A CROSS-LINKING STUDY OF THE BEEF ERYTHROCYTE MEMBRANE: EXTENSIVE INTERACTION OF ALL THE PROTEINS OF THE MEMBRANE EXCEPT FOR THE GLYCOPROTEINS

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Summary: Treatment of beef red cell ghosts with 2% glutaraldehyde for 1 hour at 0° cross-links the bulk of the protein of the membrane. Less than 10% of the protein is soluble in SDS after this treatment and even less will penetrate 5% polyacrylamide gels. Yet the glycoprotein can be quantitatively extracted as monomers either in the SDS soluble fraction or by the chloroform, methanol, water extraction procedure.[1]

Introduction

The major glycoprotein of beef erythrocytes has been purified and some of its properties characterized.[1] It has a molecular weight of about 180,000 of which 38% is protein and 62% is carbohydrate. The polypeptide portion is characterized by an abundance of serine and threonine residues.

Evidence has been presented that the glycoproteins of erythrocytes are distributed randomly and singly through the membrane,[2,3] but little is known about the state of association of these molecules with the other components of the membrane. In this communication the interactions of the glycoprotein of beef erythrocyte ghosts has been assessed by cross-linking studies with glutaraldehyde. The results suggest that the glycoprotein is not associated with other proteins of the membrane. This observation may be significant for the mobility of the molecule in the membrane.

Methods

General methods. Protein was estimated by the method of Lowry et al.[4] using bovine serum albumen as a standard. Lipid was measured as described by Chen et al.[5] Sialic acid was measured according to Warren using N-acetylneuraminic acid as a standard.[6]

Electron microscopy was performed using a Hitachi 11 DE microscope as described before.[7]

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed, and the gels were fixed and stained as described by Fairbanks et al.[8] except that 3% SDS and 5 mM mercaptoethanol was used to solubilize the samples. Densitometric tracings of the gels were performed with a Gilford linear scanning attachment to a Beckman DU spectrometer using quartz cuvettes. The molecular weights of the protein components were estimated as described by Weber and Osborn[9] using myosin, ß-galactosidase, bovine serum albumen, ovalbumen, ribonuclease and cytochrome c as the standard proteins of known molecular weight.

Preparation of ghosts. Beef erythrocyte ghosts were prepared by the method of Burger et al.[10] using 2 mM Ca^{++} to prevent disruption of the ghosts during hemolysis.

Glutaraldehyde treatment. Ghosts were suspended to 10 mg/ml in 10 mM Tris-Cl, pH 7.4, and mixed 1:1 by volume with a solution of 4% glutaraldehyde and this mixture was allowed to stir for 60 min at 0°. The reaction was terminated by diluting the reactants with 50 volumes of distilled water and this solution was centrifuged at 103,000 x g for 30 min. The supernatant was discarded and the pellet washed twice with buffer before being dissolved in 3% SDS and 5 mM mercaptoethanol or treated with chloroform-methanol, as described later.

Isolation of glycoproteins from the ghosts. Glycoproteins were isolated as described before[1] by a method similar to that developed independently and reported recently by Hamaguchi and Cleve.[11] 50 mg of erythrocyte ghosts were shaken vigorously in a solution which was 6 parts chloroform, 3 parts methanol and 1 part water. On standing, three layers developed. The bottom chloroform layer contained the lipid. The top aqueous layer contained most of the glycoprotein of the red cell (70-80% recovery), and small amounts of other protein contaminants. The bulk of the protein was retained at the interface of the aqueous and chloroform layers.

Solubilization of ghosts with Triton X-100. Ghosts suspended in 3 mM Tris-Cl, pH 7.4 were solubilized with Triton X-100 as described by Miller.[12] These conditions represented the optimum for solubilization by the method described in the above reference. However, complete solubilization was not obtained.

Results and Discussion

Glutaraldehyde was the reagent of choice for estimating the interactions of glycoproteins with other proteins in the membrane. This bifunctional reagent reacts readily with free amino groups and it was expected that closely apposed proteins would be covalently linked with this reagent.[13]

After incubation in 2% glutaraldehyde for 60 min at 0°, ghosts were very resistant to degradation. The membrane could no longer be solubilized with high levels of Triton X-100 (Figure 1) and was not disrupted by sonication, which in the absence of glutaraldehyde fixation breaks down the membrane to very small vesicles. Further, 3% dodecyl sulphate was ineffective in solubilizing the membrane to any significant extent. In fact, heating to 100°C for 1 min in a solution which was 3% in SDS and 5 mM in β-mercaptoethanol, solubilized less than 10% of the total protein of the ghost and little of this material would penetrate 5% gels (Figure 2). Thus there had been very extensive cross-linking of the proteins of the red cell, an observation consistent with the bulk of the membrane protein being organized into a protein continuum;[14] that is with long range interactions of protein molecules throughout the membrane.

Surprisingly, the protein solubilized by 3% SDS (10% of the total) contained virtually all the sialic acid of the cell, suggesting that glycoprotein had not been cross-linked by the reagent. Gel electrophoresis of the SDS soluble material on 5% acrylamide gels confirmed this and further indicated that the glycoproteins were still monomeric after the prolonged exposure to glutaraldehyde (Figure 3). The ready extractability of the glycoproteins even after almost all the other protein of the membrane had

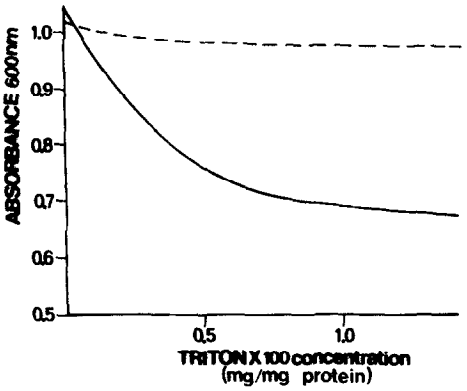

Fig. 1.

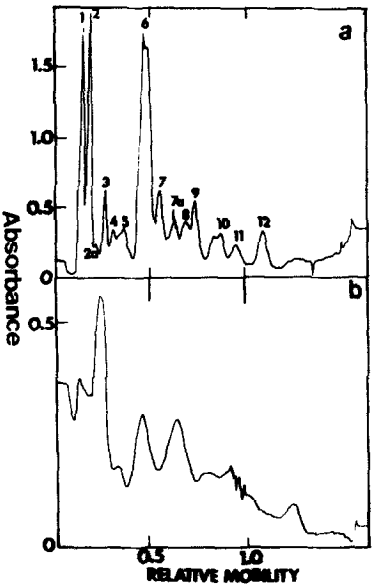

Fig. 2.

Figure 1. Triton X100 solubilization of beef erythrocyte membranes. The extent of solubilization of the membrane at different Triton X100 concentrations was followed by measuring the turbidity of the suspension (absorbance at 600 nm). The continuous line shows the dissolution of ghosts before glutaraldehyde treatment, the dashed line shows the dissolution of ghosts after incubation in 2% glutaraldehyde for 1 hr at 0°C.

Figure 2. Densitometric traces of polyacrylamide gels (5%) of erythrocyte ghosts. a. before glutaraldehyde treatment, b. the SDS soluble protein after 2% glutaraldehyde fixation. Peak numbers are those identified in reference 7. Improvement in destaining methods has allowed visualization of two extra peaks, 2a, MW 205,000 and 7a, MW 53,500.

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been cross-linked was also demonstrated by chloroform-methanol-water treatment of the ghosts. The amount of glycoprotein liberated by this treatment was about the same after glutaraldehyde treatment as before (Table 1).

The above data all indicate that the glycoproteins of beef erythrocyte ghosts are not cross-linked to other proteins in the membrane by glutaraldehyde. Further, they are not trapped within the protein domain which is stabilized by the cross-linking agent.

It is possible that glutaraldehyde does not react with glycoproteins although free amino groups are present,[1] and are available to and attacked

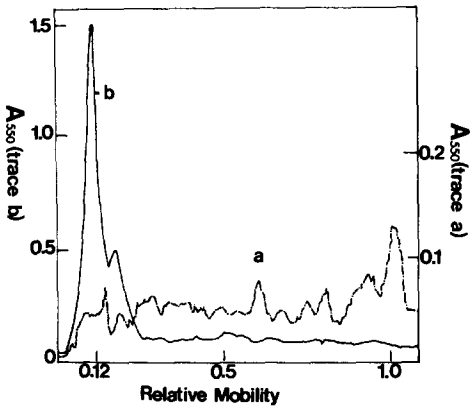

Figure 3. Densitometric traces of 5% polyacrylamide gels of the aqueous supernatant of the chloroform methanol water extraction after glutaraldehyde treatment. Stained for protein with coomassie blue trace a, and carbohydrate with Schiff reagent trace b. The glycoproteins were stained only weakly with coomassie blue. The molecular weights of the glycoproteins on 5% gels, 285,000 and 265,000, is the same as in untreated ghost preparations (1).

TABLE I

Extraction of glycoprotein from ghosts

	Before glutaraldehyde treatment			After glutaraldehyde treatment		
	% components in each phase			% components in each phase		
	Upper Supernatant	Interface	Lower Supernatant	Upper Supernatant	Interface	Lower Supernatant
Protein	7.4	90.9	1.7	4.8	93.9	1.3
Lipid	3	2	95	2	5	93
Sialic acid	84	14.8	1.2	80.6	17.5	1.9

by a number of other chemical agents, including trinitrobenzene sulfonate (TNBS).[15-17] More likely, glycoproteins, unlike the majority of membrane proteins, may not be a part of the protein domain. These molecules are highly negatively charged, (isoelectric points close to pH 2) and they may be prevented from association with other proteins by electrostatic repulsion,

much as has been suggested for rhodopsin molecules in the retinal rod membrane.[18] The great mobility of glycoprotein molecules in membranes[19-22] with diffusion rates comparable with phospholipid molecules[23] is much easier to explain if glycoproteins are floating in the lipid rather than being a part of the protein continuum.

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