Biochimica et Biophysica Acta, 330 (1973) 132–140

BBA 76515

ELECTRICAL PROPERTIES OF BLACK MEMBRANES FROM OXIDIZED CHOLESTEROL AND A STRONGLY BOUND PROTEIN FRACTION OF HUMAN ERYTHROCYTE MEMBRANES

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(Received July 16th, 1973)

SUMMARY

1. Black lipid membranes from oxidized cholesterol show an up to 10^3-fold increase in electrical conductivity following the addition of a strongly bound protein fraction from human erythrocyte membranes (obtained by depolymerisation of the membranes in 90% acetic acid after removal of the loosely bound proteins by treatment with 10% acetic acid) to the surrounding solutions. The presence of protein on both sides of the membrane is a prerequisite for this effect.

2. The major part of the interactions leading to the protein-induced conductivity change is irreversible.

3. The magnitude of the conductivity change is dependent on protein concentration and on the composition of the buffers in which the membranes are formed. In the range of conditions investigated (salt concentration $c=2$–200 mM, pH = 5.5–8.5, protein concentration $c_p=0.001$–1.0 mg/ml), membrane conductivity increases strongly with increasing salt concentration and weakly with decreasing pH; the effect of c_p shows a maximum at about 0.05 mg/ml.

4. The effect of salt concentration is about 10-fold larger in NaCl than in sodium acetate solutions. The ion transport numbers for the protein–lipid membranes were found to depend on the nature of the anion present.

5. The current–voltage curve of the membranes is nonlinear both in the presence and absence of protein.

6. It is concluded that substructures of conducting sites essentially confined to one side of the lipid bilayer are formed by hydrophobic association between the lipid membrane and molecules from the protein solution. The conductivity effects described are thought to be due to an interaction of substructures situated on different sides of the membrane.

INTRODUCTION

Black lipid membranes first described by Mueller *et al.*[1,2], can be used as a substrate for protein–lipid interaction and allow precise and unambiguous physical measurements of correlated changes in membrane properties. Stimulating investigations in this field were the synthesis of membrane structures showing electrical

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phenomena comparable to excitation in biological membranes[3,4], the reconstitution of membrane ATPase activity[5,6], and the sensitizing of lipid films to cholinergic agents[7].

Attempts to study the incorporation of proteins from erythrocyte membranes into black lipid membranes presented some difficulties. Maddy *et al.*[8] used egg lecithin–decane black lipid membranes and total proteins from bovine erythrocyte ghosts prepared by butanol extraction. They found a reduced surface pressure and an increased thickness of the membranes, but did not report any changes in the electrical properties of the black lipid membranes. Van den Berg[9], using the same method of protein preparation, briefly mentioned reistance descreases of about two decades. Cherry *et al.*[10] studied the effect of unfractionated proteins from human erythrocyte membranes upon black lipid membranes of total lipids from the same source. Addition of the protein to both or only to one side (Berger, K. U., personal communication) of the black lipid membranes caused an increase in the membrane's conductivity and optical reflectivity, but a decrease of its stability. The protein used was also prepared by the butanol method, but it had to be freed from the sialic acids and the pH of the buffer solutions had to be restricted to values around 4. Performing similar experiments with liposomes, Zwaal and van Deenen[11] proposed the necessity of attractive coulombic forces between the components for the initiation of their interaction, which were only present under the described conditions. Just before the completion of this manuscript, Tosteson *et al.*[12] described the interactions of the erythrocyte-membrane sialoglycoprotein[13] with black lipid membranes made from sheep erythrocyte lipids. In their brief paper, the authors report an increase in membrane conductivity up to a factor of 30. In the presence of the phytohaemagglutinin concanavalin A, a further 7-fold increase in membrane conductivity was found.

Recently, a new method for the preparation of protein fractions from human erythrocyte membranes was described[14,15]. The two fractions obtained by this procedure and designated as 'loosely bound' and 'strongly bound', recombine with liposomes made from total lipids of human erythrocyte membranes. In the present paper we report a study of the interactions of the 'strongly bound' fraction (consisting mainly of a protein of molecular weight 95000 and the sialoglycoprotein[15]) with black lipid membranes made from oxidized cholesterol. This material gave protein–lipid membranes of satisfying stability and reproducibility, in contrast to erythrocyte membrane lipids and egg lecithin, tested in preliminary experiments. Some preliminary results of our investigations have been reported previously[16].

MATERIALS AND METHODS

Preparation of materials

Oxidized cholesterol prepared by a technique different from that described by Tien *et al.*[17] was kindly supplied by Dr Dobias, Universität Regensburg. In this method the cholesterol (Merck, analytical grade) was dispersed in water and oxidized with molecular oxygen at about 97 °C. In addition to unoxidized cholesterol, the resulting mixture contained approximately 5–10% 7-keto- and 7-hydroxycholesterol, as was shown by infrared spectroscopy. The lipid was dissolved in octane at a concentration of 15 mg/ml. For the preparation of the strongly bound protein fraction

from human erythrocyte membranes, the method of Schubert *et al.*[14] was used. The protein was stored as an isoelectric precipitate at 4 °C and was redissolved immediately prior to use by increasing the pH value of the sample to about 10. Then the protein solution was diluted with buffer to give the desired pH, ionic composition and protein concentration. The buffers contained acetic acid–NaOH (pH 5.5), sodium–potassium phosphate (pH 7.0) and Tris–HCl (pH 8.5). Buffer concentration was 2 mM at 5, 10 and 20 mM NaCl and 10 mM at 50, 100 and 200 mM NaCl. Protein concentration of the stock solutions was determined from the absorbance difference of the samples at 280 and 320 nm. For the "differential extinction coefficient", a value of $E^{1\%}_{1\,cm} = 15.2$ was used, as determined according to Lowry *et al.*[18] using bovine serum albumin (Serva) as an arbitrary standard. Under the conditions of our measurements, this method was shown to be practically independent of the degree of light scattering of the solutions.

Experimental procedures

Membranes were formed by the usual brush technique[1,2] across a hole (1.8 mm diameter) in a teflon partition separating two aqueous solutions. They became black within 3 min or less in protein-containing and in protein-free solutions. When concentrations in the aqueous phases had to be increased, small amounts of concentrated electrolyte or protein solution were added using a calibrated syringe while stirring thoroughly. In dilution experiments, a push–pull syringe device was used. All experiments were done at room temperature (22–24 °C). Membrane conductivity was measured in a current clamp circuit (Keithley 602) while the current–voltage characteristics were recorded using a simple voltage clamp arrangement. Voltages were kept below 20 mV for routine resistance measurements. A simple Wheatstone bridge was assembled around a capacitance decade (General Radio 1412 BC) for capacitance measurements at 1 kHz. The diffusion potential measurements were made with calomel electrodes and agar bridges. In all other experiments platinized sintered Ag–AgCl electrodes were used (Annex Instruments).

RESULTS

Changes of membrane d.c. conductivity following addition, removal and enzymatic degradation of protein

Black lipid membranes formed in solutions of uncomplexed inorganic or weakly hydrophobic organic ions exhibit specific conductivities much lower than those typical of biological membranes. However, addition of μg-quantities of the strongly bound protein fraction to both buffer solutions bathing the black lipid membrane, leads to a fast and drastic increase in membrane conductivity. A very similar effect is observed if the protein is already present in the buffer solutions before film formation. In contrast to this, the conductivity of the lipid films did not decrease significantly if protein was added to one compartment only, regardless of the polarity of the applied potential V ($V \leqslant 20$ mV). When, however, after about 20 min protein was added also to the second compartment, the fast increase of conductivity described above was found. A typical time course of conductivity of such a membrane is shown in Fig. 1.

Exchange of the protein solutions bathing a black membrane, which had al-

ready reached a high value of conductance due to interactions with the proteins, against protein-free buffer solutions caused only a relatively small and very slow decrease in membrane conductivity (about a factor of 10 in approx. 4 h). Thus, the main part of the protein–lipid interactions leading to the conductivity increase described above is irreversible. Protein solutions which were incubated for 15 min with the proteolytic enzyme mixture pronase (Merck, Pronase E, 70000 proteolytic Kunitz units/g, 50–100 μg/ml) were not able to induce an increase in membrane conductivity. However, if the enzyme was added to the protein solutions bathing membranes, which had already reached a high value of conductance, membrane conductivity remained practically constant even after much longer periods of incubation. These experiments, besides confirming the irreversibility of the process of formation of conducting sites, also demonstrate that pronase is not able to degrade these conducting sites.

Time dependency of protein-induced membrane conductivity

The time dependency of membrane conductivity in the presence of protein

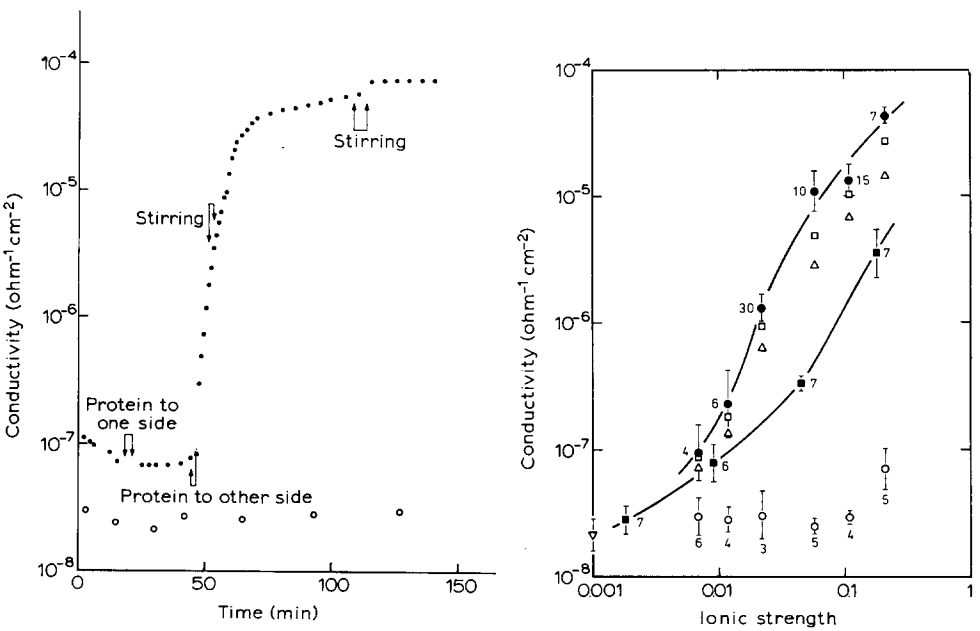

Fig. 1. Effect of protein (when added first to one side of a black film and 25 min later to the other side) on membrane conductivity (●). Control without protein (○). Aqueous solution: 100 mM NaCl, 10 mM acetic acid–NaOH (pH 5.5); final protein concentration, 33 μg/ml. During the addition of protein and in intervals indicated, the aqueous solutions were stirred vigorously.

Fig. 2. Influence of ionic strength on d.c.-membrane conductivity with predominantly NaCl (△, □, ●) and only sodium acetate (■) in the aqueous solutions. Mean values are shown at 30 (△), 45 (□) and 60 (●, ■) min after film formation in protein containing solutions. Control values without protein (○) are also shown. The point on the ordinate (▽) represents membrane conductivity in pure water. The values shown are averaged logarithms of the measurements, the bars give the mean errors of the averages, and the number of experiments is indicated. Aqueous solution: 33 μg/ml protein, 2 and 10 mM acetic acid–NaOH (pH 5.5) at 5, 10, 20, and 50, 100, 200 mM NaCl, respectively.

may be divided into two phases (Fig. 1). In the first phase, during the first 30 min after the addition of protein to both chambers or after the formation of the film in protein-containing buffers, a very rapid increase in membrane conductivity occurs. During this phase, membrane conductivity increases up to a factor of 300. In the second phase, the rate of conductivity increase is considerably smaller than in the first one. During the first 30 min of this latter phase, the increase in membrane conductivity barely amounts to a factor of 3. After this time the rate of conductivity increase becomes still smaller and in about 10% of the measurements, membrane conductivity became constant. A demonstration of the described behaviour is given in Fig. 2, which shows mean values of membrane conductivity taken at 30, 45 and 60 min after film formation in buffers containing protein and varying concentrations of NaCl.

During the fast phase of conductivity increase, the time dependency of membrane conductivity is influenced by the procedure used for the initiation of the protein–lipid interactions. If, after membrane formation, the protein was added first to one buffer compartment and about 20 min later to the second compartment, the conductivity increase observed after the second addition of protein was faster than that obtained after simultaneous addition of protein to both compartments or after membrane formation in the presence of protein. At the end of the first phase, however, these differences had practically vanished. Thus, during the second phase of conductivity increase, membrane conductivity is independent of the procedure used for the addition of the protein.

In the first phase, vigorous stirring of the buffer solutions during the whole period of measurement as compared to stirring only during the addition of protein, did not lead to significant differences in the time course of membrane conductivity. Thus, diffusion of the protein through the unstirred layers at the membrane–buffer interfaces cannot be the rate-limiting step of the conductivity increase. This is further supported by an extremely high activation energy of 43 ± 6 kcals/mole which could be estimated for the rapid phase of the conductivity change[19].

For comparative studies on the effects of various parameters on the magnitude of the protein-induced conductivity increase, the following procedure was suggested by the results described above. The membranes were formed in protein-containing solutions and conductivity values at 60 min after film formation were chosen to characterize the membrane's conductivity change. Due to the experimental scatter, conclusions on the effects of the different parameters could only be drawn from average values of a large number of measurements.

Membrane conductance as function of buffer composition, protein concentration and pH

The conductivity of black lipid membranes from oxidized cholesterol formed in solutions of 5–200 mM NaCl and/or sodium acetate depends only weakly on salt concentration. However, in the presence of the protein fraction, a strong dependence of conductivity on NaCl concentration was observed. For example, for membranes in solutions of NaCl containing 33 μg protein per ml, the conductivity increased by a factor of 100 when the concentration of NaCl was increased by a factor of 10. By performing similar experiments with sodium acetate replacing NaCl, the conductivity of the protein–lipid membranes in solutions of sodium acetate was lower for all ionic strengths. These data are shown in Fig. 2.

Variation in protein concentration also markedly influenced the magnitude of the conductivity increase of the membranes. As shown in Fig. 3, at both values of NaCl concentration tested, membrane conductivity as a function of protein concentration shows a maximum at protein concentrations around 50 μg/ml.

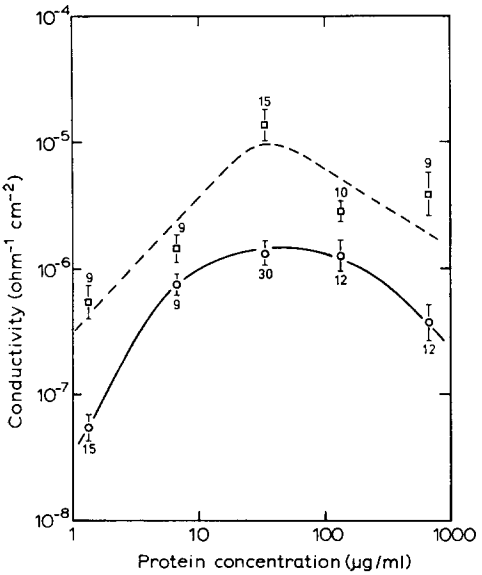

Fig. 3. Influence of protein concentration on d.c.-membrane conductivity. Buffers: 20 mM NaCl, 2 mM acetic acid–NaOH (pH 5.5) (○); 100 mM NaCl, 10 mM acetic acid–NaOH (pH 5.5) (□). Values at $t = 60$ min are shown; for averaging procedure see legend of Fig. 2.

In contrast to variations of buffer composition and of protein concentration, variation of H^+ concentration in the solutions bathing the membrane had only a weak influence on the conductivity effect induced by the protein. At constant protein concentration (33 μg/ml) and at two different NaCl concentrations (20 and 100 mM) changing the pH from 5.5 to 8.5 only led to a decrease of membrane conductivity of a factor of less than 3. Thus, titratable groups with apparent pK values in this pH range do not play a role in the process which leads to the observed increase in membrane permeability.

Current–voltage characteristic, membrane capacity and transport numbers

The normalized current–voltage characteristic ($I(U)/I(10\ \text{mV}) = f(U)$) of the black protein–lipid membranes in symmetrical aqueous solutions (50 mM NaCl, pH 5.5, 33 μg/ml protein) is nonlinear and exceeds the ohmic value at 80 mV by about 30%. It is practically the same as that obtained without protein in solutions of picric acid and other weakly hydrophobic ions[20]. In the presence of protein, the break down voltage of the black membranes was found to be approx. 90 mV compared with 180 mV in the absence of protein.

The capacitance of the membranes had a mean value of about 0.35 μF cm^{-2} both in the absence and presence of protein, even at the largest conductivity effects observed. Also the monotonous capacitance increase, which is characteristic for the

oxidized cholesterol–octane film system[21] was always found. The capacitance behaviour seems to indicate that the thickness of the low dielectric constant part of the films is not significantly influenced by the association process.

To test the ionic specificity of the conductivity effect described above, we measured diffusion potentials developed across black lipid and protein–lipid films by salt concentration gradients. From these potentials the cation transport numbers were calculated according to the liquid junction theory (Henderson and Planck, see ref. 22). In Table I, the cation transport numbers for diffusion through protein–lipid membranes are shown to differ from the transport numbers in water and in unmodified black lipid membranes. Obviously, the protein–lipid membranes in NaCl solutions are more permeable to the cations, while these membranes become more permeable to the anions in sodium acetate solutions.

TABLE I

CATION TRANSPORT NUMBERS

Transport numbers t^+ calculated according to $U_{diff} = (2t^+ - 1)\ RT/F \ln c_1/c_2$ (ref. 25). The measurements were performed at protein concentrations of 33 or 130 μg/ml and pH values of 5.5 and 7.0, respectively, in the range of salt concentration ratios c_1/c_2 from 1:3 to 1:10. In addition to the mean values, the maximum deviation from the mean and the number of measurements are given.

Salt predominantly present in aqueous solution	*Free solution**	*Black membranes of oxidized cholesterol*	
		Without protein	*With protein*
NaCl	0.39	0.65 ± 0.02 (3)	0.77 ± 0.03 (5)
Sodium acetate	0.56	0.36 ± 0.03 (6)	0.28 ± 0.04 (5)

* Values taken from Robinson and Stokes[23].

DISCUSSION

On the nature of the protein–lipid interactions

Investigations on the interactions between black lipid membranes and synthetic polypeptides[24,25] have shown that electrostatic attraction is a necessity for their association, either acting alone or facilitating or initiating hydrophobic association. Experiments with erythrocyte membrane proteins provided similar results[10]. However, the measurements described in this paper do not fit into this scheme. The black lipid membranes made from oxidized cholesterol are neutral and non-amphoteric, having neither charges to screen nor sites with which ionic groups are expected to associate[26]. Thus coulombic attraction cannot play a decisive role in the protein–lipid interactions observed. On the other hand the high stability of the complexes responsible for the conductivity increase, as demonstrated by the irreversibility of their formation, seems to rule out the predominance of forces other than coulombic or hydrophobic[27]. Thus, the interactions leading to the conductivity effects seem to be mainly hydrophobic. This is consistent with the strong increase in membrane conductivity with increasing ionic strength. A predominance of hydrophobic forces

was already observed for the binding between the strongly bound protein fraction and liposomes made from total lipids of human erythrocyte membranes, and it was also inferred for the binding of this fraction to the native erythrocyte membrane[17].

On the mechanism of the protein-induced conductivity

Some conclusions to this question can be drawn from the following results: the presence of proteins on both sides of the membranes is a prerequisite for the conductivity increase, the ion transfer numbers vary with the anions used, and the current voltage characteristic deviates from Ohm's law. In a model explaining the conductivity by the formation of pores through the membrane, it must be assumed that these pores are assembled by the interaction of substructures induced by the protein and confined to different sides of the membrane. These substructures would resemble the "half-pores" proposed by Cass *et al.*[28] for the action of nystatin and amphotericin B. The pores however cannot be regarded as wide water-filled channels which allow movement of small ions without interaction with the pore walls. Among the carrier models, the well-known model of a mobile monomeric carrier of the valinomycin type is not able to explain the results mentioned above. However, it seems possible that complicated carrier or other non-pore models consisting of at least two subunits, which are assembled on different sides of the membrane, may be applied.

A result difficult to understand is the decrease in the magnitude of the conductivity change with increasing protein concentration above 50 μg/ml (Fig. 3). It might be a consequence of increasing protein–protein interactions within the solutions and the membranes with increasing protein concentrations.

Concluding, we wish to emphasize that we are still far from an understanding of the effects described. Further studies will have to use pure proteins instead of a protein mixture, and to vary the composition and the structure of the lipid part of the membranes.

ACKNOWLEDGEMENTS

We are grateful to Professor Dr K. D. Heckmann and Professor Dr H. Passow for helpful discussions and to Mrs H. Brunner and Miss M. Hackl for skilful technical assistence. This work was supported by the Deutsche Forschungsgemeinschaft (He 378 and SFB 38).

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