Fusion of Large Unilamellar Liposomes Containing Hemocyanin with Planar Bilayer Membranes

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Large unilamellar vesicles were prepared by detergent removal from micelles containing phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Liposomes were then interacted with *Megathura crenulata* hemocyanin, a well studied channel former. Incubation of the resulting proteoliposomes on one side of a phosphatidylserine-containing planar bilayer under fusion conditions yielded strong current increases. Such increase is due to insertion of ionic channels from the liposomes into the planar bilayer. Studying the effects of Ba^{2+} on the electrical properties of the channel we could show that the protein is always inserted into a bilayer during this process, *i.e.* fusion of proteoliposomes with the artificial membrane occurs. The strong non linearity of the current-voltage curve of the hemocyanin pore could be used as a probe of the extent to which fusion preserves the orientation of the channel through the bilayer.

Introduction

Membrane fusion is at the basis of many biological processes such as neurotransmission, secretion and phagocytosis. In order to understand the mechanism of these processes much efforts have been done by researchers in studying fusion of artificial membranes such as liposomes [1, 2]. Of particular interest has become now the study of fusion of unilamellar vesicles with planar lipid bilayers, since this is a currently adopted mean to achieve reconstitution of natural channels into the widely accessible artificial planar bilayer [3–5].

Incorporation of hemocyanin molecules into artificial unilamellar vesicles and subsequent fusion of these vesicles with planar lipid bilayers can give at least three kind of informations.

First, the hemocyanin channel, which is known to bind almost irreversibly to lipid bilayers and has a large conductance [6], can be used as a conductive probe for the study of vesicle fusion into electrically accessible planar bilayers. Conditions under which fusion occurs can be optimized in this way.

Second, the hemocyanin channel has a strongly non linear I–V characteristic and binds asymmetrically to lipid bilayers [6]. Non linearity of the I–V curve can thus be used to measure the extent to which membrane fusion preserves the asymmetrical insertion of the proteic components of proteoliposomes.

Third, preparation of vesicles which are known, by fusion experiments, to contain active hemocyanin channels, can be the basis to elucidate morphologically which is the structure adopted by this protein when forming channels.

We present here evidences that hemocyanin containing proteoliposomes can be prepared and made to fuse with planar bilayers, and that this procedure preserves the asymmetry of protein through the bilayer.

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Materials and Methods

Liposome preparation

Liposomes were prepared by the detergent dialysis method [7, 8]. Lipids used were either: phosphatidylethanolamine, PE (P. L. Biochemicals), phosphatidylcholine, PC (Lipid Products), phosphatidylserine, PS (Fidia Res. Laboratories) mixed in the ratio 5:2:3, preparation 1; or: saturated PE (P. L. Biochemicals), PC (P. L. Biochemicals), PS (Fidia Res. Lab.) in the same ratio as above, preparations 2 and 3. The lipids, dissolved in chloroform, were dried at 30 °C, under N_2 flow, by evaporation performed with a Rotavapor (Büchi). They were then dispersed in a 100 mM K Cl (Carlo Erba), 0.5 mM EDTA (Merck), 10 mM Bistris (Calbiochem) acqueous solution at pH 6.5

(buffer A) to a final concentration of 25 mg/ml (preparation 1) and 20 mg/ml (preparations 2 and 3). Micelles were produced mixing *n*-octyl-β-D-glucopyranoside (SIGMA) to a final lipid detergent molar ratio of 0.3, and gently stirring at ~ 15 °C for about 6 h. Liposomes were then prepared using the flow-through dialyzer LIPOPREP (Dianorm) equipped with cellulose membranes of 10^4 molecular weight cut-off (Diachema). Dialysis was run for 20 h at 18 °C. The whole procedure is designed to give unilamellar vesicles of about 200 nm diameter [9].

Protein and proteoliposome preparation

Megathura crenulata hemocyanin (Calbiochem) A grade in 50% glycerol is stored at −20 °C. Before use the protein was dialyzed for 24 h at 4 °C against buffer A. Asymmetric proteoliposomes were prepared adding hemocyanin to the liposome solution at a concentration of 1−4 mg/ml and mixing slowly for 12 h at 4 °C. This solution was then centrifuged at ~ 30000 × *g* for 20 min at 5 °C. The pellet was then redissolved in buffer A and centrifuged again. To get free from the protein in solution this procedure was repeated 4 to 6 times and then the vesicles were used in the electrical experiments with black lipid membranes, preparations 1, 2. Since hemocyanin incorporates irreversibly and with a well defined orientation in lipid bilayers, [6] these vesicles are supposed to bear oriented protein molecules on their surface. Control experiments were run also adding to the bilayer either directly hemocyanin or the last supernatant of the above procedure.

Alternatively proteoliposomes were prepared adding hemocyanin directly to the lipid-detergent micelles solution prior to flow-through dialysis. Liposome preparation by dialysis and free protein removal by centrifugation were then performed as above, preparation 3. This procedure is supposed to give unilamellar vesicles with protein absorbed, but not strictly oriented, on their walls. Control experiments were run also in this case with the last supernatant and with protein which has been mixed with the same concentration of detergent used in the proteoliposome preparation.

Electrical measurements

Black lipid membranes (BLM) were prepared by the usual technique [10] on a circular hole, 0.5 mm diameter, in a Teflon sept between two acqueous solutions. Lipids used were either a mixture 7:3 of PE and PS (Lipid Products) or saturated PC (P. L. Biochemicals) both 25 mg/ml in *n*-decane. Electrolytic solution was 100 mM KCl, 0.5 mM EDTA, 10 mM Bistris pH 7.0 (buffer B). After complete blackening of the membrane since several minutes, proteoliposomes or free protein were added to one compartment (cis side) only. Divalent cations, $BaCl_2$ or $CaCl_2$, and glucose (all Carlo Erba RPE) were then added to the bathing solution to achieve incorporation of the protein into the bilayer. Ag−AgCl electrodes provided electrical access to the membrane and a virtual grounded operational amplifier (AD 515 K) was used as I−V converter with a $2 \times 10^8\ \Omega$, 10 pF parallel feed-back circuit. Cis compartment is taken as the reference for voltage sign. Experiments were run at room temperature.

Results

Addition of small amounts of proteoliposomes from preparation 1 to one side of a PE/PS black lipid membrane failed to increase the bilayer conductance even after 38 min. Upon subsequent symmetrical addition of $BaCl_2$ to the solutions in the range 1−4 mM a slow conductance increase which occurred in discrete steps could be observed (Fig. 1). Conductance jumps, measured at −40 mV in voltage clamp conditions, are almost all of the same size, which corresponds to the conductance of one open hemocyanin channel in these conditions [11]. Nevertheless higher jumps, whose amplitude was a multiple of the single channel conductance, could be seen sometimes. They rose as single steps at least within our experimental time resolution. Channels formation rate could be strongly increased adding 100 mM glucose to the *cis*, *i.e.* the vesicle containing, compartment, as shown in Fig. 1. Symmetrical addition of glucose to both compartments had the same effect as on the *cis* side only, in good agreement with the finding that the establishing of an osmotic gradient through the liposomes walls and not through the planar bilayer is the effect responsible for vesicles' fusion with the planar bilayer [12]. In one experiment glucose was added to a bathing solution, in which proteoliposomes have been added since 30 min, at the same concentration as above but without divalent cations present. No conductance change was observed within 55 min in

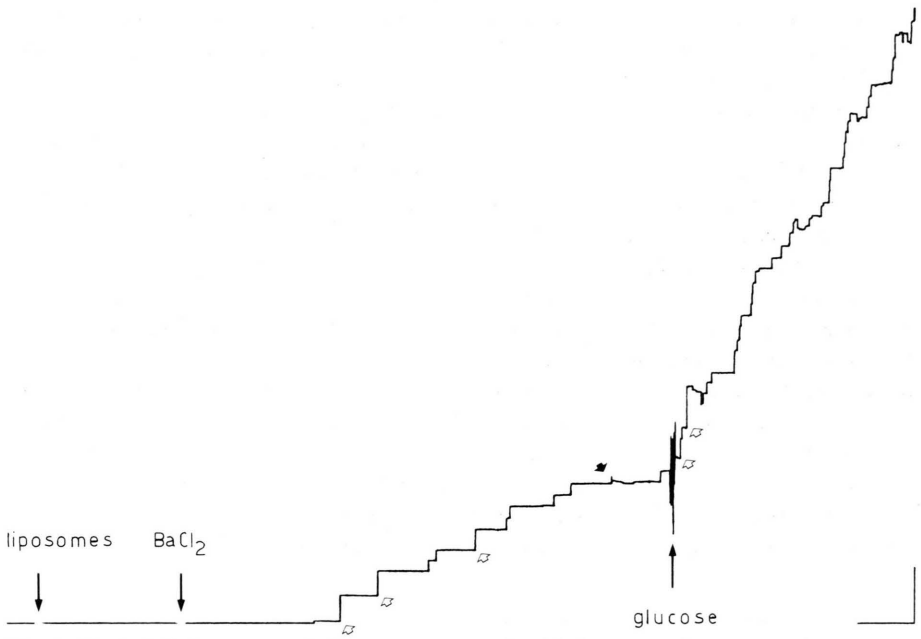

Fig. 1. Typical fusion protocol. Liposomes treated with hemocyanin, preparation 1, were added to a planar bilayer bathing solution where indicated. Addition of 1.25 mM $BaCl_2$, indicated on the figure, induced a current increase through the bilayer after a time lag. Subsequent instauration of an osmotic gradient through the vesicles with 100 mM glucose *cis*, also indicated in Fig. 1, led to a strong increase of the pore formation rate. Discrete steps are usually all of the same size, corresponding to a single hemocyanin channel. Higher steps, which correspond to the simultaneous opening of several channels, are also present in the figure and they are indicated by open arrows. The percentage of multiple steps in this experiment is higher than usual. The opening of one reversed channel, followed by transitions to lower conductance values, is also indicated, full arrow. Vertical bar 30 pA, horizontal bar 6 min.

Table I. Pore formation rates expressed as number of channels appearing per second in various conditions. The effects of three different liposomes preparations, see materials and methods, in two kind of BLM are presented. The three columns refer to pore formation rates with vesicles alone in solution, after symmetrical addition of $BaCl_2$ and after instauration of an osmotic gradient through the liposomes walls, respectively. Where the pore rate is zero the mean and s.d. of the time intervals waited, in minutes, is reported in parenthesis.

Preparation	BLM composition	Pore formation rate (Hz)			Number of experiments
		vesicles	+ Ba^{2+}	+ glucose	
1	PE/PS	0 (20 ± 10)	0 (11 ± 3)[a]	$7.6 \pm 3.6 \times 10^{-3}$	4
2	PE/PS	$9.5 \pm 0.7 \times 10^{-3}$	$1.3 \pm 0.5 \times 10^{-1}$	$1.7 \pm 0.2 \times 10^{-1}$	2
3	PE/PS	0 (14 ± 6)	$1.6 \pm 0.1 \times 10^{-2}$	8.1×10^{-2}	2
1	PC	0 (14)	0 (7)	0 (60)[b]	1
2	PC	0 (60 ± 20)	$5.7 \pm 1.9 \times 10^{-3}$	–	2

[a] In one experiment a pore formation rate of 2.4×10^{-3} Hz has been observed.
[b] Breaking the PC bilayer, painting a new one with PE/PS and restoring the osmotic gradient produced incorporation of channels in the new membrane.

these conditions. Subsequent addition of $BaCl_2$ was also ineffective, but then the restoring of the osmotic gradient through the vesicles, with 300 mM glucose in the *cis* solution, was able to induce channel incorporation into the bilayer within a few minutes.

Experiments were run also using planar lipid bilayers comprised of phosphatidylcholine alone. No conductance increase was induced by proteoliposomes of preparation 1 into a PC bilayer even after addition of $BaCl_2$ and glucose, see Table I. As a control this PC bilayer was intentionally broken

after about 2 h and a new black film was prepared with the PE/PS mixture. Also in this case restoring of the osmotic gradient, with 300 mM glucose in the *cis* side, in the presence of divalent cations, was necessary and sufficient to induce protein incorporation into the new PE/PS planar bilayer.

Use of Ca^{2+} instead of Ba^{2+} gave similar results (data not shown) but was avoided because of the great effects of Ca^{2+} on the current voltage curve of the channel which will be discussed later.

Somehow different results were obtained with preparation 2 which differs only slightly in the lipid composition of the liposomes, see materials. The incorporation rates obtained in this case are summarized and compared to those obtained with the other preparations in Table I. Briefly: slow incorporation was observed in PE/PS BLM without divalent cations; the incorporation rate was greatly increased by addition of 2 or 4 mM $BaCl_2$ but then it was only slightly enhanced by the creation of an osmotic gradient through the vesicles. Experiments with PC planar bilayers are also reported. No conductance increase was observed within at least 40 min from proteoliposomes addition, but slow increases followed addition of $BaCl_2$ to the bathing solution.

The last supernatant of each preparation was tested repeatedly and gave no or very low effects, *i.e.* few channels in the worst case and in presence of $BaCl_2$.

Further informations about the mechanisms of channel incorporation can be obtained studying the instantaneous and the steady state current voltage curves of the hemocyanin doped membranes. Instantaneous I–V characteristics are non linear and depend strongly on the electrolytic solution compo-

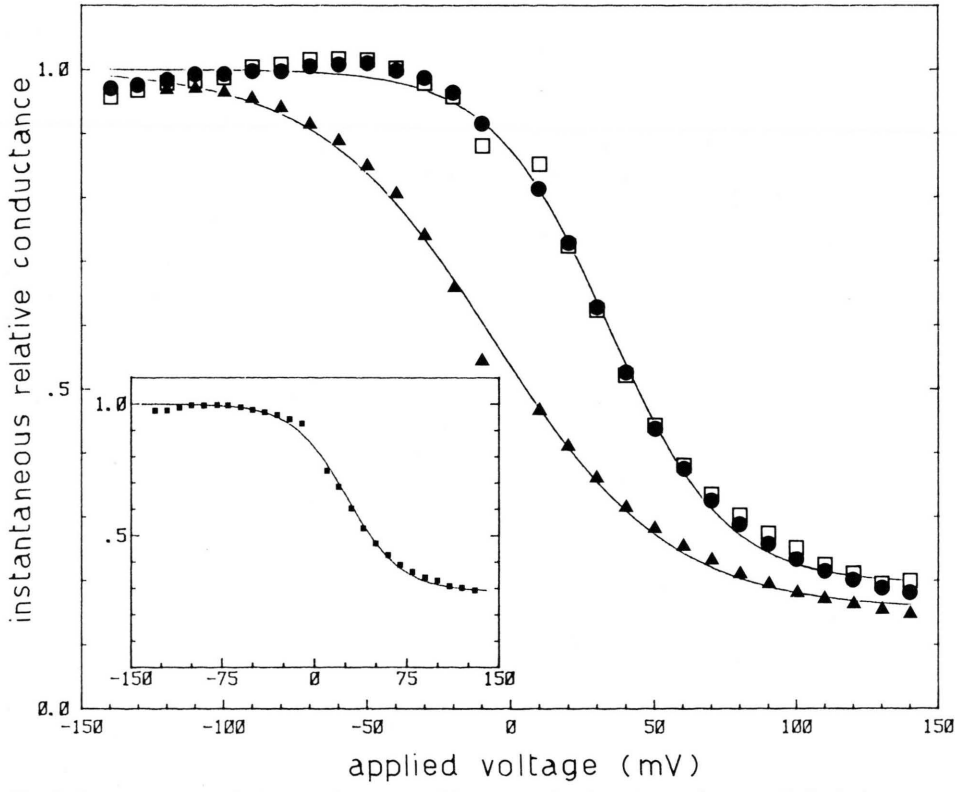

Fig. 2. Instantaneous relative conductance of hemocyanin doped membranes. Full circles represent membranes in which hemocyanin was added directly and without $BaCl_2$ in solution, whereas full triangles represent similar experiments in which 1.25 mM $BaCl_2$ was present in the solution. Open squares come from membranes doped by hemocyanin *via* proteoliposomes in the presence of 2 mM $BaCl_2$, preparation 2. Full squares in the inset are also obtained with the fusion technique but with preparation 3. Solid lines are least squares fit to the points using Eq. (4). The parameters obtained are: $B = 0.203$, $V_0 = 33.7$ mV, $Q = 0.050$ mV^{-1} (●, □); $B = 0.156$, $V_0 = -6.2$ mV, $Q = 0.032$ mV^{-1} (▲); $B = 0.288$, $V_0 = 26.8$ mV, $Q = 0.045$ mV^{-1} (■). Each point on the curves is the mean of 2–4 experiments.

sition [11, 13]. The conductance of the doped membrane can be calculated by dividing the instantaneous current value by the applied voltage. These curves are S-shaped and allow extrapolation of two asymptotical conductance values, one for large negative potentials, G_{max}, and one for large positive potentials, G_{min}.

Relative conductances, defined as:

$$G_{rel}(V) = G(V)/G_{max} \quad (1)$$

and measured in different conditions are plotted in Fig. 2 as a function of the applied voltage. Each one is an S-shaped function of voltage which can be defined by three parameters, the lower asymptote: $B = G_{min}/G_{max}$, the abscissa of the inflection point: V_0 and the slope of the curve at this point: Q. When hemocyanin is added directly to a 0.1 M KCl solution a strongly non linear $G_{rel} - V$ curve is obtained with a positive V_0 of about + 40 mV and a value of $Q \cong 0.050\ mV^{-1}$, full circles in Fig. 2. On the other hand even small amounts of divalent cations present in the bathing solution can change the $G_{rel} - V$ curve dramatically. Full triangles indicate experiments performed with free hemocyanin, in which 1.25 mM $BaCl_2$ was present in the solution from the beginning. It is apparent that the $-G_{rel}$-V curve is strongly shifted to a $V_0 < 0$ and that its slope is slightly decreased, $Q \cong 0.032\ mV^{-1}$. The same amount of Ba^{2+} failed to produce any change in the G/V curve when added to the solution after channel's incorporation, data shown in Fig. 3. This effect is known [14], it seems common to many

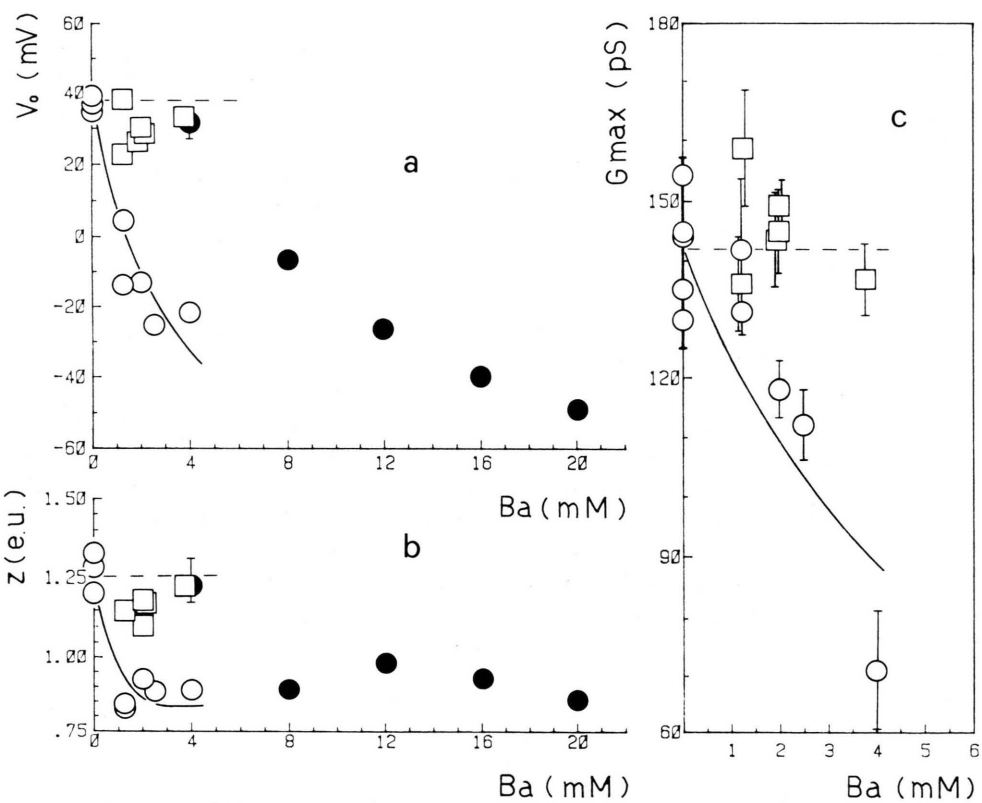

Fig. 3. Effects of Ba^{2+} on the electrical properties of the hemocyanin channel in different conditions. Open circles: hemocyanin added directly to the bilayer in the presence of $BaCl_2$. Full circles: hemocyanin added directly, but $BaCl_2$ added only after the insertion of the channels into the planar bilayer. Open squares: hemocyanin added by proteoliposomes fusion in the presence of $BaCl_2$. a) shift of the abscissa of the inflection point of the G-V curve, V_0, towards negative voltages in the presence of Ba^{2+}; b) decrease in the slope of the G-V curve at the inflection point, Q. The slope is expressed in *e.u.* in the figure making use of the relation $Q = z\,e/k\,T$ valid for a two state model [11]; c) change in the maximum conductance of the channel, G_{max}, with Ba^{2+} concentration. Each open circle or square represent a different experiment, variations of the parameters during the same experiment are within the dimensions of the symbols. Full circles come all from the same experiment, but the s.d. of three experiments at 4 mM $BaCl_2$ is indicated by the vertical bar. Dashed lines are the mean of the values measured without $BaCl_2$, whereas solid lines are drawn by eye.

plurivalent cations and it has been discussed in detail in the case of Ca^{2+} and Tb^{3+} [15]. Interestingly enough, membranes which were doped with hemocyanin via proteoliposomes, even in the presence of 2 mM $BaCl_2$, give G_{rel}-V curve which coincide within experimental error to those obtained interacting directly the protein with a BLM in the absence of divalent cations, open squares of Fig. 2. This fact indicates two things: first, incorporation of hemocyanin into a planar bilayer either directly or *via* proteoliposomes gives G-V curves with the same degree of non-linearity; second, small amounts of Ba^{2+} can strongly change the G-V curve of bilayers doped directly with hemocyanin but leave unaffected that of membranes doped *via* proteoliposomes.

The effects of Ba^{2+} concentration on the electrical properties of the hemocyanin channel in three kind of experiments are shown in Fig. 3. Open circles represent experiments in which hemocyanin was added directly to the bilayer solution in the presence of Ba^{2+}. Full circles instead are from experiments in which Ba^{2+} was added in a second time to a bilayer which incorporated hemocyanin directly but in the absence of divalent cations. A complete perfusion of the *cis* compartment before Ba^{2+} addition has been done in these cases to remove the excess of free protein present in the solution. This was necessary in order to avoid incorporation of new channels into the bilayer. In this way the effects of Ba^{2+} on channels which were already incorporated into the BLM have been tested. Finally open squares represent membranes that interacted with proteoliposomes. Fig. 3a shows the effects of Ba^{2+} on the abscissa of the inflection point of the G-V curve. With hemocyanin added directly to the bilayer V_0 was strongly shifted towards negative voltages by Ba^{2+} present in the solution from the beginning, whereas the same shift occurred at a considerably higher concentration when Ba^{2+} was added after channel opening.

Similar effects have been observed on the slope of the G-V curve at the inflection point, Fig. 3b. Also in this case Q was strongly reduced by addition of Ba^{2+} prior to channel formation, but to a much lesser extent by Ba^{2+} added after protein incorporation. Remarkably, both parameters remained unchanged with low Ba^{2+} concentrations when hemocyanin channels were incorporated *via* liposomes, *i.e.* the effects of Ba^{2+} in this case were similar to those observed when it was added after channel opening. Ca^{2+} gave stronger effects even at low concentrations, confirming that the affinity of this cation for the channel is higher than that of Ba^{2+} [14, 15].

Fig. 3c shows the effects of Ba^{2+} on the maximum conductance of the channel, G_{max}. This is strongly reduced by the divalent cation if hemocyanin is added directly to the BLM, but is left almost unchanged if the protein is incorporated *via* proteoliposomes.

Preparation 3 was similar in the lipid composition to preparation 2 and indeed incorporation rates before and after addition of $BaCl_2$ and after instauration of an osmotic gradient were comparable, see Table I. Nevertheless, since hemocyanin was present in the micelles solution during preparation of liposomes, strict insertion asymmetry was supposed to lack in this proteoliposomes. Indeed, as shown in the inset of Fig. 2, the non linearity of the G-V curve was lower with this preparation. This is exactly what one expect if some of the channels are inserted in the reversed direction, *i.e.* they reach G_{max} at high positive potentials.

Further evidence for this interpretation comes from the study of the steady-state current voltage curves. Hemocyanin doped bilayers are known to show a time dependent decrease of current when long lasting positive voltage pulses are applied [16]. This phenomenon is due to voltage dependent transitions of the channel between several conformational states which have different conductances. Negative voltages keep the channel in the most conductive state, while positive voltages favour states of increasingly lower conductance [17]. Steady state conductance defined as:

$$G_{SS}(V) = G_{t\to\infty}/G_{t=0} \tag{2}$$

are plotted *vs.* applied voltage in Fig. 4. Once again membranes doped either directly by hemocyanin or *via* proteoliposomes of preparations 1, 2 in presence of 2 mM $BaCl_2$ showed steady state conductances which were equal within experimental errors. In particular no time dependent current decrease was observed at negative voltages. On the contrary, incorporation of channels from liposomes of preparation 3 gave rise to membrane conductances which decreased for positive and for negative applied voltages. This is shown in the inset of Fig. 4.

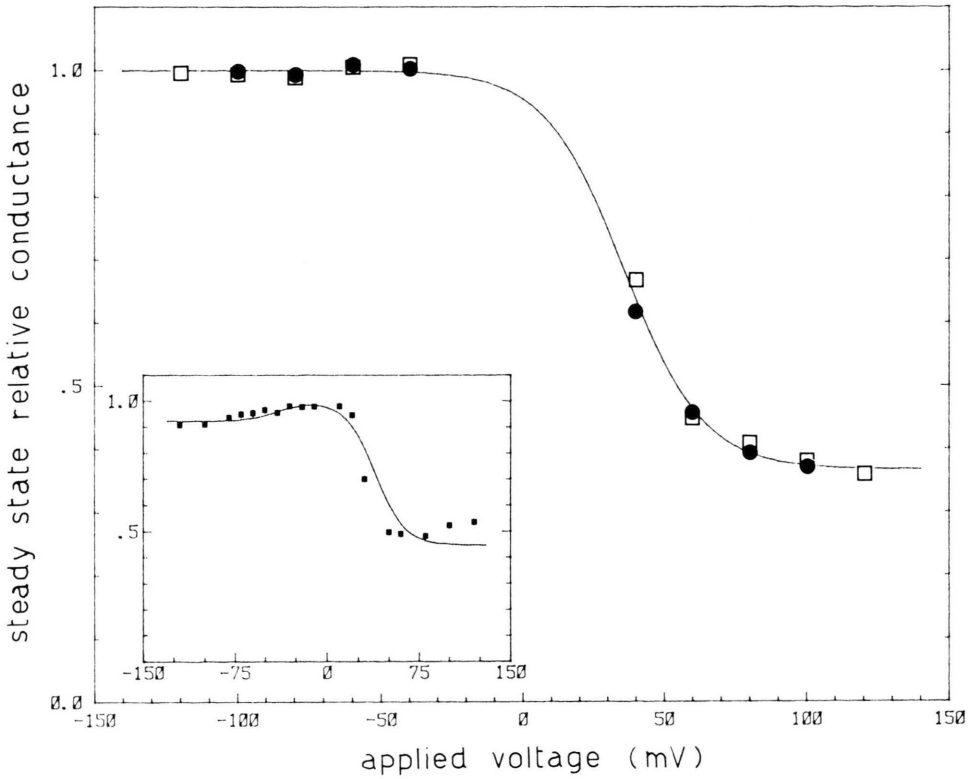

Fig. 4. Steady state relative conductance of hemocyanin doped membranes. Full circles: hemocyanin added directly to the bilayer without $BaCl_2$ in solution. Open squares: hemocyanin added by fusion of proteoliposomes of preparation 2 with 2 mM Ba^{2+} present. Solid line is a least squares fit to the points using eq. 4; the parameters obtained are: $B = 0.368$, $V_0 = 36.4$ mV, $Q = 0.076$ mV^{-1}. Inset: fusion of proteoliposomes of preparation 3 with the bilayer. Solid line is a least squares fit to the points with Eq. (5) and with the above values of B, V_0 and Q, which gives a fraction of 12.2% channels with reversed orientation.

Anyway also in this case the non linearity was much stronger with positive potentials.

Discussion

We have shown that under appropriate conditions interaction of planar lipid bilayers with large unilamellar vesicles, containing negative lipids, and doped with hemocyanin yields stepped increases of membrane conductance. These jumps are of the appropriate height to be attributed to the opening of hemocyanin channels into the bilayer [11]. We believe that incorporation of channels occurs *via* fusion of pore-containing liposomes with the bilayer. This is strongly supported by the observation that divalent cations are necessary to have substantial incorporation into a PE/PS membrane and that instauration of an osmotic gradient through the vesicles walls can enhance the incorporation rate, Fig. 1 and Table I. Both these facts are consistent with the most recent studies on membrane fusion [12, 18]. Furthermore, as one should expect [12] creation of an osmotic gradient alone, without divalent cations in solution, is ineffective for channel incorporation. Preparation of the planar bilayer with pure PC inhibits the protein incorporation completely, preparation 1, or at least by two orders of magnitude, preparation 2, which is also consistent with published data on fusion between vesicles and of vesicles with planar bilayers [19–21]. On the other hand the alternative explanation that free hemocyanin molecules are present in the vesicle solution and migrates to the bilayer through the acqueous phase in a hydrated form is made highly

improbable by the following observations. a) Addition of samples of the last supernatant of each preparation fails to give conductance increases or in the worst cases, produces only few channels after addition of Ba^{2+} to the solution, which might argue also for the presence of small proteoliposomes in the natant. b) Incorporation rate is dependent on phospholipid composition of the liposomes and on the instauration of an osmotic gradient through their walls in presence of divalent cations, Table I, which should not be the case if free hemocyanin is involved. c) Single channel conductance is strongly decreased ($\sim 50\%$) by 4 mM $BaCl_2$ if hemocyanin incorporates directly into the bilayer, whereas it remains constant at the same Ba^{2+} concentration if it is added *via* proteoliposomes, Fig. 3c. d) This last point deserves a longer discussion. Conductance voltage curves of hemocyanin treated bilayers are S-shaped and can be described by the following expression:

$$G(V) = G_{min} + (G_{max} - G_{min})/(1 + \exp Q(V - V_0)) \quad (3)$$

or, by dividing by G_{max}, and putting $G(V)/G_{max} = G_{rel}(V)$ and $G_{min}/G_{max} = B$

$$G_{rel}(V) = B + (1 - B)/(1 + \exp Q(V - V_0)) . \quad (4)$$

This expression comes from a two state model of the channel [11] but we can use it here as an empyrical one. Three parameters describe the voltage dependence of the hemocyanin channel conductance: the lower asymptote, B, the slope of the curve at the inflection point, Q, and the abscissa of this point, V_0. Fig. 2 and 3 demonstrate that the presence of $BaCl_2$ in the bathing solution strongly affects the values of the parameters V_0 and Q when free hemocyanin is added. On the other hand, no change of these parameters occur if 4 mM $BaCl_2$ is added in the solution after the incorporation of free hemocyanin has already been accomplished in a divalent cation-free solution. This indicates that Ba^{2+} at this concentration does not interact with the protein already inserted into the lipid bilayer. Notably the presence of up to 4 mM Ba^{2+} in the solution when proteoliposomes are used to incorporate hemocyanin into the BLM fails to change the parameters of the many-channel G-V curve. This gives an evidence that no free molecules are present but only molecules already incorporated into a lipid bilayer, *i.e.* those absorbed on the liposomes.

The most puzzling result to us is that incorporation usually occurred through single channel openings, and multiple simultaneous insertions were only rarely observed even when we tried to augment their probability increasing vesicle size and/or protein concentration during vesicle treatment. We think that this might be due to a destabilization of vesicles containing more than one channel due to the large size of this pore [22].

Divalent cations-induced fusion of PE/PS containing vesicles is thought to occur through a bilayer to hexagonal phase transition of the lipid mixture at the contact region [19, 23]. This transition introduces a local disorder into the bilayer. Accordingly it is interesting to see if fusion of protein-containing vesicles with a planar bilayer can save protein orientation, and to what extent. Hemocyanin treated liposomes are suitable from this point of view, because hemocyanin channels have a strongly non linear I-V curve, and are known to incorporate all with the same orientation into the bilayer and to maintain this orientation with time [6]. An upper limit of 2% reversed channels has been given for incorporation of hemocyanin into a bilayer in asymmetrical conditions [6] like those adopted for preparations 1, 2.

Given the almost perfect coincidence of the conductance-voltage curve of membranes doped adding the protein directly to the solution and membranes in which fusion with hemocyanin-containing liposomes was achieved, Fig. 2, we can say that fusion preserved orientation within the initial margin of error, the outer face of the liposomes remaining on the *cis* side of the planar bilayer after fusion. That this method can detect the presence of reversed channels is shown by the effects of proteoliposomes from preparation 3. As shown in the inset of Fig. 2, G-V curves with this preparation, which is not strictly asymmetrical, have a higher value of the lower asymptote B, which is consistent with the presence of 8–10% reversed channels in those experiments. A more quantitative analysis is given in Fig. 4, where the steady state properties of the modified membranes are presented. The steady state relative conductance, Eq. (2), of the hemocyanin channel can also be described empirically by the sigmoid curve of Eq. (4). As in the case of the instantaneous G-V curve incorporation of free hemocyanin or of proteoliposomes of preparations 1, 2 into a BLM gives steady state conductances

which are coincident within experimental error, Fig. 4. This allows us to obtain the parameters B, Q and V_0 by a least squares fit of Eq. (4) to these points. Proteoliposomes of preparation 3 instead show a slight non linearity at negative voltages besides that large at positive ones, inset of Fig. 4. Assumig that this is due to the presence of reversed channels in the membrane one can write the following expression for the steady state conductance in this case:

$$G_{SS} = (1-f)[B + (1-B)/(1 + \exp Q(V - V_0))] + f[B + (1-B)/(1 + \exp Q(-V - V_0))] \quad (5)$$

where f is the probability to have a reversed channel.

Introducing the values of B, Q and V_0 calculated above into Eq. (5) one can calculate f by a least squares fit to the points on the inset of Fig. 4. This procedure gives $f = 0.12$ in good agreement with the value deduced from the instantaneous conductance values.

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