HUMAN ERYTHROCYTE MEMBRANE SIALOGLYCOPROTEINS: A STUDY OF INTERCONVERSION

Jean K. Tuech and Martin Morrison

Laboratories of Biochemistry

St. Jude Children's Research Hospital

Memphis, Tennessee 38101

Received May 20,1974

SUMMARY. The sialoglycoproteins of human erythrocyte membranes can be separated into four molecular weight classes by gel electrophoresis in the presence of sodium dodecyl sulfate. Two of these components participate in an aggregation/disaggregation equilibrium favoring the lower molecular weight form at higher temperature. The two other forms do not participate in this interconversion. Partially purified sialoglycoproteins contain high molecular weight aggregates which involve all species and dissociate at high temperature. Most important is the finding that interconversion does not take place in phosphate buffered gels but does occur on gels employing Tris buffer systems.

Although the glycoproteins of the normal human erythrocyte membrane have been the subject of numerous studies there is still no agreement on their size. Early sedimentation studies suggested that the major glycoprotein of the red cell membrane had a molecular weight of 31,000 (1,2). More recently, Marchesi and his collaborators have concluded on the basis of SDS[1] acrylamide gel electrophoresis that this protein has a molecular weight of 55,000 (3). These workers have investigated the protein structure and presented a partial primary sequence based on this size (4,5). Recently two other groups have questioned the larger molecular weights from SDS acrylamide gel data and presented evidence that this larger molecular weight represents an aggregated form of the glycoprotein (6-8).

It was the object of the present investigation to clarify the

[1]Abbreviations: SDS, sodium dodecyl sulfate; PAS, periodic acid-Schif; TBTS, Tris borate/Tris sulfate.

observation concerning the size of these glycoproteins as determined by electrophoresis in SDS acrylamide gels.

METHODS. Erythrocyte membranes were prepared by the procedure of Dodge *et al* (9) from whole blood collected in citrate phosphate dextrose solution. Sialoglycoproteins of intact erythrocytes were labeled with ^{125}I using lactoperoxidase as previously described (10,11). The sialoglycoproteins were partially purified by the procedure of Hamaguchi and Cleve (12).

The proteins were separated into molecular weight classes on SDS gels using both cylindrical (10 x 0.5 cm) and slab (7.5 x 10 x 0.4 cm) gels. The gels were 10% T, 3.3% C, and 0.1% SDS with a Tris borate/Tris sulfate (TBTS) buffer system, pH 9, described by Miner (13). Samples were solubilized in 1% SDS, 1% 2-mercaptoethanol in dilute gel buffer by heating at 37° for 20-30 min or at 100° for 3 min. Alternatively 0.1M phosphate buffer, pH 7.4, was used for SDS gel electrophoresis with other gel parameters unchanged. Bromphenol blue was used as tracking dye and fluorescent-labeled bovine serum albumin and carbonic anhydrase as internal protein standards.

Glycoproteins were located by staining gels with the periodic acid-Schiff procedure (14) and proteins with Coomassie Brilliant Blue. In order to determine the distribution of iodine label the gels were cut into 2-mm segments and the radioactivity in each slice determined with a gamma spectrometer.

RESULTS AND DISCUSSION. The glycoproteins derived from erythrocyte stroma were separated by the TBTS SDS gels system into a number of molecular weight classes. The relative intensities of the PAS stain in these classes varied with the temperature at which the preparation was treated prior to electrophoresis. Figure 1 illustrates the differences observed. There was a shift in intensity of PAS stain

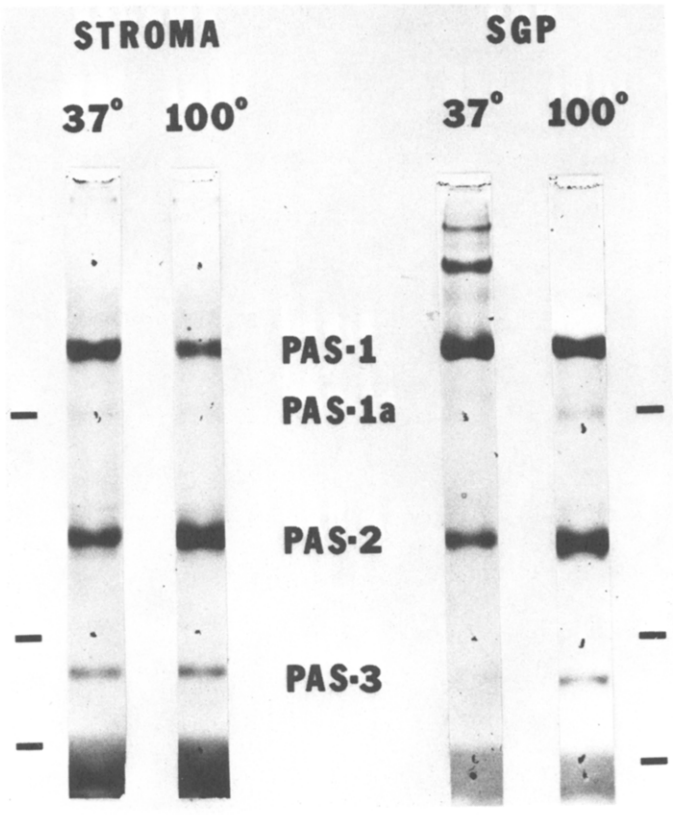

Fig. 1. Sialoglycoproteins from stroma and soluble glycoprotein preparations (SGP) were separated by SDS gel electrophoresis after treatment at 37° or 100° and the gels stained for glycoproteins. Black stab marks and short horizontal lines locate internal standards bovine serum albumin and carbonic anhydrase and the dye front.

from the region of PAS-1 to that of PAS-2 when the glycoprotein was heated at the higher temperature as reported by Marton and Garvin (6). This was seen in both stroma and soluble preparations of glycoprotein but less dramatically in the latter.

Soluble glycoprotein preparations treated at 37° also had several high molecular weight forms which were aggregates involving all species. This indicates that the solubilized glycoproteins aggregate readily. These high molecular weight aggregates were not observed in gel patterns obtained directly from stroma.

In order to investigate the relationship between PAS-1 and PAS-2

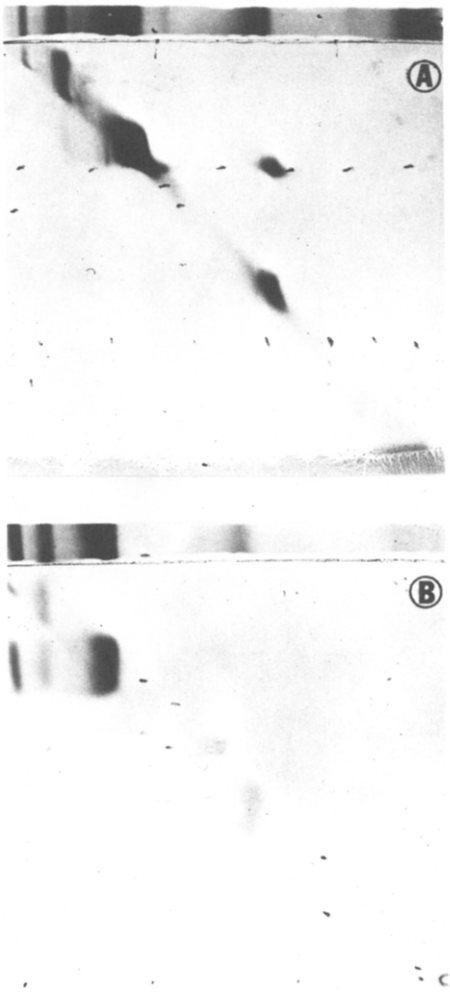

Fig. 2. Two-dimensional electrophoresis of soluble glycoprotein preparations. A portion of the gel was fixed and stained after the first separation and is positioned above the two-dimensional gel for reference. (A) Original sample treated at 100°; repeated in second dimension after 3 hours at 20°. (B) Original sample treated at 37°; repeated in second dimension after 20 hours at 20°.

a two-dimensional electrophoresis procedure was used which did not involve extraction of the protein from the gel. A sample treated at 37° or 100° was electrophoresed in one direction on a slab. A strip from this slab containing all of the separated species was then placed on top of a second slab. After equilibration with solubilizing sample buffer for at least an hour at room temperature electrophoresis

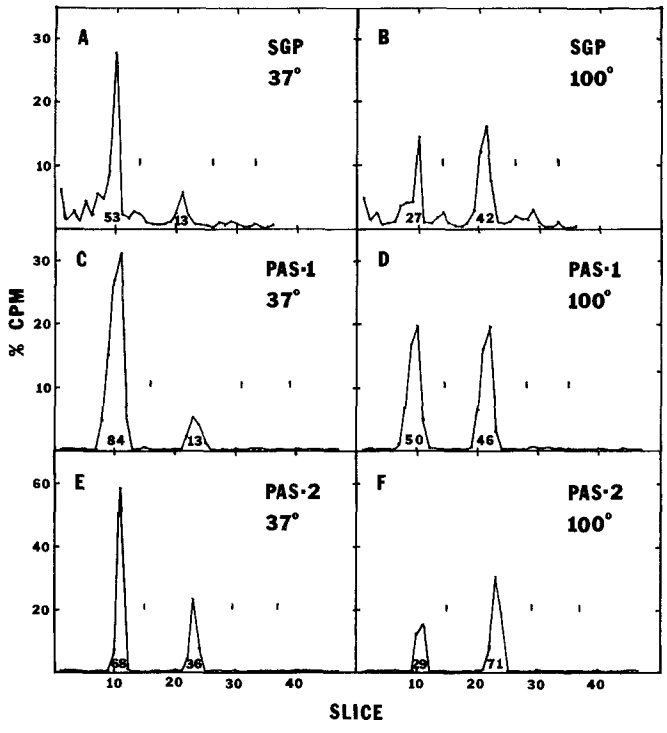

Fig. 3. Distribution of glycoprotein after SDS gel electrophoresis and re-electrophoresis of ^{125}I-labeled glycoprotein. (A) Glycoprotein treated at 37°. (B) Glycoprotein treated at 100°. (C) Slice from PAS-1 region of A treated at 37°. (D) Slice from PAS-1 region of A treated at 100°. (E) Slice from PAS-2 region of B treated at 37°. (F) Slice from PAS-2 region of B treated at 100°. Short vertical lines locate internal protein standards and the dye front. Numbers under curves represent the approximate percent of total counts under the curve.

---

was carried out as usual with the strip containing the sample. In such a system components which are homogeneous should migrate in the second electrophoresis as single entities. The separation of the components of a soluble glycoprotein sample treated at 100° prior to electrophoresis is shown at the top of Fig. 2A. This gel was allowed to stand for 3 hours at 20° and the separation repeated in the second dimension. Most of the glycoproteins migrated as homogeneous entities with the same apparent molecular weights as in the first dimension. Part of PAS-2, however, migrated as PAS-1 indicating an aggregation of material in the Pas-2 region to form the larger molecular weight material.

Figure 2B shows the two-dimensional electrophoretic pattern of a sample treated at 37° before the first electrophoresis. This favored the aggregated forms (see Fig. 1). The highest molecular weight aggregate moved in the second dimension as in the first and also gave rise to the lower molecular weight PAS-1. The next highest molecular weight aggregate on re-electrophoresis gave rise to PAS-1 and PAS-2.

The aggregation of PAS-2 to form PAS-1 was shown clearly by the two-dimensional technique (Fig. 2A). The disaggregation of PAS-1 to PAS-2, however, is not favored at room temperature and did not occur in sufficient quantity to be detected.

In order to make quantitative observations regarding aggregation and disaggregation and to increase the sensitivity of detection ^{125}I-labeled glycoproteins were used. The co-migration of stain and radioactivity was established by cutting and counting PAS-stained gels.

Electrophoresis of labeled glycoprotein after treatment at 37° or 100° showed the same aggregation/disaggregation pattern seen by staining (Fig. 3A,B). Slices from regions of PAS-1 and PAS-2 were treated at 37° or 100° and again subjected to electrophoresis. The results of several typical experiments are shown in Fig. 3C-F. Slices from the PAS-1 region showed radioactivity migrating as both PAS-1 and PAS-2 regardless of the temperature of treatment (Fig. 3C,D). The proportion which migrated as PAS-1 was greater, however, when the slice was treated at 37°. Similarly, PAS-2 material migrated as both forms (Fig. 3E, F). Again, the proportion of PAS-1 was greater after the 37° treatment.

Slices from the PAS-1 and PAS-2 regions of a second electrophoresis gel (Fig. 3D) were electrophoresed a third time. The PAS-1 region still yielded material migrating as PAS-2 indicating that the material at PAS-1 in Fig. 3D was not interconversion-resistant.

The PAS-2 region migrated as both PAS-1 and PAS-2 indicating that whatever the process by which this material had been produced from PAS-1, it was reversible. In separate experiments involving the serial re-electrophoresis of only PAS-1, at the end of four separations all involving treatment at 100° the amount of material still migrating as PAS-1 was reduced to an estimated 6% of that which had migrated as PAS-1 during the first electrophoresis. With each successive re-electrophoresis the percentage of counts shifting to the PAS-2 mobility was greater, indicating that there was no nonparticipating population and also that the equilibrium was concentration-dependent.

From these results it is clear that PAS-1 and PAS-2 have at least some PAS-positive, iodinatable component in common but whether the association is between identical subunits has not been determined. It also seems clear that PAS-1a and PAS-3 are not participants in this equilibrium; we have never detected radioactivity on the PAS-1a or PAS-3 regions after re-electrophoresis of PAS-1 or PAS-2. Treatment at 100° and second electrophoresis of the high molecular weight aggregates showed that they contained all the lower molecular weight forms. The presence of such aggregates in soluble glycoprotein preparations has been previously observed (15, 16). The present results demonstrate their existence and relation to the lower molecular weight forms.

A number of variables which might affect the PAS-1/PAS-2 interconversion such as 4M urea, 1 mM EDTA, 1 mM $CaCl_2$ slow or rapid freezing, and sample pH have been investigated by observing the temperature-associated shift in stain intensities. Nones of these factors appeared to influence the interconversion.

The observation of one factor which does influence the interconversion is highly significant. Interconversion cannot be shown by electrophoresis on SDS gels using the 0.1 M phosphate buffer system. This is

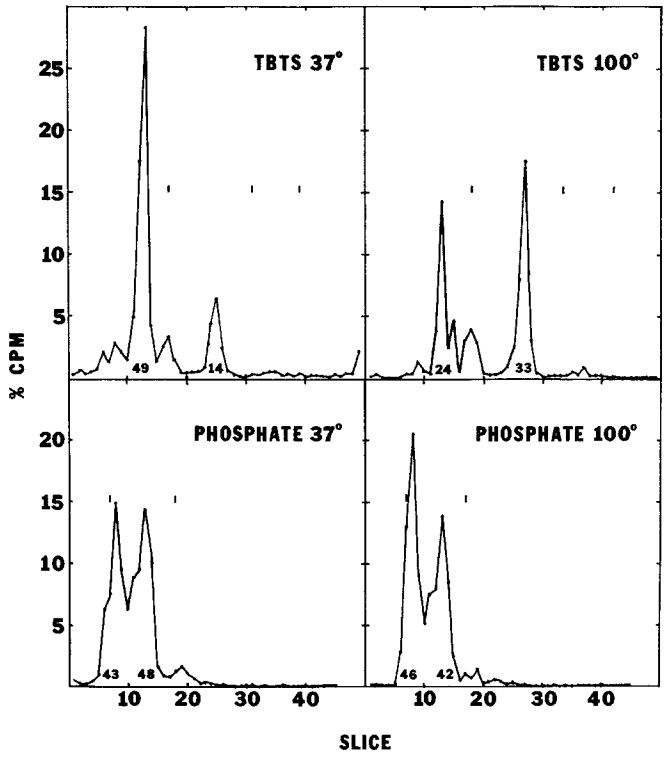

Fig. 4. Distribution of glycoprotein after SDS gel electrophoresis of labeled soluble glycoprotein treated at 37° or 100° and separated on gels buffered with TBTS or phosphate. Short vertical lines locate internal protein standards and the dye front. Numbers under the curves represent the approximate percent of total counts under the curve.

illustrated in Fig. 4. The same labeled glycoprotein preparation was electrophoresed on TBTS gels and phosphate gels after treatment at 37° or 100°. Although the shift was seen clearly on the TBTS gels (upper panels) there was little if any difference in the patterns on the phosphate gels (lower panels).

When PAS-1 and PAS-2 peak slices from TBTS and phosphate gels were re-electrophoresed on the same or the alternate gel type, interconversion occurred in TBTS but not in phosphate gels. PAS-1 to PAS-2 conversion occurred when material from the PAS-1 region of a phosphate gel was electrophoresed on a TBTS gel.

Interconversion has been observed in staining patterns of t o other SDS gel systems: Tris/Tris glycine, pH 8.8, 8.3, and Tris acetate, pH 7.4 (6). The influence of phosphate and Tris buffers on the aggregation/disaggregation equilibrium is under investigation.

ACKNOWLEDGMENTS: This investigation was supported in part by NIH grants GM 15913, CA 13534, and CA 05176. We also acknowledge the technical assistance of Ms. Jan Graham.

REFERENCES

1. Kathan, R.H., Winzler, R.J., and Johnson, C.A. (1961) J. Exp. Med. *113*,37-45
2. Morawiecki, A. (1964) Biochim. Biophys. Acta *83*, 339-347.
3. Segrest, J.P., Jackson, R.L., Andrews, E.P., and Marchesi, V.T. (1971) Biochem. Biophys. Res. Commun. *44*, 390-395.
4. Segrest, J.P., Kahane, I., Jackson, R.L., and Marchesi, V.T. (1973) Arch. Biochem. Biophys. *155*, 167-183.
5. Jackson, R.L., Segrest, J.P., Kahane, I., and Marchesi, V.T. (1973) Biochemistry *12*, 3131-3138.
6. Marton, L.S.G., and Garvin, J.E. (1973) Biochem. Biophys. Res. Commun. *52*, 1457-1462.
7. Azuma, J., Janado, M., and Onodera, K. (1973) J. Biochem. *73*, 1127-1130.
8. Janado, M., Azuma, J., and Onodera, K. (1973) J. Biochem. *74*, 881-887.
9. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) Arch. Biochem. Biophys. *100*, 119-130.
10. Morrison, M., Bayse, G.S., and Webster, R.G. (1971) Immunochemistry *8*, 289-297.
11. Phillips, D., and Morrison, M. (1971) Biochemistry *10*, 1766-1771.
12. Hamaguchi, H., and Cleve, H. (1972) Biochim. Biophys. Acta *278*, 271-280.
13. Miner, G.D. (1973) In: Application Note AN 32A, pp. 39-40, Ortec, Inc.
14. Zacharias, R.J., Zell, T.E., Morrison, J.H., and Woodlock, J.J. (1969) Anal. Biochem. *31*, 148-152.
15. Springer, G.F. (1967) Biochem. Biophys. Res. Commun. *28*, 510-513.
16. Blumenfeld, O.O., Gallop, P.M., Howe, C., and Lee, L.T. (1970) Biochim. Biophys. Acta *211*, 109-123.