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EFFECTS OF TERBIUM ON THE HEMOCYANIN PORE FORMATION RATE IN PHOSPHATIDYLCHOLINE PLANAR BILAYERS

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Megathura crenulata **hemocyanin forms ionic channels in planar lipid bilayer membranes. It was found that hemocyanin is more potent as a channel former if $TbCl_3$ is added to the bathing solution. Furthermore membranes separating symmetrical $TbCl_3$ solutions show a pore formation rate which depends exponentially on the applied voltage, positive potentials favouring the insertion of new channels. The slope of this voltage dependence, which gives a measure of the effective charge displaced during the incorporation of one channel, increases and saturates with $TbCl_3$ concentration. The dose response curve indicates that binding of Tb^{3+} to the phosphatidylcholine bilayer is involved in creating the effective charge.**

Introduction

Megathura crenulata hemocyanin is known to form ionic channels in black lipid membranes [1], thereby increasing the bilayer conductance up to several orders of magnitude [2]. The extent of this increase, that is the number of channels formed in the membrane at steady state, depends strongly on the protein concentration [3] but also on the electrolytic solution composition; decreasing the pH and increasing the ionic strength at a fixed protein concentration have both the effect to raise the final conductance [4]. It was suggested that the decrease in electrostatic repulsion between the protein and the membrane due to screening of negative fixed charges present on both was responsible of the observed effect.

On the other hand it has been reported that in the presence of mono- and divalent cations the pore formation rate does not depend on the transmembrane voltage [5]. I present here evidence that when $TbCl_3$ is present in the bathing solution, not only the steady state conductance increases by increasing the ionic strength, but also the pore formation rate becomes voltage dependent in a way similar for example to that of alamethicin, a well studied pore forming antibiotic [6].

The dose response curve, and comparison with recently published NMR data on the interaction between lanthanides and phosphatidylcholine vesicles [7,8], suggest that this effect is due to specific binding of Tb^{3+} to the phosphatidylcholine bilayer.

Materials and Methods

Black lipid membranes were obtained by the usual technique [9] on a circular hole of about 0.4 mm diameter on a Teflon sept separating two aqueous solutions. The lipid used was saturated egg phosphatidylcholine (PC), more than 99% pure, purchased from P.L. Biochemicals, dissolved in *n*-decane at a final concentration of 50 mg/ml.

Abbreviation: Bistris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)-1,3-propanediol.

Electrolytic solutions were prepared either with KCl (Carlo Erba) or with $TbCl_3$ (Riedel-De Haën) and buffered at pH 7.0 with Bistris (Calbiochem) at a concentration 5% of that of the main electrolyte. *M. crenulata* hemocyanin, A grade in 50% glycerol was purchased from Calbiochem and stored at −20°C. Small amounts of a 4 mg/ml aqueous stock solution were added to one chamber only, *cis* side, when the membrane was completely black for at least 15 min. The membrane was connected through Ag-AgCl electrodes to the voltage source and to a virtual grounded current to voltage converter (AD 515K) with feedback resistance 10^7 Ω and feedback capacitance 200 pF. Potentials refer to the cis side. The experiments were run at room temperature.

Results

Additions of *M. crenulata* hemocyanin to the acqueous phase bathing a bilayer results in the irreversible formation of ionic channels which, under voltage-clamp conditions, appear as current jumps of fixed amplitude [4]. As already shown [5] after at least one hour from the addition of the protein, when hundreds to thousands channels are already present, the rate of increase in conductance becomes slow but steady (e.g., in Fig. 1 approx. 6 nS/h). It has been also shown that in the presence of KCl the magnitude of the hemocyanin doped membrane conductance in this steady state is strongly dependent on protein concentration [3], ionic strength and pH [4].

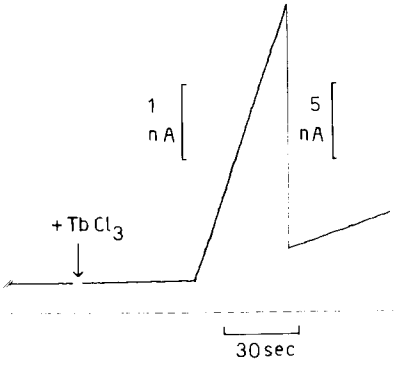

Fig. 1. Conductance increase after the addition of $TbCl_3$ to an hemocyanin doped PC bilayer bathed by 0.1 M KCl. Hemocyanin had been added about 50 min before this trace was recorded at a concentration of 100 μg/ml. About 300 channels were present at the beginning of this trace, recorded at −10 mV, and the conductance increased very slowly. Within 1 min from the addition, indicated by the arrow, of 2.5 mM $TbCl_3$ to the cis solution a great conductance increase developed. The trace has been interrupted to avoid electrical noise due to the insertion of a pipette. Note the change in the current scale after about 30 s of conductance growth. – – – –, zero current.

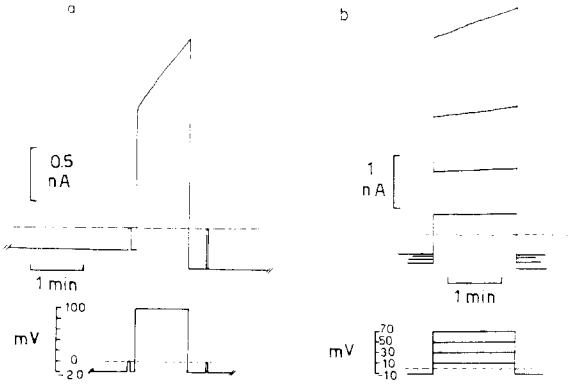

Fig. 2. Voltage dependence of the hemocyanin pore formation rate in the presence of a symmetrical 0.02 M $TbCl_3$ solution. (a) Hemocyanin was added about 70 min before the start of this trace at a concentration of 10 μg/ml. About 100 channels were present and the conductance at −20 mV was practically constant. Upon reversal of the potential to +100 mV a strong conductance increase is observed with time, which is irreversible as one can see returning to −20 mV. Once again, at the negative potential the conductance remained nearly constant. The dashed line indicates zero current and in the lower part is the pulse protocol used. (b) Superposition of consecutive current traces obtained at increasing positive potentials shows that the rate of growth of conductance increases with the applied voltage. Between one positive pulse and the other the membrane was held at −10 mV. Since the conductance increases are irreversible the amplitude of the current trace at −10 mV grows with time. Zero current and pulse protocol are indicated as in part (a).

In Fig. 1 I present the effect of adding a small amount of $TbCl_3$ to the cis compartment, filled with 0.1 M KCl, for an aged membrane.

Within few seconds from the addition of Tb^{3+} a strong increment in the rate of conductance increase is clearly observed. Control experiments (data not shown) indicate that addition of $TbCl_3$ after that hemocyanin has been removed by perfusion of the cis compartment with protein-free solution, does not induce any change in the conductance of the hemocyanin doped membrane. These facts lead to the straightforward interpretation that

the conductance increase is due to incorporation of new channels into the bilayer. In other words, hemocyanin is more potent as a channel former when $TbCl_3$ is present in the bathing solution.

These results are confirmed and extended by experiments in which the membrane separated symmetric solutions of $TbCl_3$ alone. 2.5 μg/ml of protein were sufficient in symmetrical 0.1 M $TbCl_3$ to have approximately the same steady-state conductance as with 100 μ/ml hemocyanin but 0.1 M KCl symmetrical. Furthermore in the presence of $TbCl_3$ on both sides, the rate of formation of new channels becomes voltage dependent. This is shown in Fig. 2. Part (a) demonstrates that application of a +100 mV square pulse leads to the irreversible opening of new channels at a much higher rate than at −20 mV, whereas in part (b) the conductance growing rates at four different positive potentials are compared.

From traces like those in Fig. 2b it is possible to calculate the rate of conductance increase as a function of the applied voltage. By dividing by the single channel conductance, obtained measuring the amplitudes of the first incorporation steps [4], the pore formation rate is derived. These data are

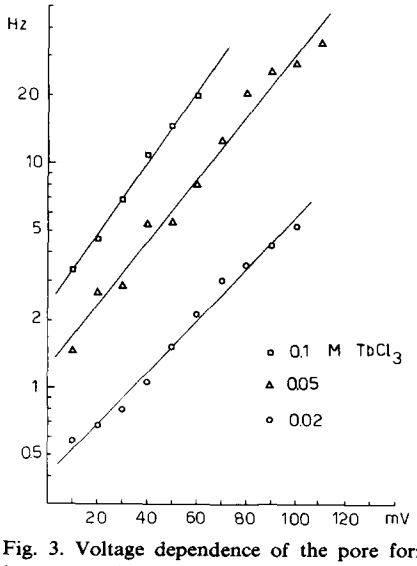

Fig. 3. Voltage dependence of the pore formation rate. The frequency of appearance of new channels is reported in a semilogarithmic scale versus the applied potential at three different $TbCl_2$ concentrations: 0.02, 0.05 and 0.1 M. Protein concentration was 10, 6 and 2.5 μg/ml respectively. Straight lines are least-squares fit to the points according to Eqn. 1 in the text.

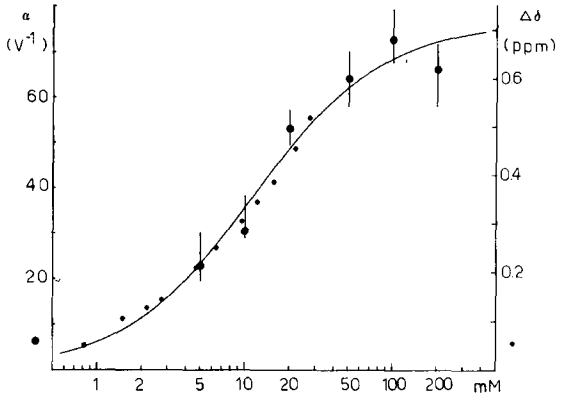

Fig. 4. $TbCl_3$ dose response curve relative to the slope of the pore formation vs. voltage exponentials (full points, left-hand scale). Error bars are obtained rocking the straight lines of Fig. 3 around the experimental points by eye and taking the maximum and minimum slope. The asterisks, righ-hand scale, are replotted from Fig. 1 of Ref. 7 and represent changes in chemical shift $\Delta\delta$ (ppm) of the $POCH_2$ (choline) proton resonances of egg yolk PC vesicles in ^2H_2O induced upon addition of $EuCl_3$. Solid line is a least-squares fit to the full points with Eqn. 3, which represent the titration of a single binding site for Tb^{3+} of binding constant 87.8 M^{-1}. The same procedure applied to the NMR data gives an apparent binding constant of 83.1 M^{-1} for Eu^{3+}.

presented in Fig. 3. Here the frequency of appearance of new channels is plotted in a semilogarithmic scale against the applied voltage for three different $TbCl_3$ concentrations. Straight lines are fairly good fit to the measured pore formation rates, which means that they depend exponentially on the applied voltage.

The slope of these lines increases increasing $TbCl_3$ concentration. A dose response curve for this parameter is shown in Fig. 4. On the same figure, for comparison, are also reported published NMR data of change in chemical shift of ^1H from PC vesicles in presence of different concentrations of $EuCl_3$ [7]. This change in chemical shift is due to a specific binding of lanthanides to the phosphatidylcholine headgroups.

Discussion

The efficiency of hemocyanin as a pore former in black lipid membrane depends on its concentration [3], but also on the composition of the solution [4]. In the presence of KCl, an equilibrium situation is reached within few hours from the

addition of the protein to one compartment, where the pore forming rate is very slow. As shown in Fig. 1, subsequent addition to the same compartment of a small amount of $TbCl_3$ induces a strong increase in the conductance, which grew a factor of 15 within 2 min from the addition of the lanthanide in that experiment. Since the single channel conductance is only a little decreased by the presence of Tb^{3+} in traces on the cis side (Menestrina, G., unpublished result) and since addition of Tb^{3+} after remotion of free hemocyanin from the solution has little or no effect at all, I conclude that the observed conductance growth is due to the insertion of new channels into the bilayer. In other words, the presence of Tb^{3+} changes the partition equilibrium between lipid and solution of hemocyanin, favouring the hydrophobic phase.

Fig. 2 shows that channels formation is irreversible and occurs at a rate which depends on the applied potential, the higher the positive voltage across the bilayer the higher the pore formation rate.

Measured pore formation rates, plotted in Fig. 3, depend exponentially on the applied voltage V, i.e., one can write:

$$\mu = \mu_0 \exp(\alpha V) \tag{1}$$

where μ is the pore formation rate, μ_0 is the pore formation rate in the absence of applied voltage and α is a constant which depends on Tb^{3+} concentration. Eqn. is completely analogous to the expression for the pore formation rate of alamethicin [6] and indicates that the insertion of one channel into the bilayer involves the movement of an effective charge q_{eff} across the applied voltage, with q_{eff} given by:

$$q_{eff} = \alpha \cdot kT \tag{2}$$

where k and T have their usual meaning.

The effective charge displaced depends upon $TbCl_3$ concentration showing a saturation, which suggests that Tb^{3+} is responsible in forming the charge. A dose response curve for this effective charge upon increasing $TbCl_3$ concentration is shown in Fig. 4. Experimental points are well fitted by a single binding titration curve, that is:

$$\alpha = \alpha_{max}(1 + (1/k_b c))^{-1} \tag{3}$$

where α_{max} is the saturating value of α at very high concentration of $TbCl_3$, k_b is an apparent binding constant and c is the concentration of $TbCl_3$; q_{eff} may be obtained substituting Eqn. 3 in Eqn. 2. A comparison with published NMR data of binding of $EuCl_3$ (Fig. 4 replotted from Ref. 7) to egg yolk PC vesicles, shows that there is striking correspondence between the apparent binding constant measured for Tb^{3+} in this work, 87.8 M^{-1}, and that of Eu^{3+}, 83.1 M^{-1}, under similar experimental conditions. Furthermore different lanthanides such as La^{3+}, Tb^{3+} and Eu^{3+} gave much similar binding curves to PC vesicles [8]. These facts suggest that the voltage dependence of the pore formation rate is due to adsorption of Tb^{3+} ions onto the PC bilayer and rules out, in my opinion, the influence of a possible binding of Tb^{3+} to the protein itself, since this has a binding constant of $(33\text{–}100) \cdot 10^2$ M^{-1} [10] about two orders of magnitude larger than that observed.

I will try to suggest here one possible explanation of how adsorption of $TbCl_3$ to the PC bilayer can induce a voltage-dependent pore formation rate as expressed by Eqns. 1 and 3. Binding of Tb^{3+} to the bilayer produces the appearance of a positive charge on the surface of the membrane, whose density σ is given by:

$$\sigma = \sigma_{max}(1 + (1/k_b c))^{-1} \tag{4}$$

where σ_{max} is the positive surface charge density when all sites are occupied, k_b is the apparent binding constant of Tb^{3+} and c its concentration. Let me further assume that the channel inserted into the bilayer is asymmetrical and occupies an area A_t at the trans side of the membrane, whereas only an area $A_c < A_t$ at the cis side. It is worth remembering that it is known from current-voltage curves that hemocyanin is asymmetrically oriented into the bilayer [1]. If this is the case, incorporation of a new channel into the membrane will cause the disappearance of a charge $\sigma \cdot A_t$ at the trans side and $\sigma \cdot A_c$ at the cis side. Of course the lipid in excess on each side does not disappear, an equivalent amount is rather supposed to get into

the torus surrounding the membrane, thereby losing its orientation. Since A_t is larger than A_c, this is equivalent to move a charge:

$$q_{eff} = (A_t - A_c) \cdot \sigma/2 \quad (5)$$

from the trans to the cis side. From this point of view a positive voltage will favour channel insertion into the bilayer because this implies the movement of a positive charge down the potential gradient. This give rise to a voltage dependence of the pore formation rate μ of the type:

$$\mu = \mu_0 \exp(q_{eff} V/kT) \quad (6)$$

where μ_0 is a constant related to the change in free energy of the system during the incorporation of the channel. Provided that:

$$\alpha_{max} = (1/2\,kT)\,(A_t - A_c)\,\sigma_{max} \quad (7)$$

Eqns. 6 and 4 are equivalent to Eqns. 1 and 3 used to fit the experimental data.

From Fig. 4 a maximum value for q_{eff} of approx. 2 e.u. can be estimated; since a maximum loading of about one lanthanide cation for each nine PC molecules has been derived from NMR data [7], one can give a rough value of 12 lipid molecules comprising the charge $(A_T - A_c) \cdot \sigma$. On the other hand, A_c has been directly measured by electron microscopic studies [11] and it corresponds, using the approximate value of 70 Å^2 for the cross-sectional area of a PC molecule, to about 110 phosphatidylcholine molecules. The model then demands that A_T is roughly 10% larger than A_c.

Finally also the zero-voltage pore formation rate μ_0 increases with $TbCl_3$ concentration, as is apparent from Fig. 3, probably for a decrease in electrostatic repulsion between the protein and the bilayer as already proposed to explain the effects of pH and ionic strength [4].

Acknowledgments

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