DIFFERENTIAL SOLUBILIZATION OF HUMAN ERYTHROCYTE CELL MEMBRANE PROTEINS BY MALEIC ANHYDRIDE

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SUMMARY

A method is described to differentially solubilize the proteins of erythrocyte membranes using maleic anhydride. Based on this solubilization, the two groups of proteins appear to be distinct in their molecular organization within the membrane structure. From the operational criteria of peripheral and integral proteins of biological membranes, it is suggested that maleic anhydride by itself solubilizes mostly the peripheral proteins, while, in the presence of urea, it solubilizes almost all the proteins. It is suggested that maleic anhydride could be used to solubilize, fractionate and characterize the membrane proteins. Since maleyl groups can be removed from proteins, reconstitution of membrane structure from solubilized components could be studied.

INTRODUCTION

Recent investigations on biological membranes seem to support the fluid mosaic model for membrane structure (1,2). These studies indicate that the proteins of biological membranes are organized at two distinct levels, namely, 1) the integral proteins and 2) the peripheral proteins. This information was, in general, obtained mainly by two lines of investigation; one in which a single membrane protein is solubilized and then its characteristics defined, and a second in which the membranes as a whole are solubilized using various agents and the protein fraction is then characterized.

Many solubilizing agents, ranging from phenol-acid mixtures, organic solvents, high salt concentrations, both non-ionic and ionic detergents, have been used. However, proteins isolated by most of these methods are not suitable for studying the process of reconstitution of membranes.

In this article we describe a method to differentially solubilize membrane proteins as two distinct categories, using the reagent maleic anhydride. Maleic

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anhydride has been introduced into protein chemistry by Butler et al. (3) as a specific modifying agent for the ε-NH_2 groups of lysine residues. As a result of the net negative charge introduced by maleyl-lysyl residues, the proteins become very soluble in aqueous media at neutral pH values.

MATERIALS AND METHODS

Fresh blood samples were collected, from student volunteers, into ACD (acid citrate dextrose) solution and used almost immediately. Removal of serum and buffy coat, followed by isolation of red blood cell membranes was carried out at pH 7.4 as described by Dodge et al. (4).

For the maleylation reaction, membranes were suspended in 0.1 M borate buffer, pH 9.0. Maleylation itself was carried out according to Butler et al. (3). In this procedure, maleic anhydride, which was freshly redistilled, was dissolved in a minimal volume of acetone. Maleic anhydride solution at 0° was added in small aliquots (5-10 µl at a time), to the membrane suspension which was being stirred at 0°. The pH of the reaction mixture was maintained between 8.5 and 9.0 by the addition of 1 N NaOH. At the end of the addition, the reaction mixture was allowed to stand for an additional 15 minutes.

At the end of this time, the mixture was centrifuged in a Hitachi model 65P ultracentrifuge at 100,000 g for 2 hours at 0°. The supernatant was decanted and the pellet suspended in 0.1 M borate buffer, pH 9.0.

Protein concentrations of the original membrane sample, the 100,000 g supernatant and the precipitate were determined by the method of Lowry et al. (5). Organic phosphorous content of the samples was determined by the method of Bartlett (6). Total lipids were determined by the gravimetric method as described by Rose and Oklander (7). Phospholipid content was computed from total organic phosphorous values, according to Redman (8).

RESULTS AND DISCUSSION

The results shown in Table 1 make it clear that the membrane samples used were of consistent composition. These values agree well with the results of Dodge et al. (4). The total protein to total lipid ratio for these membrane

TABLE I

COMPOSITION OF INTACT ERYTHROCYTE MEMBRANE BEFORE SOLUBILIZATION

(Membrane samples were isolated by the method of Dodge, Mitchell and Hanahan (1963).)

SAMPLE	TOTAL PROTEIN	TOTAL LIPID	TOTAL ORGANIC PHOSPHORUS	PHOSPHO LIPID	PROTEIN TOTAL LIPID RATIO	PROTEIN PHOSPHO LIPID RATIO
1	7.4×10^{-3}g	6.4×10^{-3}g	10.0×10^{-6}g	2.5×10^{-4}g	1.2	29.6
2	7.4 "	5.0 "	9.0 "	2.3 "	1.5	32.1
3	7.4 "	5.7 "	10.5 "	2.6 "	1.3	28.4
4	7.4 "	7.0 "	8.0 "	2.6 "	1.1	37.0
5	7.4 "	6.5 "	10.7 "	2.7 "	1.2	27.0
6	4.7 "	3.8 "	5.8 "	1.5 "	1.2	27.5
7	4.7 "	4.0 "	5.9 "	1.5 "	1.2	31.3
8	4.7 "	3.9 "	5.8 "	1.5 "	1.2	31.3
9	4.7 "	4.1 "	6.0 "	1.5 "	1.2	31.3
10	4.7 "	4.0 "	5.0 "	1.5 "	1.2	31.3
11	4.7 "	3.7 "	5.6 "	1.5 "	1.2	31.3

preparations is about 1.2, while the total protein to phospholipid ratio is about 30.

Figure 1 shows the turbidity changes in relation to the concentration of maleic anhydride. The original membrane suspension has a turbidity value of 0.8 OD units. With the addition of increasing amounts of maleic anhydride, the turbidity drops and soon attains its lowest value. Increase in maleic anhydride concentration to very high levels does not have any further effect on the turbidity.

Similar results were observed when the amount of protein released into the 100,000g/2 hr. supernatant fraction was measured as a function of increasing concentrations of maleic anhydride. As shown in Figure 2, the protein con-

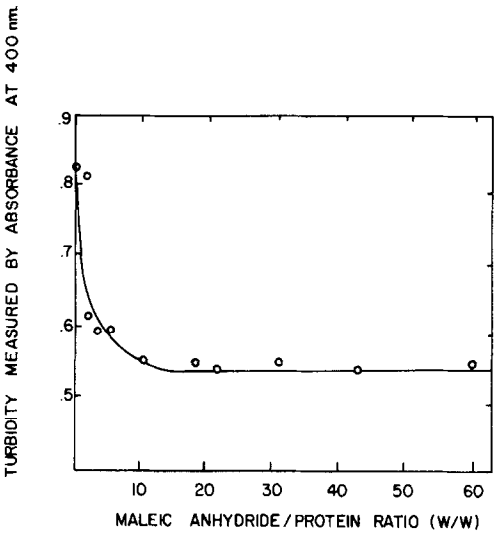

Fig.1 The effect of maleylation on the turbidity of human erythrocyte membrane suspension treated with different amounts of maleic anhydride

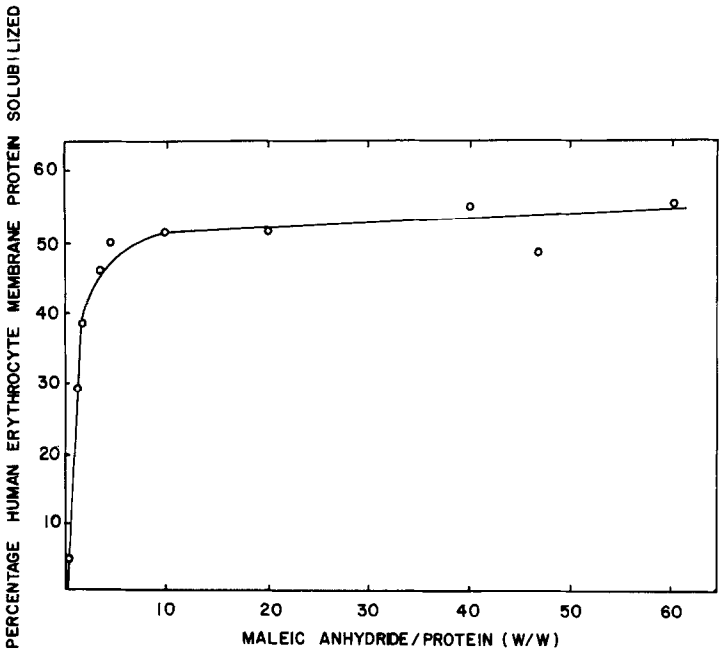

Fig.2 Percentage of human erythrocyte membrane solubilized as a function of maleic anhydride/protein ratio

centration in the supernatant is very low in the beginning and increases with increasing concentrations of maleic anhydride. However, the protein values attain a plateau at a maleic anhydride to protein ratio of about 10:1 (w/w). Any further increase in maleic anhydride concentration does not increase the amount of protein released.

Table 2 shows the amounts of protein solubilized by a constant amount of maleic anhydride with different membrane preparations. These values are

TABLE II

PERCENTAGE OF ERYTHROCYTE MEMBRANE PROTEIN SOLUBILIZED BY REACTION WITH MALEIC ANHYDRIDE
(Protein to Maleic anhydride ratio = 1:30 w/w)

EXPERIMENT NO.	TOTAL PROTEIN IN 100,000xg SUPERNATANT	TOTAL PROTEIN IN 100,000xg PELLET	PERCENTAGE PROTEIN SOLUBILIZED
1.	2.5×10^{-3}g	2.5×10^{-3}g	50
2.	2.5 "	2.4 "	51
3.	3.2 "	3.0 "	52
4.	3.5 "	2.6 "	57
5.	2.3 "	2.0 "	54
6.	2.5 "	3.2 "	44
7.	2.3 "	2.1 "	52
8.	3.6 "	2.4 "	52

consistent, and average just over 50% of the total membrane proteins.

These results indicate that half of the membrane proteins are readily accessible to maleic anhydride, and hence are released by reaction with maleic anhydride, while the other half are not so readily accessible. If all the membrane proteins were to be accessible, then one would expect solubilization

and release of almost all the proteins into the 100,000g/2 hr. supernatant fraction, since it is known that maleic anhydride, by reacting with ε-NH_2 groups of lysine, renders proteins soluble in neutral aqueous solutions (3). In addition, we found that if 8 M urea were to be present during maleic anhydride reaction, more than 95% of the membrane proteins are solubilized and released into the supernatant fraction. Similar results were reported by Moldow et al. (9) using succinic anhydride reaction with red blood cell membranes.

Analysis of the 100,000 g precipitate for total lipid and phosphorous shows that the total protein to total lipid ratio is only 0.42, compared with 1.2 for the original membranes. Similarly, the total protein to phospholipid ratio is about 11.3, compared with 30 for the original membranes (Table 3). These results indicate that maleic anhydride reaction solubilizes half of the protein but only 1/6 of the lipids.

TABLE III

COMPOSITION OF NON-SOLUBILIZED ERYTHROCYTE MEMBRANE PREPARATION

(100,000 x g/2 hrs. precipitate fraction)

EXP'T NO	TOTAL PROTEIN	TOTAL LIPID	TOTAL ORGANIC PHOSPHORUS	PHOSPHO LIPID	PROTEIN TOTAL LIPID RATIO	PROTEIN PHOSPHO-LIPID RATIO
1.	1.9×10^{-3}g	3.8×10^{-3}g	5.6×10^{-6}g	1.4×10^{-4}g	0.47	13.6
2.	2.3 "	5.3 "	8.4 "	2.1 "	0.43	11.0
3.	1.9 "	4.3 "	6.8 "	1.7 "	0.44	11.2
4.	2.0 "	5.2 "	8.0 "	2.0 "	0.39	10.0

These results suggest that maleic anhydride reacts mainly with those proteins which do not directly interact with the lipids of the membrane, namely the peripheral proteins, and releases them into solution.

This implies that the lipid bilayer structure remains intact with its integral proteins. If this were to be the case, one would expect that most of the maleic anhydride reacted would be associated with the soluble fraction, while only a negligible amount would be associated with the precipitate fraction. When the amount of maleic anhydride bound to the soluble protein fraction and the precipitate protein fraction was measured, it was found that most of the maleic anhydride is indeed associated with the solubilized proteins (Vadlamudi and Larway, unpublished results). Recently Obaid et al. (10) reported that maleic anhydride treatment of red cell ghosts, only alters the ion permeability characteristics of the membrane, which implies that at least the lipid bilayer structure is intact.

Thus it is clear that maleic anhydride differentially solubilizes membrane proteins as two groups. It appears possible that those proteins solubilized by maleic anhydride alone are mostly peripheral proteins, while those proteins solubilized by maleic anhydride in the presence of urea are peripheral and integral proteins.

Since maleic anhydride can be removed from proteins under mild conditions (3), the solubilized proteins can be fractionated, demaleylated and further characterized. The reconstitution of membrane structure can then be conveniently studied. This is not possible with several other acid anhydrides including succinic anhydride, since they are not easily removed.

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