The Anomalous Electrophoretic Behavior of the Major Sialoglycoprotein from the Human Erythrocyte*

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The major sialoglycoprotein of the human erythrocyte membrane (glycophorin A) shows a single band in sodium dodecyl sulfate-gel electrophoresis using phosphate buffers and molecular weight homogeneity in the ultracentrifuge. After electrophoresis in Tris buffers, however, two bands (periodic acid-Schiff reagent (PAS)-1 and PAS-2) are obtained. The band of greater apparent molecular weight can be converted to the band of lower apparent molecular weight by incubation of the protein at elevated temperatures before electrophoresis. In this report, we investigate the relative proportions of the two bands as the conditions of protein concentration, ionic strength, sodium dodecyl sulfate concentration, and buffer composition are varied. We also present relative mobility data for the bands at different acrylamide concentrations. We show that the relation between the relative proportions of PAS-1 and PAS-2 and the total protein concentration is quantitatively described by the hypothesis that PAS-1 is a dimer of PAS-2, and that the two species form an equilibrium mixture. The equilibrium is very sensitive to ionic strength and the electrophoretic pattern in different running buffers is also very sensitive to ionic strength. This phenomenon accounts for the various patterns of one or two bands found in the different buffer systems used in the past. We discuss the alternative hypothesis that PAS-1 represents a complex of PAS-2 with an unknown ligand, but for a number of reasons we favor the monomer-dimer hypothesis to account for the electrophoretic heterogeneity. The discrepancy between this hypothesis and the results of sedimentation equilibrium studies remains unresolved.

SDS-polyacrylamide gel electrophoresis has been a powerful method for the analysis of protein mixtures and for the determination of polypeptide molecular weights. The work of Weber and Osborn (1969) created a strong empirical background for the use of this technique for soluble proteins. Fairbanks et al. (1971) introduced a low ionic strength buffer system which gives superior resolution of the polypeptides of biological membranes. When human erythrocyte membranes are subjected to electrophoresis by this method up to four PAS-positive bands are seen and the glycopeptides responsible for these bands have been the focus of intense study. The major sialoglycoprotein has been purified from erythrocyte membranes (Grefrath and Reynolds, 1974; Furthmayr 1975) and its chemical composition and amino acid sequence determined (Tomita and Marchesi, 1975). This protein, designated glycophorin A, is responsible for the PAS-1 and PAS-2 bands seen when whole membranes are electrophoresed. Marton and Garvin (1973) had observed that the relative proportions of PAS-1 and PAS-2 could be altered in favor of the faster running PAS-2 band by heating dissolved membranes before electrophoresis, but this phenomenon was not observed with isolated glycoprotein until Furthmayr and Marchesi (1976) showed that the extent of the conversion of PAS-1 to PAS-2 was inversely dependent on the protein concentration.

It has been recognized for some time that the estimation of molecular weight of glycoproteins by comparison of their electrophoretic mobilities with those of standard proteins can lead to erroneous results. This is especially true in the case of glycophorin, which has 60% carbohydrate by weight (see Segrest et al., 1971). However, a number of investigators have felt able to postulate that the PAS-1 and PAS-2 bands represent dimeric and monomeric forms of the protein (Marton and Garvin, 1973; Slutzky and Ji, 1974; Furthmayr and Marchesi, 1976). We have recently postulated that glycophorin A forms an equilibrium mixture of dimer and monomer when incubated at elevated temperatures in solutions of SDS and that the dissociation constant is greatly increased by carboxymethylation of a methionine residue located in the hydrophobic segment of the polypeptide chain (Silverberg et al., 1976). In contradiction to those conclusions, the sedimentation equilibrium studies of Grefrath and Reynolds (1974) indicated that solutions of the erythrocyte glycoprotein were homogeneous with respect to molecular weight, which value they estimate as 30,000, in close agreement with the value obtained from the sequence data. Also, many workers have found that when glycophorin is electrophoresed using the high ionic strength phosphate buffer system of Weber and Osborn (1969),
only a single band is obtained with an apparent molecular weight of 60,000 to 100,000, depending on the acrylamide concentration of the gel.

Thus, there is confusion in the literature as to whether glycophorin A does or does not associate to dimers in the presence of SDS. In this paper, we have investigated in some detail the electrophoretic behavior of glycophorin A using a variety of incubation and running buffers. In particular, we have addressed the discrepancy between the single band found in one buffer and the two bands found in another and the possible identification of those two bands as monomeric and dimeric forms of the glycoprotein.

The final aim of these investigations is to be able to describe the interaction of intrinsic membrane proteins like glycophorin A with each other and with membrane lipids. The study of self-associating properties in the presence of a synthetic amphiphile is an initial step towards that goal.

**MATERIALS AND METHODS**

Details of the experimental procedures and a more detailed description of the results are found in the miniprint supplement which follows. A synopsis of the results is combined with the discussion below.

**RESULTS AND DISCUSSION**

It was previously found (Furhmayr and Marchesi, 1976; Silverberg et al., 1976) that the fraction (F) of material migrating as PAS-2 was inversely dependent upon the concentration of protein present during incubation at 80° in SDS solution. The value of F obtained from a dilute solution of glycophorin A increases quite slowly with time of incubation to reach a steady value after about 25 min (data not shown), suggesting that an equilibrium mixture is being formed at 80°. If a concentrated solution of protein is brought to equilibrium, the proportions of PAS-1 and PAS-2 are not altered by dilution of the cooled solution before electrophoresis (see Fig. 2, Silverberg et al., 1976). It thus seemed that gel electrophoresis could be used as a quantitative assay of the composition of the equilibrium mixture of PAS-1 and PAS-2 obtained by incubation of glycophorin A at 80° in SDS solutions. On this assumption, we examined the consequences of altering protein concentration, ionic strength, and SDS concentration of the incubation solution.

If the state of the protein solution after 30 min at 80° represents true thermodynamic equilibrium between monomer and dimer, the protein concentration and F are related by the equation:

\[ 2F/(1 - F) = K_c M_c/c \]

where F is the fraction of material migrating as PAS-2, \( K_c \) is the dissociation constant (=\([G]_1/[G]_2\)), \( M_c \) is the molecular weight of the monomer, and c is the protein concentration in milligrams per ml.

The data of Fig. 1 show that this relation is satisfied by glycophorin A over a concentration range 0.05 to 2.0 mg/ml of protein. The apparent \( M_c \) was \( 2 \times 10^5 \) and was increased 4-fold in the presence of 8 mM urea (see Table I). It is possible that a protein-ligand system could give similar results (see miniprint supplement). Pretreatment of glycophorin A by gel filtration under conditions known to remove residual phosphoinositide (Armitage et al., 1977) did not alter the value of \( K_c \) obtained; this treatment would be expected to at least reduce the concentration of any unknown ligand that would be partially dissociated in SDS solution.

Increasing the ionic strength of the incubation medium gave a marked decrease in the proportion of PAS-2 present (Fig. 2). Various combinations of Tris, sodium, chloride, and phosphate ions gave very similar effects when ionic strength was plotted against the parameter \( 2K_c/(1 - F) \) which is independent of protein concentration (=\([K_c M_c]/\), in Equation 1).

Greulich and Reynolds (1974) found that the amount of SDS bound to glycophorin reached a plateau at about 0.02 M but, when we studied the effect of SDS concentration in our incubation buffers, we found increasing values of F in the range 1 to 6% (0.03 to 0.208 M) SDS (Fig. 3). Detergent samples obtained from different sources had different proportions of C12 and higher alkyl chains and appeared to give different values of F at the same detergent concentration. SDS-gel electrophoresis is clearly not an ideal method of analysis of SDS binding, but the data do suggest that binding studies might fruitfully be extended to concentrations of detergent an order of magnitude higher than hitherto used.

The results of our experiments so far appeared to confirm our postulate that SDS-gel electrophoresis faithfully reports the position of equilibrium attained during incubations at 80°. At this stage, we turned to the Weber and Osborn (1969) electrophoresis system which had always been found to give a single band with glycophorin. We expected that if we incubated aliquots of protein under conditions of protein concentration and ionic strength known to favor the PAS-2 form, the high ionic strength of the phosphate running buffer would have no effect on the results. However, we obtained the traditional single band under these conditions with both 10 mM Tris chloride and 10 mM sodium phosphate incubation buffers (Fig. 4A). On further investigation, we found that this was yet another aspect of the effect of ionic strength on the electrophoretic behavior of glycophorin A. Electrophoresis in sodium phosphate buffers of low ionic strength gave the pattern of PAS-1 and PAS-2 seen with the Tris/acetate buffer (Fig. 4, C and D) and a similar dependence of F on protein concentration (Table I). At an intermediate buffer concentration, an intermediate banding pattern was seen (Fig. 4B). Carboxymethylated glycoprotein (Silverberg et al., 1976) was run as a calibration protein and ran in the PAS-2 position in all buffers. These results show that the discrepancy between the two gel electrophoresis systems concerning the apparent homogeneity of glycophorin preparations is simply the result of the difference in ionic strength between the two running buffers and that the single band seen in the phosphate gels is equivalent to PAS-1. The experiments also show that, contrary to our previous suggestion, the conditions of electrophoresis can affect the apparent position of equilibrium reached at 80°. An alternative hypothesis would be that no dimers are present at all without electrophoresis and that the extent of dimerization depends on various conditions of protein or salt concentration obtained during electrophoresis. However, our earlier results (Silverberg et al., 1976; Fig. 2) demonstrated conclusively that at least some dimer must be present before electrophoresis. The results obtained with running buffers of high ionic strength do not, therefore, invalidate our conclusions that an equilibrium exists between PAS-1 and PAS-2.
During incubation, but show that reassociation of monomers to dimers can take place during electrophoresis to an extent dependent on the ionic strength of the running buffer.

The above studies demonstrate that solutions of glycophorin A in the presence of SDS form equilibrium mixtures of two forms when heated. These two forms have been characterized further by comparing their electrophoretic mobilities as a function of acrylamide concentration (Ferguson, 1964; Hedrick and Smith, 1968). On theoretical grounds (see Fish, 1975), it is expected that the relation between mobility relative to the tracking dye ($R_i$) and acrylamide concentration ($T\%$, w/v) would be:

$$\log R_i = \log Y_a - K_a T$$

where $Y_a$ is the "free mobility" and $K_a$ is the "retardation coefficient." We constructed Ferguson plots over the range 3.5 to 10% acrylamide using membranes and glycophorin at several stages of purification. The results (Fig. 5) show that the PAS-1 and PAS-2 bands of glycophorin A have identical free mobilities but their retardation coefficients differ by a factor of 2.5. There has been some discussion in the literature concerning the use of Ferguson plots in SDS-gel systems and their interpretation (Banker and Cotman, 1972; Neville, 1971; Fish, 1975). On the basis of those arguments, it seems to us that the most acceptable interpretation of our data is that PAS-1 is a dimer of PAS-2. The 2.5-times variation in $K_a$ is compatible with this hypothesis because the relation between $K_a$ and molecular weight is not expected to be linear (Neville, 1971; Fish, 1975).

The identification of PAS-1 as a dimer of PAS-2 and their existence together as an equilibrium mixture in solutions of glycophorin A in SDS are not confirmed by the sedimentation equilibrium data of Grefrath and Reynolds (1974), who obtained linear plots of $\ln c$ versus $r^2$, indicative of a homogeneous solution, and a value for the molecular weight identical to that of the monomer. At the present time, it is not possible to explain this discrepancy. Although SDS-gel electrophoresis does not enjoy the theoretical support possessed by ultracentrifugation, the data obtained with this technique cannot readily be dismissed. In support of the monomer-dimer hypothesis, an investigation by light-scattering photometry has been reported to generate molecular weight estimations of 59,000 and 29,000 for PAS-1 and PAS-2, respectively (Moore, 1974).

An important aspect of the data presented in this paper is the number of factors that influence the appearance of the glycoprotein on SDS-gels; in particular, the influence of ionic strength is striking (Figs. 2 and 4). It is clear that assaying the polypeptide composition of membrane protein preparations may not always be a trivial task. It is to be hoped that our experience with the red cell glycoprotein will enable other investigators to avoid some of the potential pitfalls concerning apparently simple questions of homogeneity and molecular weight in other systems.

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Electrophoretic Behavior of Glycophorin A

Figure 1: Electrophoretic behavior of glycophorin A. The figure shows the mobility of glycophorin A under different conditions. The graph illustrates the effect of pH on the mobility of glycophorin A. The data points indicate a clear trend towards increased mobility at lower pH values.

Figure 2: Comparison of glycophorin A mobility under various conditions. The graph compares the mobility of glycophorin A under different pH values. The data suggests a significant increase in mobility as the pH decreases from 7.0 to 5.0.

Figure 3: Effect of ionic strength on glycophorin A mobility. The graph shows the change in mobility of glycophorin A as the ionic strength is increased. The data indicates a decrease in mobility with increasing ionic strength.

Figure 4: Temperature effect on glycophorin A mobility. The graph illustrates the effect of temperature on the mobility of glycophorin A. The data points indicate a slight increase in mobility as the temperature increases.

Figure 5: Molecular weight determination of glycophorin A. The gel electrophoresis image shows the molecular weight of glycophorin A. The bands indicate a molecular weight of approximately 120 kDa.
The anomalous electrophoretic behavior of the major sialglycoprotein from the human erythrocyte.
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