Eur Biophys J (1985) 12 · 33–41

European Biophysics Journal

On the interaction of hemocyanin with lipid bilayer membranes

F. Pasquali and G. Menestrina*

Dipartimento di Fisica, Università degli Studi di Trento, I-38050 Povo (TN), Italy

Received July 10, 1984/Accepted December 17, 1984

Abstract. Osmotic jump experiments were used to measure the ionic permeability induced in lipid vesicles by *Megathura crenulata* hemocyanin. It was found that this protein strongly increases the conductance of K^+ and Cl^- through these membranes but not that of $SO_4^=$. These effects were attributed to the formation of ionic channels in the vesicles. We have found that a simple first-order binding model can explain the dependence of the number of pore-containing vesicles both on the time after exposure to hemocyanin and on the protein concentration. Milder effects were attributed to a non-specific adhesion of the protein to the membrane surface. Consistent with the hypothesis of reversible association, vesicles which retained hemocyanin after step sucrose density gradient centrifugation at low ionic strength, lost most of the protein upon recentrifugation at high ionic strength. Consistent with the hypothesis of channel formation both the above vesicle preparations transferred voltage-dependent hemocyanin channels into planar bilayers when they were made to fuse with them. It is concluded that hemocyanin can interact both specifically, by forming pores within the hydrophobic core of lipid membranes, and non-specifically, probably by means of electrostatic interaction with the surface of the same membrane.

Key words: Hemocyanin, lipid vesicles, planar bilayer, ion permeability, osmotic jump, membrane fusion, ionic channel

* To whom reprint requests should be sent

Abbreviations: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine, DOC, sodium deoxycholate

Introduction

Megathura crenulata hemocyanin interacts with black lipid membranes (Pant and Conran 1972) and forms ionic channels through them (Latorre and Alvarez 1981), even though it is widely recognized as a highly soluble protein (Van Holde and Miller 1982). In a recent attempt to clarify the structure of the hemocyanin channel, using electron microscopy, McIntosh et al. found that hemocyanin associated with lipid structures in the form of "70 Å annuli" (McIntosh et al. 1980). Since most of these experiments were performed with lipid vesicles we have tried to demonstrate the formation of hemocyanin pores in this same model system, which differs from the black lipid membrane at least in the complete absence of any organic solvent.

We have thus studied the permeability of lipid vesicles, which were exposed to hemocyanin, by the light-scattering changes caused by osmotically induced volume changes. A decrease in the light-scattering signal is expected to parallel the formation of channels in the liposomes, since vesicles bearing a pore equilibrate immediately, without changing their volume, in gradients of ions which can permeate through the pore. In this way, we have seen that hemocyanin strongly increases the permeability of vesicles for K^+ and Cl^- but not for $SO_4^=$, which is consistent with the selectivity of the channel measured in planar bilayers (Cecchi et al. 1982). We have also found that a simple first-order binding model can be used to describe the reaction of channel formation in this system. As a further step, vesicles exposed to this protein were purified by sucrose density gradient centrifugation at low and high ionic strength and then made to fuse with planar bilayers. This procedure was adopted to ensure that active hemocyanin channels were indeed present in the vesicles, and that they were anchored to the lipid matrix by hydrophobic interactions.

Materials and methods

Reagents and buffers

All reagents used, including glucose and sucrose, were from Carlo Erba, analytical grade. EDTA was purchased from Merck and Hepes from Sigma. Solutions normally contained 100 mM KCl, 1 mM EDTA and 5 mM Hepes, which will be referred to as Hepes buffer; the pH was adjusted with KOH and will be specified in the text. Solutions of different composition, when used, are indicated in the text.

Light-scattering experiments

Liposome volume changes induced by osmotic imbalances have been used to study their ionic permeability in the presence of a pore former (Kometani and Kasai 1978). Liposomes were prepared as follows: 120 mg egg PC (BDH) dissolved in chloroform was dried at room temperature in a rotary evaporator (Büchi) under a stream of nitrogen and was then connected to high vacuum for several hours; 6 ml of Hepes Buffer, pH 8.0, containing DOC was added to the dry film in a 1 : 1 lipid : detergent molar ratio, and the solution was gently stirred until clear (6–12 h). DOC was then removed from this mixed lipid-detergent micelle solution by 16–18 h dialysis at 18° C against 600 volumes of Hepes Buffer, pH 8.0, in a flow-through dialyzer, LIPOPREP (Dianorm), equipped with cellulose membranes of 10^4 molecular weight cut-off (Diachema). As previously reported, flow-through dialysis is much more efficient for detergent removal than conventional dialysis (Milsman et al. 1978; Zumbuhel and Weder 1981). Volume changes after osmotic jumps were detected by measuring the absorbance at 310 nm in a Perkin-Elmer 551 spectrophotometer connected to an x-y plotter. It was assumed that all changes in adsorption were due to variations in light-scattering deriving from shrinking or swelling of the vesicles (Van Zoelen et al. 1976; Bangham et al. 1974). Typical experiments were run by adding 900 µl of either Hepes Buffer, pH 7.0, or the same medium plus 1 M KCl or plus 0.5 M $K_2 SO_4$ to a 1 cm optical path length quartz cuvette, thermostatted at 35° C, containing 90 µl of liposome solution. After thorough mixing of the solution, which took about one second, absorbance was recorded continuously. To test the effects of the pore former on vesicle permeability, *M. crenulata* hemocyanin (Calbiochem), dialyzed overnight against Hepes Buffer, pH 7.0, and centrifuged at 10,000 rpm for 20 min, was added to the liposome stock solution (time 0) at a final protein concentration between 2 and 4 mg/ml. Osmotic jump experiments were then performed, as described above, at successive time intervals.

Preparation of purified proteoliposomes for fusion experiments

Liposomes were prepared by detergent removal as described above, except that the lipid composition was changed to a more fusogenic one (Miller et al. 1976; Düzgünes and Ohki 1981) i.e., 15 mg/ml PC, PE, PS in a molar ratio 2 : 5 : 3. Proteoliposomes were prepared by mixing 2 ml of this solution with 1 ml of *M. crenulata* hemocyanin prepared as above. Two procedures were then followed: in preparation 1, vesicles consisting of PC (Lipid Products), PE (Fidia Res. Lab.), PS (Calbiochem) were mixed with hemocyanin at a final protein concentration of 4 mg/ml and after one hour were made 40% in sucrose and further equilibrated for several hours against Hepes Buffer, pH 7.0, with 40% sucrose; in preparation 2, vesicles consisting of PC (Lipid Products), PE (Fidia Res. Lab.), PS (Lipid Products) were mixed with hemocyanin at a concentration of 2 mg/ml, were made 5% in sucrose and then equilibrated by dialysis, as described above, in 5% sucrose.

Proteoliposomes were purified by centrifugation on a discontinuous sucrose gradient made of three layers of Hepes Buffer, pH 7.0, containing 5%, 40%, 50% sucrose, from top to bottom, with a volume ratio of 2 : 1 : 1 in the same order. Proteoliposomes of preparations 1 and 2 were layered in the middle and top layer respectively; centrifugation was run to equilibrium at 42,000 rpm in a Sorvall Ti-865 rotor for 15 h at 4° C. Five fractions, numbered from the bottom, of approximately the same volume were collected by puncturing the tubes. Fractions 1–3, which were clear, were dialyzed extensively against Hepes Buffer, pH 7.0; fractions 4 and 5, which were turbid, against the same buffer plus 2% sucrose. Absorption spectra from 500 to 200 nm were then taken for each sample in quartz cuvettes of appropriate optical pathlength. Parts of these fractions were used for bilayer experiments as described in the following sections. As a further purification step, some of fraction 4 of preparation 1, containing vesicles, was made 1.1 M in KCl and 5% in sucrose, layered on a sucrose density gradient and centrifuged again as above except that the 40% and 50% sucrose layers also contained 1.1 M KCl, (preparation 3). Fractions were collected, dialyzed, and tested as above.

Planar bilayer experiments

Black lipid membranes were prepared by the usual technique (Szabo et al. 1969) on a circular hole of 0.5 mm diameter in a Teflon septum between two compartments of equal volume (~ 4 ml) filled with

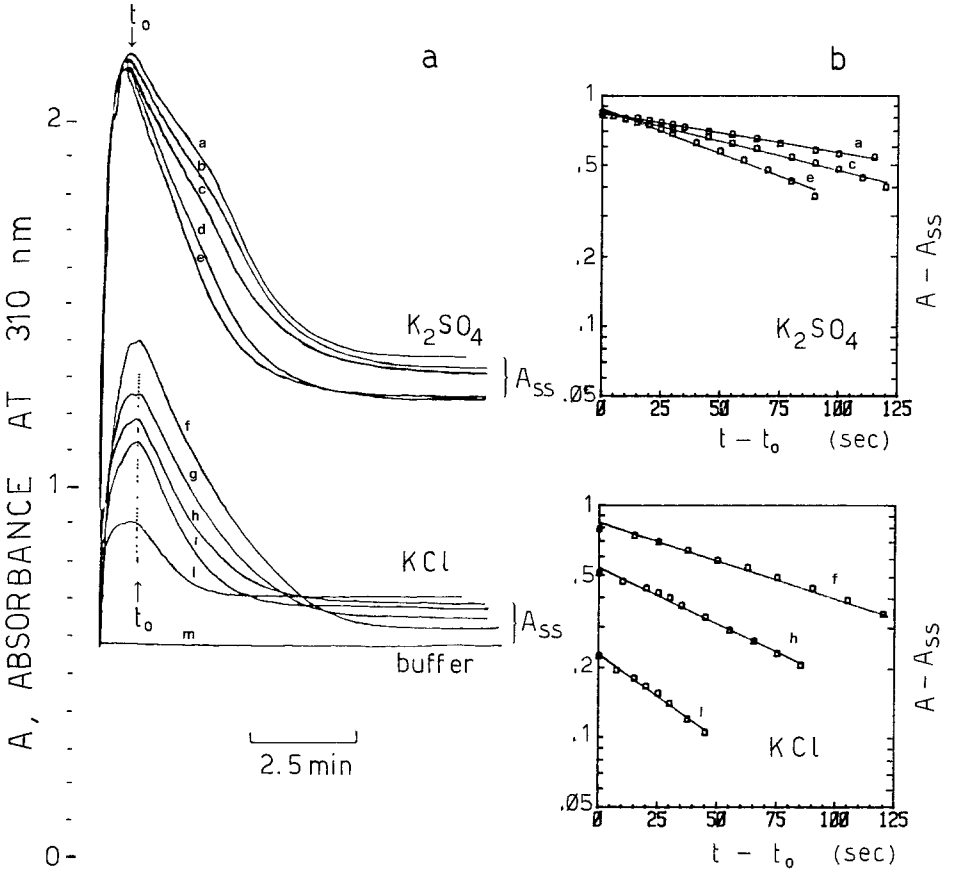

Fig. 1. Changes in absorbance at 310 nm due to variation in size of lipid vesicles diluted into different media. **a** Vesicles were diluted into 10 volumes of either the same buffer, *curve m*, or of a KCl rich solution, *curves f–l*, or of a K_2SO_4 rich one, *curves a–e*, as described in methods. *Curves b, c, d, e* and *g, h, i, l* were taken 20, 55, 118, 188, and 10, 45, 105, 175 min after the addition of 3 mg/ml hemocyanin to the vesicle solution. *Curves a, f, m* were control experiments with protein-free liposomes. **b** The transient amplitude, A-A_{ss} (where A_{ss} is the absorbance at the end of the transient, indicated in the figure), is plotted semi-logarithmically versus the time elapsed from the instant of maximal absorbance, t_0, also indicated for the traces in (**a**). Regression lines give a permeation time of 250, 174, 120 s for *curves a, c, e* respectively, and of 132, 90, 60 s for *curves f, h, l*

Hepes Buffer, pH 7.0. The lipid mixture used was: saturated egg PC (P.L. Biochemicals) and PS (Lipid Products), w/w ratio 2 : 1, dissolved at 47.5 mg/ml in n-decane. Samples from the different fractions of the preparations described above were added to one compartment only, cis side, after complete formation of the bilayer. Current flowing through Ag-AgCl electrodes was converted into voltage by a virtual ground operational amplifier (AD 515 K) with 10^8 Ω, 20 pF in the feed-back loop. Voltages, applied by a DC variable generator, are referred to the cis compartment. Experiments were run at room temperature.

Results

The time course of the absorbance of PC vesicles diluted in different media is shown in Fig. 1. Upon resuspending them in a hyperosmotic solution of either K_2SO_4, curves a–e, or KCl, curves f–l, a large increase in absorbance is observed, followed by a decrease; this behaviour should be compared to the constant absorbance recorded when the vesicles are diluted into the buffer medium in which they are stored, trace m. As indicated by Kometani and Kasai (1978) these absorbance variations can be interpreted as follows: the fast rising phase can be attributed to an increase in the scattering intensity caused by the shrinkage of the vesicles due to the outflow of water through the membranes, which is driven by the osmotic pressure difference. The subsequent, decreasing phase is caused by the increase of vesicle volume due to the inflow of water which is accompanied by the influx of salt ions which is driven by the chemical potential difference. The rate of reswelling is then determined by the ion pair permeability, i.e., it is limited by the rate of inflow of the less permeable ion species.

Experiments run at successive intervals after the addition of hemocyanin to the liposome stock

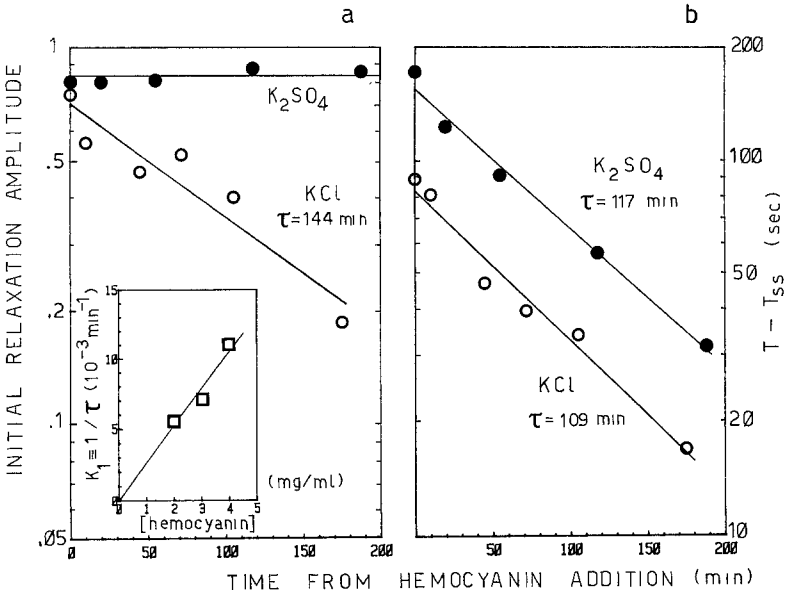

Fig. 2. Time course of permeability parameters after the addition of hemocyanin to PC vesicles. **a** The initial amplitude of the absorbance transient, calculated as in Fig. 1, is plotted versus the time elapsed from protein addition both for K_2SO_4 and for KCl. While the K_2SO_4 amplitude remains constant, the KCl amplitude decreases exponentially with time as required by Eq. (4), with the time constant indicated. The dependence of the inverse of the time constant, i.e., of the rate k_1 of Eq. (4), on the protein concentration is shown in the inset. The straight line is the best fit for Eq. (3) with $k_2 = 2.63 \times 10^{-3}$ l/min · g. **b** The difference between the permeation time, T, at various times after protein addition, and its steady-state value, T_{ss}, measured 16 h after the beginning of the reaction, is plotted against time. T_{ss} was 80 and 50 s for K_2SO_4 and KCl solutions, respectively. With both salts the permeation time decreased exponentially towards its steady-state value; straight lines are least-squares fit to the points which give the time constants indicated. Note that the permeation time of K_2SO_4 was always larger, by approximately a factor of two, than that of KCl

solution indicate that the interaction with protein results in a large decrease of the amplitude of the transient signal in the KCl medium, curves f–l, but not in the K_2SO_4 one, curves a–e.

In both media, hemocyanin induces an increase in the rate of the reswelling phase. This is shown more clearly in the two insets, where the early part of the transient, starting at the time of maximal absorbance, indicated as t_0 in the figure, are replotted on a semilogarithmic scale. As shown these plots can be fitted reasonably well by a straight line, which allows us to calculate two parameters: the slope of the line, whose reciprocal gives the permeation time of the ion pairs, and the intercept at $t = t_0$ which gives the amplitude of the relevant transient. These parameters are plotted, as a function of the time elapsed from the addition of the pore former to the vesicle solution (Fig. 2). In Fig. 2, (part a) we have plotted, on a semilogarithmic scale, the value of the intercept with both K_2SO_4 and KCl, and, Fig. 2. (part b) shows a similar plot for the values of the permeation times of the two electrolytes, from which the respective steady-state values, measured 16 h after the addition of the protein, have been subtracted.

Both the permeation times decrease exponentially with time towards their steady-state values, with approximately the same time constant. In contrast, the amplitude of the relaxation decreases exponentially only in the case of KCl, remaining constant with K_2SO_4. Varying the hemocyanin concentration has an effect on the kinetics of the process as shown in the inset to Fig. 2a, where the reciprocal of the time constant for the decrease of the relaxation amplitude observed with KCl is plotted as a function of protein concentration.

In an attempt to clarify the nature of the interaction of hemocyanin with vesicles we purified liposomes, which were incubated with this protein, by means of sucrose density gradient centrifugation as described in the methods section. Fractions, collected and numbered from the bottom of the tubes, were tested for the presence of vesicles, by measuring the absorbance arising from light-scattering at 310 nm and for the presence of protein, by measuring the protein the absorbance at 280 nm, corrected for light scattering effects (Mimms et al. 1981). The results are shown in Fig. 3 for the three preparations described in the methods section.

In preparation 1, most of the protein sedimented in the bottom fractions while the vesicles floated in the top fractions; however, measurable quantities of protein floated with the vesicles. Preparation 2, which

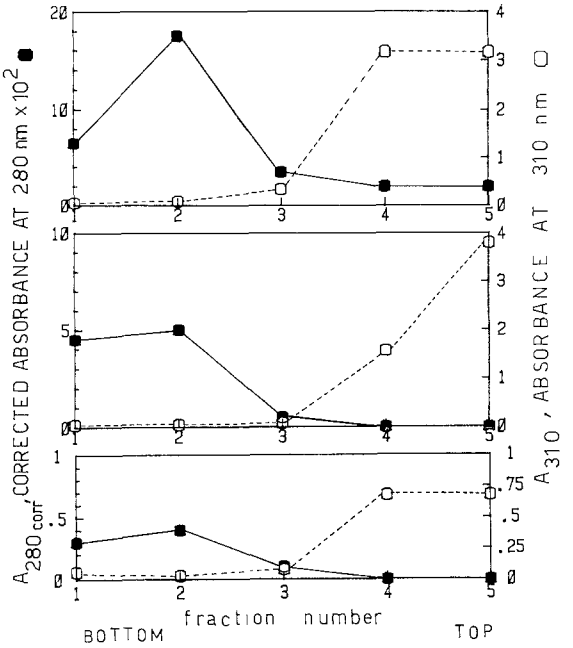

Fig. 3. Content of vesicles and protein for different fractions after sucrose density gradient centrifugation. Purifications were performed as described in methods; protein concentration was measured by absorbance at 280 nm, A_{280}, and corrected for light-scattering as follows: $(A_{280})_{corr} = A_{280} - {}^1/_2(A_{250}+A_{310})$, i.e., assuming a linear contribution of light scattering between 250 and 310 nm; vesicle concentration was measured by absorbance at 310 nm, A_{310}, arising from light scattering. The three panels refer to preparations 1–3 from top to bottom. Absorbance values are for a 2-mm optical pathlength

differed in the lower concentration of hemocyanin used and in the centrifugation procedure used, gave essentialy the same results. To test if the protein which floated with the vesicles in preparation 1 was retained by means of a hydrophobic interaction with the lipid membranes, or, less strongly, by electrostatic interactions (Bhakdi and Tranum-Jensen 1980; Doufourcq and Foucon 1978), we resuspended a part of fraction 4 in a medium of high ionic strength and recentrifuged it to give preparation 3, bottom panel of Fig .3. Most of the protein was released from the vesicles by this treatment; in fact, the concentration of vesicles in fractions 4 and 5 of preparation 3 is decreased by a factor of about four with respect to the starting material (fraction 4 of preparation 1) whereas the protein concentration in the same fractions is decreased by a factor of 100 and hemocyanin is found at the bottom of the tube. These facts indicate a non-specific interaction between the protein and the lipid vesicles which is reversible at high ionic strength.

Nevertheless it is known that hemocyanin forms ionic channels in planar lipid bilayers, and that this interaction is virtually irreversible, i.e., that pores never detach from the membrane once they open (Latorre and Alvarez 1981). Since the light scattering experiments presented above suggest that hemocyanin can also form ionic channels in unilamellar vesicles one has to take into account the possibility that small amounts of protein, which represent irreversibly bound channels, are also retained by liposomes of preparation 3, and excape detection by absorption measurements because of the very low concentration.

To test this hypothesis we used a different, more sensitive, technique i.e., we induced fusion of the purified vesicles with planar bilayers and monitored the change in electrical conductance which followed this event. The results of this kind of experiment are shown in Fig. 4, traces a–d. In trace a), liposomes of fraction 4 of preparation 2 were added to a solution containing Ba^{2+} ions. No change in conductance was observed until 400 mM glucose was added to the cis compartment, but a large current increase followed this osmotic jump within 1 minute. Trace b) shows a similar experiment except that glucose was added symmetrically to both compartments; in this case also, no conductance increase was induced by liposomes, even in the presence of divalent cations and after long time periods, but the current increased immediately after the glucose was added to the solution. Discrete jumps and non-linear, instantaneous current-voltage curves ensured that the conductance induced by vesicles was due to the insertion of hemocyanin channels into the bilayer (Latorre and Alvarez 1981; Cecchi et al. 1981). Relative conductances obtained by dividing the instantaneous current by the applied voltage and by the maximum conductance are plotted in the inset of the figure for the two experiments in a) and b). Besides showing the typical sigmoidal shape (Menestrina and Antolini 1982) the two curves also show the effects that glucose on the trans side has, when it is added in the presence of Ba^{2+} cations, i.e., to shift the current-voltage curve towards negative potentials. This effect, which can also be observed on directly incorporating hemocyanin into the planar bilayer without using fusion of vesicles, will be fully described elsewhere (Menestrina and Pasquali 1985). We have performed similar experiments using sucrose, instead of glucose, with essentially the same results (data not shown), which indicates that an increase in osmolarity, and not the specific presence of glucose, is required for the current increase.

The incorporation patterns in traces a) and b) strongly suggest that vesicles fuse with the planar bilayer, thus transferring their ionic channels into the host membrane. In fact, our results are entirely consistent with similar experiments performed with multilayer vesicles containing porin (Cohen et al. 1982). They demonstrated that an osmotic gradient

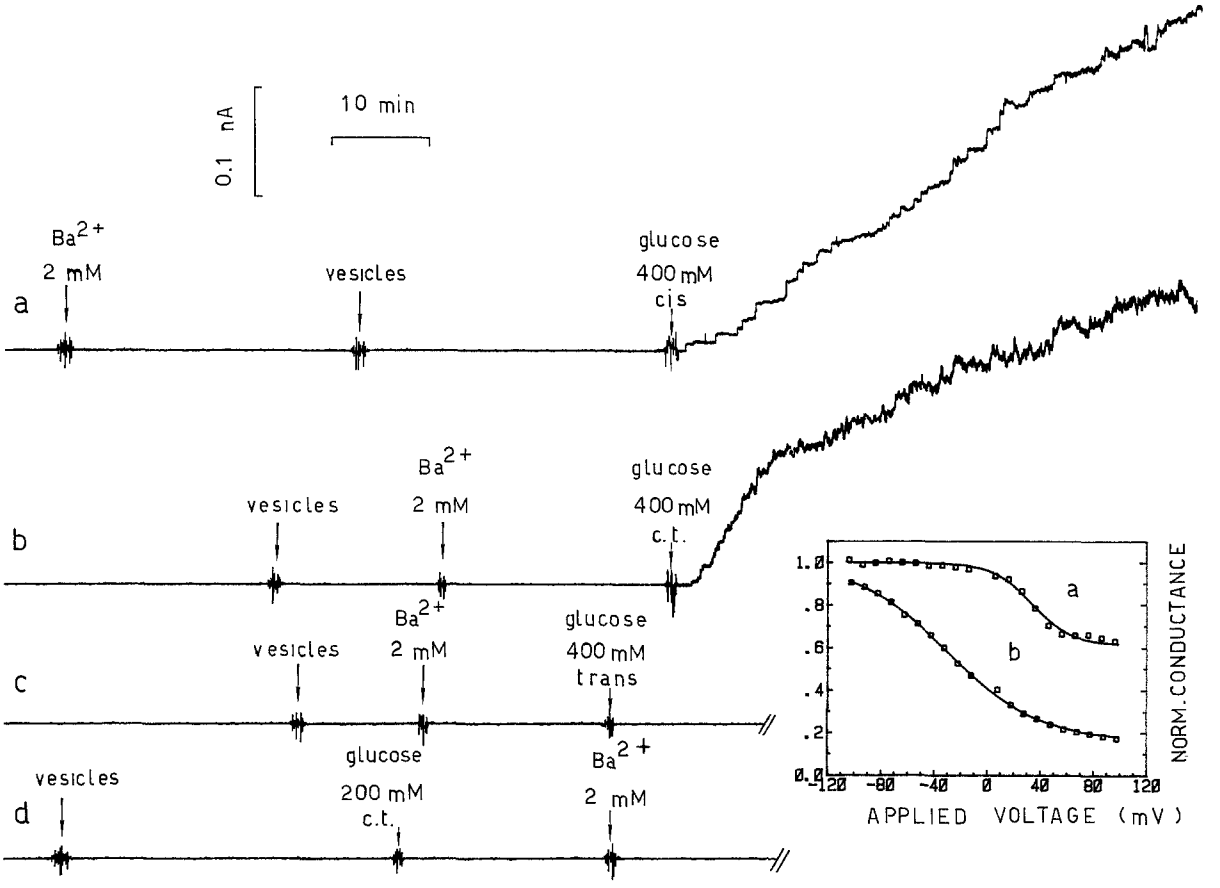

Fig. 4. Time courses of membrane current after addition of vesicles under different experimental conditions. *Trace a:* a conductance increase follows an osmotic jump in the cis compartment containing vesicles and divalent cations. *Trace b:* a similar conductance increase also follows the simultaneous increase of osmolarity in both compartments in the presence of vesicles and Ba^{2+}. *Trace c:* an osmotic jump in the trans compartment has no effect on the membrane conductance, other conditions, the same as in *a* and *b*. The conductance increased in the usual way 2 min after the addition of 400 mM glucose on the cis side, not shown. *Trace d:* no conductance increase is observed in one experiment similar to *trace b* but in which the order of addition of glucose and Ba^{2+} has been inverted. The conductance increased immediately only after a new addition of 400 mM glucose on both sides, since Ba^{2+} was already present at this time, not shown. Vesicles used were: *trace a*, fraction 4 of preparation 2; *traces b–d*, fraction 4 of preparation 1. Clamp-voltage was −40 mV. Current and time scales are the same for all traces. In the inset the normalized instantaneous conductance-voltage curves of membranes in *a* and *b* are reported. Solid lines are least-squares fits of a two-state model described elsewhere (Menestrina and Antolini 1982) for the hemocyanin channel

through the vesicles walls, and not through the planar bilayer, is necessary to promote fusion with a BLM of those liposomes which were kept in close contact with the planar bilayer by divalent cations. That this is also the case here is demonstrated, not only by traces a and b, but also by the two control experiments in c and d. Trace c shows that glucose on the trans side was ineffective in inducing current increases even in the presence of liposomes and divalent cations. Trace d, on the other hand, shows that glucose cannot induce any current increase by itself, i.e., if divalent cations are not present, nor can the addition of Ba^{2+} induce the current increase when the osmotic jump has already occurred. When free hemocyanin or fractions 1 and 2 of the preparations were added to the bath solution in control experiments, channels incorporation was observed even in the absence of divalent cations and glucose.

Finally we tested the effects of vesicles of fraction 4 of preparation 3, to see if they also contain active channels. Indeed, in an experiment performed exactly as that in trace b, we observed a current increase as soon as glucose was added to the solution. The conductance at 10 min and 30 min after glucose addition was 1.2 and 3.2 ns respectively, which should be compared with the values of 2.7 and 4.5 ns for trace b, obtained at the same time intervals after glucose addition for fraction 4 of preparation 1. One should notice that despite the two orders of magnitude decrease in the protein concentration after the high ionic strength treatment, see Fig. 3, the vesicles retained, almost completely, their ability to transfer channels to the BLM by fusion, which suggests that the pores cannot be washed out by this treatment.

Discussion

Osmotic jump experiments, followed by light-scattering changes, as shown in Fig. 1, are suitable for measuring the permeability of lipid vesicles to electrolytes. In particular, the analysis of the simple exponential decay of the first part of the absorbance transient, due to reswelling of the vesicles, as shown in the insets of Fig. 1, gives both the amplitude of the transient and the permeation time of the ion pair (Kometani and Kasai 1978). The results of this procedure, applied to vesicles at different times after their exposure to the pore former hemocyanin, are reported in Fig. 2. We think that at least two kinds of interaction between hemocyanin and lipid vesicles are needed to explain the experimental findings. The first, which accounts for the results of Fig. 2a, is the formation of ionic pores through the membranes. From the recent report of Cecchi et al. (1982), we know that formation of one hemocyanin channel in a planar bilayer is accompanied by a large increase in its K^+ permeability, about 160 pS at 0.1 M K^+ activity, and by a smaller, but still sizeable, increase in its Cl^- permeability, which can be estimated to be about 40 pS under the same conditions. There is, however, no measurable change in its $SO_4^=$ permeability. Since our method is sensitive to the ion pair permeability, i.e., it is rate limited by the ion species of lowest conductance (White and Miller 1981) we would expect that the formation of hemocyanin channels in lipid vesicles modifies their osmotic swelling in a KCl medium but not in a K_2SO_4 one. In particular, we can calculate that a PC vesicle the size of ours, average radius 30–40 nm (Milsman et al. 1978; Zumbuhel and Weder 1981), containing only one ionic channel, equilibrates a KCl osmotic imbalance in less than 300 ms (White and Miller 1981) i.e., less than our mixing time. It follows that vesicles containing hemocyanin pores do not contribute further to the absorbance transient after an increase in the external KCl concentration, while retaining their usual behaviour after the addition of K_2SO_4. Hence, we expect that a decrease in the reaction amplitude parallels the formation of hemocyanin channels in KCl experiments but not in K_2SO_4 and this is what is indeed observed, Fig. 2a. The decrease in the reaction amplitude, which reflects a reduction in the number of unmodified vesicles, can therefore serve as a criterion for the progress of the reaction of formation of the channels, allowing the presentation of a simple kinetic model for this process.

In the course of this reaction, unilamellar lipid vesicles, V, interact with hemocyanin, H, to form channel-containing vesicles, VH; the rate of the reaction

$$V + H \xrightarrow{k_2} VH \tag{1}$$

is given by

$$-\frac{d[V]}{dt} = k_2 [V] [H] . \tag{2}$$

Assuming that the concentration of hemocyanin effectively available for pore formation remains unchanged throughout the course of the reaction (under our conditions, we have about 50 hemocyanin molecules for each vesicle and we expect[1] to have not more than one or two channels per vesicle at steady-state), we can put:

$$k_2 [H] = k_1 \tag{3}$$

and

$$\ln [V] = - k_1 t + c . \tag{4}$$

We believe that the linear time-dependence of the logarithm of the absorbance transient amplitude, Fig. 2a, is indicative of such pseudo first-order kinetics for channel formation, its slope reflecting the pseudo first-order kinetic constant of the reaction, k_1. Equation (3) implies that k_1 depends linearly on the pore former concentration, and this is what we have found, inset to Fig. 2a. From the straight line fit to the points in that inset, the true rate constant k_2 can be estimated as 15 $s^{-1} \cdot M^{-1}$ (where we have used the molecular weight of dissociated hemocyanin, about 3.5×10^5 (Van Holde and Miller 1982), because the dissociated form is believed to form the channel [Latorre and Alvarez 1981; McIntosh et al. 1980)].

The second kind of interaction could account for the results reported in Fig. 2b, i.e., that hemocyanin induces a slight increase in the permeability of all vesicles with a half-time of about 80 min. We think that this effect is not due to channel formation, otherwise it could not be detected in KCl osmotic jump experiments, but rather to a non-specific adsorbtion of hemocyanin molecules onto the vesicle surface. Such adsorbtion can increase their permeability; as is the case, for example, with cardiotoxin (Doufourcq and Foucon 1978), and may be due, as in that case, primarily to a reversible electrostatic

1 This expectation is based on the fact that each vesicle usually transfers just one channel, and only very seldom two or more, to a planar bilayer during one fusion event, see Fig. 4 trace a) and for more details Pasquali et al. (1984). It is also completely consistent with the findings of McIntosh et al. (1980), who observed, under similar experimental conditions, a density of about one to two annuli, the proposed hemocyanin channel structure, per 10^4 nm^2 of vesicle surface, which gives an average number of 0.5–1 pores for each of our vesicles, using a radius of 40 nm

interaction between protein charged groups and the zwitterion polar heads of PC molecules. This hypothesis seems to be consistent with the findings in Fig. 3, where the results of sucrose density gradient centrifugation of lipidic vesicles, which were incubated with hemocyanin, have been reported. We have found that a large part of the protein which was retained by the vesicles at low ionic strength, was released upon prolonged incubation and recentrifugation in a high ionic strength medium, indicating that the interaction is mainly electrostatic (Bhakdi and Tranum-Jensen 1980; Doufourcq and Foucon 1978). On the other hand, both vesicle preparations, before and after recentrifugation, also contained active hemocyanin channels as was confirmed by incubating them on one side of a planar bilayer in the voltage-clamp experiments shown in Fig. 4. Under appropriate conditions large conductance increases were observed, which were due to the insertion of hemocyanin channels from the vesicles into the planar lipid membrane, as was clear from the typical shape of the current-voltage characteristic, shown in the inset of Fig. 4. The special set of conditions necessary for this transfer of protein strongly suggests that it occurs via fusion of the proteoliposomes with the planar bilayer. In fact, negatively charged lipids and divalent cations were absolutely required, but not sufficient, to observe the conductance increase; it is known, in fact, that they only promote the vesicle-planar membrane adhesion process (Miller et al. 1976; Düzgünes and Ohki 1981). The subsequent production of an osmotic gradient through the vesicles walls was also required, consistent both with the observation of Cohen et al. (1982) that fusion of vesicles with planar bilayers is induced by their osmotic swelling, and with the recent report that fusion between two adjacent planar lipid bilayers occurs only when an osmotic gradient, necessary to remove all of the residual water from the narrow gap between the two membranes, is applied through at least one of the two films Fisher and Parker 1984). It should be noted that vesicles which were recentrifuged at high ionic strength gave conductance increases, in fusion experiments, comparable to those of vesicles prepared at low ionic strength, even though the protein concentration in the vesicle fractions was strongly decreased by this treatment (Fig. 3). This is consistent with the idea that ionic channels were formed in the vesicles by a hydrophobic anchoring of the protein to the lipid matrix, i.e., that after pore formation, hemocyanin behaves like an intrinsic protein which cannot be removed by high ionic strength washing procedures.

In conclusion, purification of proteoliposomes by sucrose density gradient centrifugation and reconstitution into planar bilayers confirmed the findings of osmotic jump experiments that hemocyanin interacts with lipid vesicles both specifically, forming ionic pores strongly anchored to the lipid matrix, and non-specifically, adhering to their surface in a reversible way.

It is worth comparing our results with the previous electron microscopy study of McIntosh et al. (1980). As shown in Fig. 3 of that work, liposomes prepared by phospholipids and mixed with hemocyanin carry on their surface both whole hemocyanin molecules, which are randomly oriented appearing either in the "side" view or in the "end on" view, and a few smaller annuli all oriented perpendicularly to the plane of the membrane and supposed to be the real channel structures. Our results are entirely consistent with this interpretation and suggest that whole molecules are reversibly coordinated by the lipid without forming pores, whereas the annuli, the true pores, are bound irreversibly.

Acknowledgements. We wish to thank Ms. M.T. Giunta for her patient work in typing this manuscript. This work was supported in part by the Italian CNR and Ministero di Pubblica Istruzione. F.P. is the recipient of a grant from the Istituto di Ricerca Scientifica e Tecnologica (TN).

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