSO}_4} (1 - \bar{v}_{\text{NaDodSO}_4} \rho)$$

We have measured \bar{v}_{GP} directly by densimetry, obtaining a value of 0.68 ± 0.01 cm³/g. The calculated value, based on the amino acid and carbohydrate composition is 0.67 cm³/g. The value of $\bar{v}_{\text{NaDodSO}_4}$ has been previously reported as 0.87 cm³/g (15).

Table 3 presents the molecular weight of the glycoprotein and the polypeptide chain determined in the ultracentrifuge at two different ionic strengths. The agreement between sedimentation equilibrium data and molecular weight determined from a measurement of R_s and S is very good. There is also good agreement between the results obtained at two different salt concentrations where the cm_c (critical micelle concentration) of the detergent and the characteristic binding isotherms (Fig. 1) are very different. It should be noted that at detergent binding ratios less than 1.8 g of NaDodSO₄ per g of glycoprotein, the glycoprotein appears to be aggregated and is heterogeneous in apparent molecular weight. At all binding ratios greater than 1.8 g of NaDodSO₄ per g of glycoprotein, the complex is homogeneous with respect to molecular weight. Our values of 13,000 for the polypeptide component and 29,000 for the entire glycoprotein are in agreement with amino-acid data showing 14,000 for the polypeptide if it contains two methionine residues per chain.

DISCUSSION

In the presence of high concentrations of NaDodSO₄ above the critical micelle concentration, the glycoprotein from

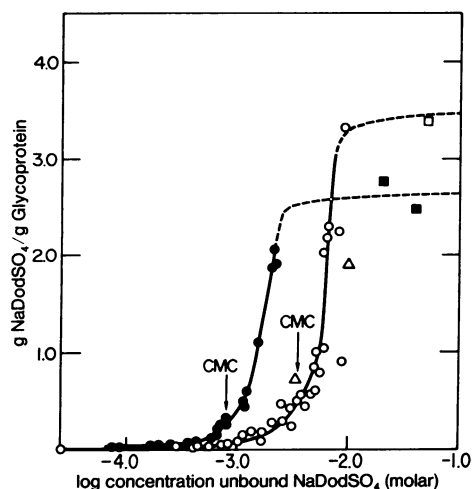


FIG. 1. Binding of NaDodSO₄ to the major glycoprotein ●, in the presence of 0.261 M salt, equilibrium dialysis measurements; ■, in the presence of 0.261 M salt, sedimentation equilibrium measurements; ○, in the presence of 0.027 M salt, equilibrium dialysis measurements; △, in the presence of 0.027 M salt, column chromatography measurements; □, in the presence of 0.027 M salt, sedimentation equilibrium measurements. Measurements by sedimentation equilibrium were based on the assumption that no further dissociation of the protein occurs above the highest concentration at which direct binding measurements were possible. The critical micelle concentration (CMC) at the two salt concentrations used is shown.

human erythrocyte membranes dissociates to a 29,000 molecular weight species. One does not expect *a priori* that all proteins will be dissociated to a monomer by this detergent since strong inter-molecular forces between protein molecules can stabilize a self-aggregated state which is of lower free energy. However, the amino-acid analysis and the observation of three cyanogen bromide fragments implies that the glycoprotein is monomeric in NaDodSO₄.

A more important point to be made, however, is that any molecular weight determined by NaDodSO₄ gel electrophoresis for this glycoprotein is suspect. Since this technique demands that (i) the g of NaDodSO₄ bound per g of protein must be the same for the glycoprotein as for standard water soluble proteins and (ii) the relationship between the hydrodynamic size of the glycoprotein and its molecular weight must be identical to that relationship for water soluble proteins, any agreement between molecular weights obtained on NaDodSO₄-gels and the true molecular weight is purely fortuitous.

The binding of detergents to intrinsic membrane proteins is frequently anomalous (27, 28). The presence of large hydrophobic regions on a protein can lead to mixed micelle formation which manifests itself as micellar binding above the critical micelle concentration. This phenomenon has never been observed with water soluble proteins which are used as standards in the empirical molecular weight method of NaDodSO₄-gel electrophoresis.

Glycoproteins present special problems since the presence of large amounts of carbohydrate can affect both detergent binding and the hydrodynamic behavior of the detergent-protein complex. The precise manner by which so much NaDodSO₄ is bound to this particular protein is unknown and is being investigated. The saturation level seen in Fig. 1 (2.5–3.4

TABLE 3. Molecular weight of the glycoprotein in NaDodSO₄ by sedimentation equilibrium*

Conc. added salt (molar)	Conc. unbound NaDodSO ₄ (molar)	$\delta_{\text{NaDodSO}_4}$ g/g of glycoprotein	Molecular weight	
			Poly-peptide	Glyco-protein
0.027	0.01	1.9	12,200†	27,300†
0.027	0.0048	0.59	14,200	31,500‡
0.26	0.0022	1.9	12,600	27,900
0.26	0.0016	0.85	14,300	31,800‡

By sedimentation velocity and stokes radius

Conc. added salt (molar)	Conc. unbound NaDodSO ₄ (molar)	R_s Å	$s_{20,w}$	Molecular weight	
				Poly-peptide	Glyco-protein
0.027	0.01	46 ± 2§	3.23¶	13,000	28,900

* All sedimentation equilibrium determinations were carried out at several rotor speeds (12,000–24,000 rpm).

† Slight curvature was noted in this experiment which may result from heterogeneity in the carbohydrate portion of the glycoprotein or heterogeneity in detergent binding.

‡ Molecular weights determined at the top of the cell (meniscus value). Sedimentation equilibrium plots were curved due to aggregation at these relatively low values of NaDodSO₄ binding.

§ R_s determined by gel chromatography on Sepharose 4B.

¶ $s_{20,w}$ determined at 0.40 mg/ml.

g of NaDodSO₄ per g of glycoprotein) would correspond to more than 5–7 g of NaDodSO₄ per g of protein if the detergent is bound *solely* to the polypeptide portion.

The most important point of this paper is that anomalous binding or the presence of carbohydrate do not interfere with the determination, in a rigorous manner, of a correct molecular weight by sedimentation equilibrium.

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- Kathan, R. H., Winzler, R. J. & Johnson, C. A. (1961) *J. Exp. Med.* **113**, 37–45.
- Winzler, R. J. (1969) in *Red Cell Membrane, Structure and Function*, eds. Jamieson, G. A. & Greenwalt, T. J. (J. B. Lippincott, Co., Philadelphia, Pa.), pp. 157–171.
- Reynolds, J. A. (1973) *Fed. Proc.* **32**, 2034–2038.
- Marchesi, V. T., Jackson, R. L., Segrest, J. P. & Kahane, I. (1973) *Fed. Proc.* **32**, 1833–1837.
- Segrest, J. P., Jackson, R. L., Marchesi, V. T., Guyer, R. B. & Terry, W. (1972) *Biochem. Biophys. Res. Commun.* **49**, 964–969.
- Jackson, R. L., Segrest, J. P., Kahane, I. & Marchesi, V. T. (1973) *Biochemistry* **12**, 3131–3138.
- Segrest, J. P., Kahane, I., Jackson, R. L. & Marchesi, V. T. (1973) *Arch. Biochem. Biophys.* **155**, 167–183.
- Springer, G. F., Nagai, Y. & Tegtmeier, H. (1966) *Biochemistry* **5**, 3254–3271.
- Segrest, J. P., Jackson, R. L., Andrews, E. P. & Marchesi, V. T. (1971) *Biochem. Biophys. Res. Commun.* **44**, 390–395.
- Hamaguchi, H. & Cleve, H. (1972) *Biochim. Biophys. Acta* **278**, 271–280.
- Banker, G. A. & Cotman, C. W. (1972) *J. Biol. Chem.* **247**, 5856–5861.
- Fukuda, M. & Osawa, T. (1973) *J. Biol. Chem.* **248**, 5100–5106.

13. Zuilichovsky, B., Gallop, P. M. & Blumenfeld, O. O. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1234-1243.
14. Janado, M., Azuma, J. & Onodera, K. (1973) *J. Biochem.* **74**, 881-887.
15. Tanford, C., Nozaki, Y., Reynolds, J. A. & Makino, S. (1974) *Biochemistry* **13**, 2369-2376.
16. Bray, G. A. (1960) *J. Anal. Biochem. USSR* **1**, 279-282.
17. Ray, A., Reynolds, J. A., Polet, H. & Steinhardt, J. (1966) *Biochemistry* **5**, 2606-2616.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-270.
19. Winzler, R. J. (1955) *Methods Biochem. Anal.* **2**, 279-311.
20. Graff, M. M., Greenspan, E. M., Lehman, I. R. & Holechek, J. J. (1951) *J. Lab. Clin. Med.* **37**, 736-740.
21. Svennerholm, L. (1957) *Biochim. Biophys. Acta*, **24**, 604-611.
22. Reynolds, J. A. & Trayer, H. (1971) *J. Biol. Chem.* **246**, 7337-7342.
23. Steinhardt, J. & Reynolds, J. A. (1969) *Multiple Equilibria in Proteins* (Academic Press, New York).
24. Gibbons, R. A. (1966) in *Glycoproteins*, ed. Gottschalk, A. (Elsevier, Amsterdam.) pp. 29-95.
25. Cohn, E. J. & Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides* (Reinhold Publishing Corp., New York).
26. Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P. & Scott, R. E. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1445-1449.
27. Helenius, A. & Simons, K. (1972) *J. Biol. Chem.* **247**, 3656-3661.
28. Robinson, N. C., Nozaki, Y. & Tanford, C. (1974) *Fed. Proc.* **33**, 826.
29. Edelhoch, H. (1967) *Biochemistry* **6**, 1948-1954.