

# Variability of Conductivity Changes in Black Phosphatidylserine Membranes Induced by Proteins from Erythrocyte Membranes

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The electrical conductivity of black phosphatidylserine membranes, in solutions of 100 mM NaCl, 10 mM sodium phosphate (pH 7.1), is strongly increased by the intrinsic proteins ("strongly bound" protein fraction) from human erythrocyte membranes. The magnitude of the conductivity increase is highly dependent on the maximum pH-value pH\* used during the preparation of the protein ( $8.0 \leq \text{pH}^* \leq 11.8$ ). For each pH\*, membrane conductivity  $\lambda_f$  and protein concentration  $c$  are linked by the equation  $\lambda_f = k \cdot c^s$ ,  $k$  and  $s$  being functions only of pH\*. The value of  $s$  varies between 1.0 (pH\* 8) and 4.0 (pH\* 10). It is assumed that the protein-induced conducting sites, at least for protein pretreatment at  $\text{pH}^* \leq 10$ , are assembled from four protein subunits. The incorporation of the subunits into the lipid bilayer is supposed to occur either as the final tetramer (pH\* 8) or as monomers (pH\* 10) and possibly dimers (pH\* around 9).

## Introduction

Protein-lipid membranes formed by association of solubilized membrane proteins with black lipid membranes (BLM) have become a frequently used model system in studies on biomembrane ion permeability. Generally, these protein-lipid membranes were found to have a much higher electrical conductivity than their parent lipid membranes, the difference reaching up to a factor of  $10^4$  (e. g.  $10^{-5}$ ). Little is known, however, about the influence of a possibly important parameter of the experimental system: the way of isolating and handling the protein before addition to the film compartments. Apparently, most authors have assumed that the effect on BLM conductivity is a unique property of the protein used.

As an extension of earlier work on BLM-protein systems<sup>3, 5</sup>, we have tried to study the interactions between black phosphatidylserine (PS) membranes and the "strongly bound" protein fraction from human erythrocyte membranes<sup>6, 7</sup>. This protein fraction consists essentially of the two integral proteins of the erythrocyte membrane: the protein(s) of molecular weight 100,000 ("band 3-protein"<sup>8</sup>) and "glycophorin", the main membrane sialo-protein, the band 3-protein being the predominant component<sup>7</sup>. During our studies, we found that slight variation of the conditions of protein prep-

aration could lead to profound changes in the properties of the BLM-protein system. On the other hand, it showed up that this variability of the experimental data can yield informations on the structure and assembly of the conducting sites in the membrane. Our findings are described below.

## Materials and Methods

### Lipid stock solution

The lipid: monosodium phosphatidylserine from bovine spinal cord, was purchased from Lipid Products, South Nutfield (Surrey), England (solution in chloroform: methanol, "grade I"). According to the data of the manufacturer and to thin layer chromatography in our laboratory, its purity was above 99%. It was dried under a nitrogen stream and then dissolved in one part of chloroform and ten parts of decane at a final concentration of 9 mg/ml. The solution was then warmed up for about 10 min to 45 °C. Afterwards, it was stored at room temperature and used within 4 hours.

### Protein stock solution

The preparation of the "strongly bound" protein fraction from human erythrocyte membranes was performed as described earlier<sup>6, 7</sup>. However, dialysis of the protein after gel filtration in 90% acetic acid was done against 10 mM acetic acid instead of water. After dialysis, the protein was brought to pH 7–8 by addition of 0.1 M NaOH (thus crossing the isoelectric pH range of the protein<sup>6</sup>). Then, 100 mM glycine was added to a concentration of

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10 mM, and the pH was made more alkaline by further addition of NaOH up to a value  $\text{pH}^*$ . Protein concentration in these stock solutions, as determined from the absorbance difference at 280 and 320 nm<sup>3</sup>, was about 300  $\mu\text{g}/\text{ml}$ .

Before being used for the BLM experiments, the protein was kept at the pH value  $\text{pH}^*$  for at least 10 min. At  $\text{pH}^* \leq 11.0$ , this incubation time could be extended to more than 3 h, without significant influence on the results of the BLM measurements. At  $\text{pH}^* 11.8$ , however, it had to be restricted to no more than 20 min.

After gel filtration and dialysis against 10 mM acetic acid, the protein could be stored for up to one week, without noticeable influence on the results described below. However, when stored as an isoelectric precipitate<sup>6</sup> (as was done in our earlier studies<sup>3</sup>), storage for more than 3 days distinctly impaired the ability of the protein to increase BLM conductivity.

#### Membrane formation and measuring technique

Black membranes were formed from the lipid stock solution by the brush technique<sup>9</sup>, across a hole (approx. 1 mm diameter) in a teflon partition separating two buffer compartments ( $V = 6$  ml). The compartments were filled with 100 mM NaCl, 10 mM sodium phosphate (pH 7.1) to which, before membrane formation, up to 0.05 volumes of the protein stock solution had been added. Buffer temperature was 28–29 °C. Membrane conductivity was measured in a current clamp circuit using Ag–AgCl electrodes. Voltages were kept in the linear range of the current-voltage characteristic (normally below 10 mV for  $\lambda > 5 \times 10^{-7} \Omega^{-1} \text{cm}^{-2}$ , up to 100 mV for smaller values of  $\lambda$ ).

#### Chemicals

All chemicals were obtained from Merck (Darmstadt). *n*-Decane was of reagent grade (purity 99%), the other chemicals were of analytical grade.

### Results

Black lipid membranes made from PS, at pH values near 7 and an ionic strength of approx. 0.1 M, in our hands have a specific electrical conductivity of  $\lambda = 1 - 5 \times 10^{-10} \Omega^{-1} \text{cm}^{-2}$ . This conductivity value, which is distinctly lower than those given by other investigators<sup>10,11</sup>, is practically time-independent. However, if the membranes are formed in solutions containing the strongly bound protein fraction, an increase with time in membrane conductivity is observed which is very rapid during

the first minutes but vanishes or at least becomes negligibly small after about 20 minutes. This effect, together with its experimental scatter, is demonstrated in Fig. 1. The highest values of  $\lambda$  thus obtained were around  $1 \times 10^{-6} \Omega^{-1} \text{cm}^{-2}$ . Conditions promising still higher conductivity values in nearly all cases led to membrane rupture.

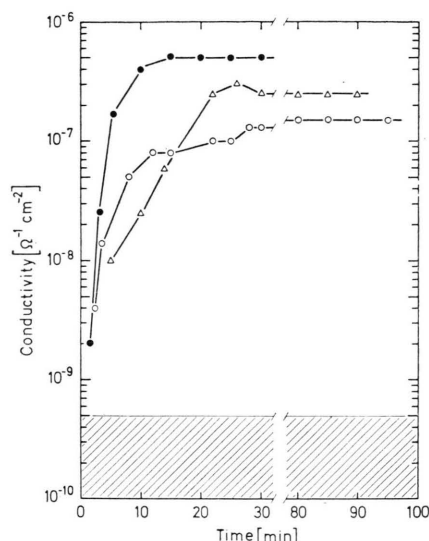


Fig. 1. Typical time course of membrane conductivity  $\lambda$  in the presence of the strongly bound proteins. Conditions: protein concentration  $c = 5.0 \mu\text{g}/\text{ml}$ ;  $\text{pH}^* = 10.0$ . Out of 8 films studied, one with an "average" behaviour, the one with the highest and that with the lowest final value of  $\lambda$  are shown. Dotted area: range of  $\lambda$  in the absence of protein.

The final value  $\lambda_f$  of BLM conductivity which is induced by the protein increases with increasing protein concentration  $c$ . It is, however, also influenced by another parameter: the highest pH-value  $\text{pH}^*$  applied during the preparation of the protein. This is demonstrated in Fig. 2. As can be seen from this figure, variation of  $\text{pH}^*$  at a fixed value of  $c$  can lead to variations of  $\lambda_f$  (as measured at pH 7.1) up to a factor of 100. Apparently, in the range  $\lambda_f = 10^{-9} - 10^{-6} \Omega^{-1} \text{cm}^{-2}$ , the data for each value of  $\text{pH}^*$  in the double-logarithmic plot of Fig. 2 can be fitted by straight lines, *i.e.* in this range the relationship between  $\lambda_f$  and  $c$  can be described by the equation

$$\lambda_f = k \cdot c^s \quad (1)$$

Interestingly, not only the constant  $k$  but also the slope  $s$  of these straight lines are dependent on  $\text{pH}^*$ . The variation of  $s$  with  $\text{pH}^*$  is shown in Fig. 3.

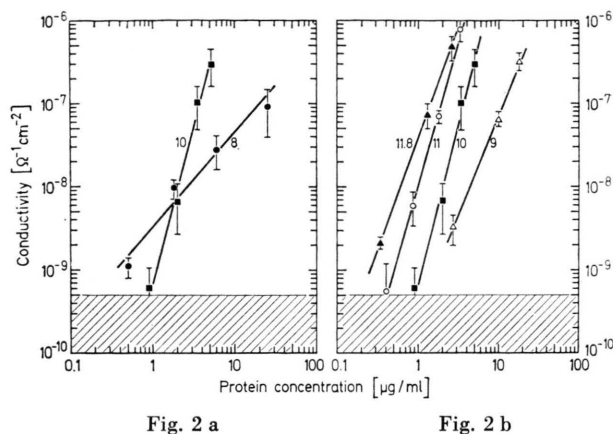


Fig. 2. Final membrane conductivity  $\lambda_f$  as a function of protein concentration  $c$  and of  $\text{pH}^*$ . The values of  $\text{pH}^*$  are 8.0 (●) and 10.0 (■) [Fig. 2a]; 9.0 (△), 10.0 (■), 11.0 (○) and 11.8 (▲) [Fig. 2b]. Each data point is the mean of at least 5 measurement. Bars: standard deviation of a single measurement. For reasons of comparison, the data for  $\text{pH}^* 10$  are shown in both drawings.

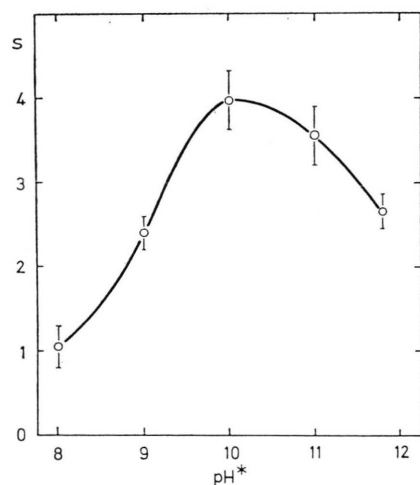


Fig. 3. The exponent  $s$  from equation (1) (i.e. the slope of the straight lines in Fig. 2) as a function of  $\text{pH}^*$ . Bars: estimated maximum error.

## Discussion

The results described in this paper confirm the earlier finding that the strongly bound protein fraction of the human erythrocyte membrane is able to recombine with black lipid membranes, thus inducing a strong increase in membrane electrical conductivity<sup>3,12</sup>. However, they also demonstrate that the properties of the system BLM-strongly bound proteins are highly affected by the pretreatment of the protein. The distinct response of the

BLM system to changes in the parameters connected to this pretreatment may, however, also help in studying the structure and assembly of the protein-induced conducting sites in the membrane.

Concerning the latter aspect of our findings, the data shown in Fig. 2 seem to be especially useful. Equation (1), which is obeyed by these data, is equivalent to that describing the formation of a complex of  $s$  molecules. Therefore, one interpretation of the results is that  $s$  protein particles must combine with each other in order to assemble a conducting site. However, according to Fig. 3  $s$  is not a fixed number, but, depending on the maximum  $\text{pH}$ -value  $\text{pH}^*$  used during protein preparation, varies between approx. 1.0 ( $\text{pH}^* 8$ ) and 4.0 ( $\text{pH}^* 10$ ). The pretreatment of the protein preceding the insertion into the lipid membrane at  $\text{pH} 7.1$  thus seems to lead to the formation of different types of building blocks of conducting sites, the relative amount of these building blocks depending on  $\text{pH}^*$ . Apparently, there must be at least two types of building blocks, and it seems probable that there is also more than one type of conducting site. The following hypothesis is consistent with the experimental data:

(1) After pretreatment at  $\text{pH}^* 8$  to  $\text{pH}^* 10$ , only one predominant type of conducting site is formed at  $\text{pH} 7.1$ . It is an aggregate of four subunits (which may or may not be identical and which may be aggregates themselves, the number of polypeptide chains per site thus being  $\geq 4$ ). These tetrameric sites can be assembled from different kinds of building blocks: after pretreatment at  $\text{pH}^* 10$ , the building blocks passing from the aqueous buffer into the membrane are the subunits, the tetrameric conducting site thus being assembled only in the membrane. This gives rise to  $s=4$ . After  $\text{pH}^* 8$ -pretreatment, the tetrameric aggregate already exists in the buffer and passes into the membrane without changes in its state of aggregation ( $s=1$ ). After treatment at  $\text{pH}^* 9$ , both subunits and tetramers can act as building blocks, a contribution of dimers also being possible.

(2) After pretreatment of the protein at  $\text{pH}^* 11$  and 11.8, in addition to the tetramer discussed above, a second type of conducting site occurs. These sites consist of only one or two subunits, thus leading to a decrease of  $s$ . Their occurrence may be a consequence of unfolding of the polypeptide chains at high  $\text{pH}$ .

Obviously, the essential assumption in this hypothesis is that the conducting sites formed after protein pretreatment at pH\* 8 and pH\* 10 are identical to each other. It is clear that, in the absence of data confirming this assumption, our hypothesis is only one of various possibilities.

Concerning the structure of the "subunits" mentioned above, we have recently shown that, after pH\* 10-pretreatment, that part of the protein which is responsible for the conductivity effects has an  $s_{20}$ -value of approx. 4.3<sup>13</sup>. This value agrees with that reported for the monomer of the band 3-protein<sup>14</sup>, the main component of the protein fraction used by us. In addition, preliminary BLM experiments using purified band 3-protein (purity approx. 97%)<sup>15</sup> instead of the strongly bound protein fraction have given results very similar to those of the latter protein sample (pH\* 10). Thus, the hypothesis described above can be extended such that the con-

ducting sites assembled after protein pretreatment at pH\* 8–10 are assumed to be tetramers of the band 3-protein. Interestingly, Wang and Richards have claimed that this protein exists as a tetramer in the native erythrocyte membrane<sup>16</sup>. (Other authors, however, could detect only dimers of the protein in the membrane as well as after membrane solubilization by Triton X-100<sup>17,18</sup>.) Since the band 3-protein most probably is involved in anion transport through the erythrocyte membrane (*e.g.*<sup>19–21</sup>), it seems worthwhile to study in more detail the properties of the conducting sites described, especially with respect to their ion specificity and its relation to that of the "conductance pathway" in the erythrocyte membrane<sup>22</sup>.

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