Biochimica et Biophysica Acta, 727 (1983) 56–62
Elsevier Biomedical Press

BBA 71468

EFFECTS OF pH AND DIETHYLPYROCARBONATE ON THE CONDUCTANCE STATES OF PLANAR LIPID BILAYERS CONTAINING HAEMOCYANIN

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(Received August 23rd, 1982)

Key words: Ion channel; Membrane conductance; Hemocyanin; pH; Lipid bilayer

The addition of haemocyanin from *Megathura crenulata* to the aqueous phase bathing a bilayer lipid membrane resulted in the formation of ionic channels. With an applied voltage biased negative with respect to the haemocyanin-containing side, a single conductance state was observed above pH 7.0. Below pH 7.0 several conductance states were manifested, and the maximum conductance observed for a single channel decreased with decreasing pH. Extensive treatment of the haemocyanin with diethylpyrocarbonate, which reacts primarily with histidine residues, completely prevented the formation of ionic channels; however, milder treatment produced a chemically modified haemocyanin that was capable of forming ionic channels with modified conductance properties. Each channel conductance was typically much lower than that of the channels formed from unmodified haemocyanin, and there was now substantial variation in conductance from channel to channel. Following the use of hydroxylamine to remove the carbethoxy groups from the modified haemocyanin, it formed ionic channels that were restored to the original unit channel conductance.

Introduction

Chemical manipulation of the conductance state of channel-forming molecules offers an experimental approach by means of which the properties and function of membrane ionic channels may be investigated. Using planar lipid bilayers into which the channel-forming protein haemocyanin has been incorporated, we have attempted to detect changes in conductance, both as a function of pH and in response to chemical modification of the protein. Haemocyanin is a respiratory pigment of the blood of arthropods and molluscs, which binds oxygen through complex formation with copper ions [1]. Under physiological conditions, gastropod haemocyanins are associated with multimeric structures, each with a sedimentation coefficient of about 100 S [2] and a molecular weight of approx. 9 000 000 [3]. Above approximately pH 8.0, measurements of sedimentation coefficients, diffusion coefficients and molecular dimensions as revealed by electron microscopy, show that gastropod haemocyanins dissociate into submultiples: firstly into halves and then further into tenths and twentieths [4,5]. A recent study of the haemocyanin from the giant keyhole limpet, *Megathura crenulata*, showed that the undissociated molecules exist in vivo in several states of aggregation which are multiples of half of the 100-S structure, while on dialysis into pH 8.9 buffer containing EDTA the haemocyanin dissociates into two electrophoretically distinct types of subunits of one-twentieth the size of the 100-S particles [6].

Engelborghs and Lontie [7] reported that reaction of either α- or β-haemocyanins from *Helix pomatia* with diethylpyrocarbonate (a reagent used

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to modify histidine residues specifically and reversibly [8]) results in dissociation into halves and tenths under appropriate conditions of pH. No modifications of tyrosine residues were observed spectrophotometrically, and hydrolysis with hydroxylamine resulted in reassociation of the particles. The authors have proposed, therefore, that dissociation induced by the reaction with diethylpyrocarbonate is due solely to modification of histidine residues.

Pant and Conran [9] have reported that an interaction between *M. crenulata* haemocyanin and planar lipid bilayers results in a decrease in bilayer resistance by several orders of magnitude. Alvarez et al. [10] described the appearance of ionic channels resulting from the incorporation of *M. crenulata* haemocyanin into black lipid membranes. The channel was found to be asymmetric in conductivity with respect to the applied voltage. With an applied voltage biased negative with respect to the side of the membrane exposed to the haemocyanin-containing solution, a single conductance state was observed for the channels, whereas several conductance states were detected when the voltage was applied with the opposite bias [11]. Antolini and Menestrina [12] have studied the ion selectivity of the channel under an applied voltage of -40 mV with respect to the protein-containing side, from which it appeared that the channel discriminates against anions and shows very little selectivity between cations.

Ionic channel formation has also been reported using haemocyanins from *Busycon canaliculatum, Helix pomatia, Paludina vivipara* [13] and an unidentified snail [11]. Recently the annular structure (70 Å outer diameter) of the *M. crenulata* haemocyanin channel has been shown by electron microscopy [14] to resemble the nicotinic acetylcholine receptor channel [15] and the 'connexon' of gap junctions [16].

In this paper we report changes with pH in the conductance properties of the ionic channel formed by the interaction of *M. crenulata* haemocyanin with bilayer lipid membranes. Similar reversible changes in the haemocyanin channel properties are noted on treatment of haemocyanin with diethylpyrocarbonate.

Materials and Methods

Lipid bilayer membranes were prepared from either soybean lecithin (phosphatidylcholine, type IIS, commercial grade, Sigma Chemical Co.) dissolved in decane (Koch-Light, Puris grade) at 20 $mg \cdot ml^{-1}$ or glycerol monooleate (Nu-Check Prep. Inc., Elysian, MN, U.S.A.) dissolved in decane at 3 $mg \cdot ml^{-1}$. The method described originally by Mueller et al. [17] was used to make planar lipid membranes of bimolecular thickness across a 1 mm diameter circular aperture in a PTFE septum (approx. 0.2 mm thick) separating the two aqueous compartments. The bathing solutions contained 0.1 M KCl and were buffered with borate, phosphate or acetate (5.0 mM in each case), potassium being the only metal cation present. Experiments were all carried out at room temperature (18–20°C).

To form ionic channels from *M. crenulata* heamocyanin, 5–50 μl haemocyanin solution (0.4 $mg \cdot ml^{-1}$, supplied as grade A by Calbiochem-Behring Corp., CP Laboratories Ltd., Bishops Stortford, U.K.) was added with gentle stirring to the bathing solution (total volume 20 ml) on one side of the bilayer. A constant voltage of 50 mV (negative with respect to the haemocyanin exposed side of the bilayer) was applied across the bilayer using Ag/AgCl electrodes in contact with the bathing solution on either side of the membrane. In this way, the appearance of haemocyanin channels was monitored. Transmembrane current was measured using a FET operational amplifier (type AD 515J, Analogue Devices, Ltd., East Molesey, Surrey, U.K.) with a current feedback loop, and the output was recorded on a chart recorder. Capacitance was measured by applying an a.c. voltage to one side of the membrane, connecting the other side to an operational amplifier (type 714) with standard capacitors in the feedback loop. The polarity of voltage applied to the bilayer was always referred to the compartment of the experimental chamber containing haemocyanin.

Modification of haemocyanin with diethylpyrocarbonate was carried out essentially as described by Miles [8]. Haemocyanin at 1 mg/ml in phosphate buffer (5 mM) pH 7.0 was treated for several hours with diethylpyrocarbonate, added from a 20 mM solution in Analar ethanol to a concentration

of 2.0 mM (extensive modification) or 1.0 mM (partial modification). The reaction was followed by running ultraviolet spectra, since modification of histidine residues results in increased absorbance around 240 nm. Finally the ethanol was removed by dialysis.

The modifying groups were removed by the addition of 0.3 ml NH_2OH (1.0 M, made up as the hydrochloride and neutralized to pH 7.0 with KOH) to each 1.0 ml of haemocyanin solution, and ultraviolet spectra were again run to note the corresponding decrease in A_{240}. The protein solution was left overnight to complete the reaction. Following this treatment the ultraviolet spectrum once more resembled that of the native protein, which indicated that the carbethoxy groups had been removed.

Results

The addition of *M. crenulata* haemocyanin (0.1–1.0 $\mu g \cdot ml^{-1}$) to the aqueous solution bathing the bilayer lipid membrane formed from soybean lecithin in decane resulted, invariably, in the formation of conductance channels (Fig. 1), although usually there was a time lag of 60–90 min before channels were detected. The properties of the channels at pH 7.0 were dependent on the applied voltage. When a voltage was applied negative with respect to the haemocyanin-containing compartment, the resistance was ohmic. By contrast, with a positively-biased applied voltage several states were observed for a single channel and after the application of the voltage the output observed decreased in steps through several conductance states, in agreement with the work of Latorre et al. [11]. We investigated in detail the conductivity of channels at a constant applied voltage of -50 mV.

The unitary (single channel) conductance from bilayers treated with haemocyanin was 180 ± 20 pS in potassium phosphate (5.0 mM) pH 7.0 containing 0.1 M KCl. Because of the duration of the experiments (2–5 h), controls using bilayers with no added haemocyanin were essential in order to check that the bilayers did not become leaky or mimic channel behaviour in any way during the same period. Bilayers were formed in the same buffer and the current was measured for the same

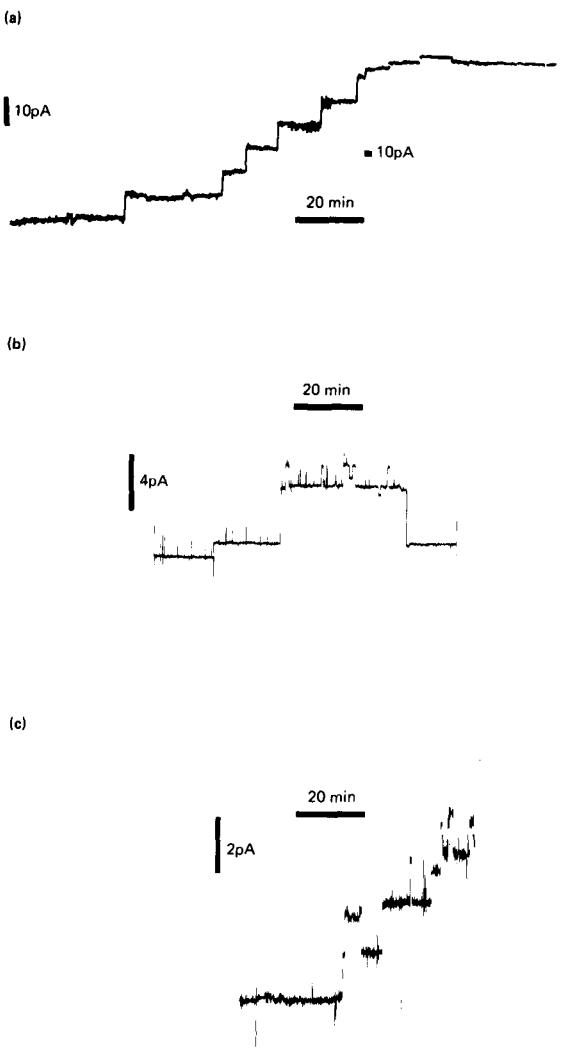

Fig. 1. Effects of pH on haemocyanin channels. Current steps induced by the addition of haemocyanin (from *Megathura crenulata*) to one side of a planar bilayer formed from soybean lecithin in decane at 18–20°C. The potential is -50 mV and the haemocyanin concentration approx. 0.8 μg/ml. The aqueous phase contains 0.1 M KCl and the traces were obtained at: (a) pH 7.00; (b) pH 5.95; (c) pH 5.40.

applied voltage over the space of seven hours. During this time there was no detectable change in membrane resistance. The bimolecular thickness of the membrane was checked periodically by measuring the total capacitance from the area of the membrane. The lipid film was observed with a monocular microscope ($\times 10$ magnification) and the area of the membrane was estimated by means of an eyepiece graticule. During long experiments,

the total capacitance remained constant at circa 0.39 $\mu F \cdot cm^{-2}$.

Changing the pH of the bathing solution resulted in altered channel properties. Over the pH range 7.0–9.0 (buffers used: phosphate at pH 7.0 and pH 8.0; borate at pH 9.0 (all solutions contained 0.1 M KCl)), the unitary conductance of the channel was unchanged at 180 pS. On reducing the pH below 7.0, however, a marked change in channel properties was detected. For example, on application of a voltage of −50 mV, multistate channels were seen rather than the unitary conductance observed at pH 7.0. No regular pattern of appearance of the different conductance states was observed. This contrasted with the observations at pH 7.0, but using instead a positive applied voltage, when the highest conductance state always preceeded other states [10,11]. Fig. 1 shows multistate channels at pH 5.4 and pH 5.95. In general the maximum conductance observed for the haemocyanin channels was lower at lower pH values, no conductance step as high as 180 pS being observed at pH 6.5 and below (Table I). At pH values less than 5.0 the trend continued, but the experiments were not very reproducible as the bilayers were less stable, and measurements on haemocyanin channels were correspondingly more difficult.

TABLE I

AMPLITUDES OF THE VARIOUS CONDUCTANCE STATES AT DIFFERENT pH VALUES

Recorded from planar lipid bilayers formed using soybean lecithin in decane at 19–21°C.

pH	Conductance states observed (pS)	pH	Conductance states observed (pS)
5.0	54	6.0	132
	28		120
	16		115
	6		104
5.4	105		90
	69		22
	59	6.9	175
	32	8.0	175
		9.0	180

Haemocyanin channels were also observed in bilayers formed from glycerol monooleate in decane. However, a detailed study was precluded because, although it was possible occasionally to observe single haemocyanin channels as a result of incorporation of haemocyanin from one side of the bilayer only, these bilayers generally broke down within 15 s. It was then possible to reform much more stable bilayers containing channels

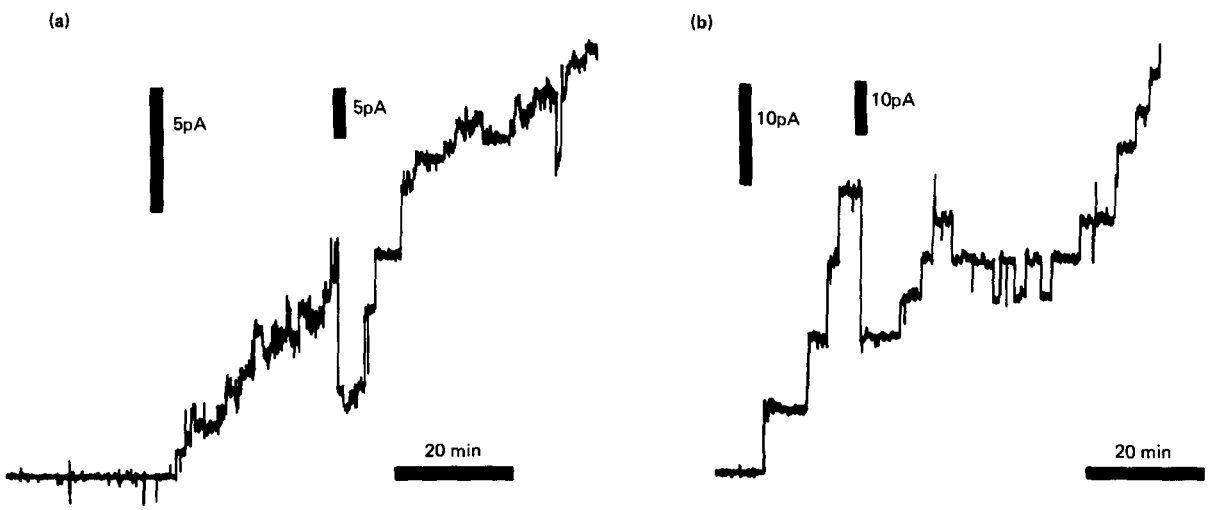

Fig. 2. Current steps induced by the addition of haemocyanin (from *Megathura crenulata*) to one side of the planar bilayer formed from soybean lecithin in decane at pH 7.0 and 21°C. (a) Following chemical modification of histidine residues by diethylpyrocarbonate (1.0 mM). (b) After removal of carbethoxy groups on treatment with hydroxylamine, as described in the text.

from the remaining lipid hydrocarbon material, which would not be polarized exclusively in one direction. In addition glycerol monooleate bilayers were not stable above pH 6.5, but as far as the pH dependence of channel conductivity could be observed in glycerol monooleate it seemed to follow the same general trend as in soybean lecithin.

Upon modification of haemocyanin with diethylpyrocarbonate an increase in absorbance at 240 nm was observed, which corresponds to a specific carbethoxylation of histidine residues. Within one hour of incubation with hydroxylamine the ultraviolet spectrum had returned to its original form. Following modification and removal of carbethoxy groups with hydroxylamine, lipid bilayers of soybean phospholipid were treated with the protein. Extensive modification (as described in Methods) prevented the formation of haemocyanin ionic channels in the lipid bilayers. This capacity to form channels returned after the treatment with hydroxylamine prior to attempted incorporation experiments. Milder treatment with diethylpyrocarbonate resulted in the formation of channels of various conductivities at pH 7.0 up to a maximum of 180 pS, whereas again removal of the modifying groups with hydroxylamine resulted in a protein that showed channel properties indistinguishable from those of the unmodified haemocyanin (Fig. 2).

Discussion

The values obtained for the unitary (single channel) conductance of soybean lecithin and glycerol monooleate bilayers containing *M. crenulata* haemocyanin (at pH 7.0) are found to be intermediate between the values of 200 pS reported by Latorre et al. [11] and 145 pS noted by Antolini and Menestrina [12] in oxidised cholesterol bilayers. Reducing the pH below 7.0 results in both an attenuation of single channel conductivity and the appearance of multistate channels. The pronounced effects of pH implicate the dissociation of an amino acid residue with a pK_a between 6 and 7 in the conductance changes. A number of studies have demonstrated a pH-dependence of sodium and potassium channel permeability in nerve membranes [18–20]. Of particular interest in the present context are the findings by Clark and Strickholm (1971) [21] for the crayfish axon membrane of a pH dependence of the axon cable impedance which closely resembled a protein dissociation curve. Transitions at pH 6.3 and pH 8.5 were tentatively identified as resulting from histidine and sulphydryl groups. More recently, the findings of Smart and Constanti [22] on the pH dependence of 4-aminobutyric acid activated chloride channels also point to a role for imidazole groups of histidine residues in modulating the conductance state of these anionic channels. In consideration of these results on biological membranes, and in view of the role of histidine residues in haemocyanin subunit dissociation [7,8] diethylpyrocarbonate was chosen as a reagent in the present study to modify histidine residues selectively, and the result of partial modification was a change in the multi-state properties of the channels. This was completely reversible.

Histidine residues have been implicated in the aggregation properties of haemocyanin in solution in a study by Engelborghs and Lontie [7] using chemical modification with diethylpyrocarbonate. These authors have shown that modification of *H. pomatia* haemocyanin with diethylpyrocarbonate results in dissociation (to different extents with α- and β-haemocyanin from *H. pomatia*) under the same conditions. We have verified this result in our own laboratory using *Lymnaea stagnalis* haemocyanin (Griffin, M.C.A. and Sattelle, D.B., unpublished observations). It appears, therefore, that there may be a connection between the loss of aggregative properties on modification of histidine residues and the appearence of less conducting types of haemocyanin channel.

Similarly to haemocyanin, the linear polypeptide, alamethicin [23], also shows several conductance levels [24]. The appearance of different conductance levels in the case of alamethicin has been explained either in terms of conformational or positional changes within oligomeric channel-forming clusters of fixed size [25], or as the result of variations in the number of monomers forming the channel oligomer [26]. Infrared attenuated total reflection spectroscopy of alamethicin incorporated into lipid multilayers has provided some support for the first model [27]. Clearly, with either model for ionic channels, changes in the ability to aggregate in the bilayer which might

arise from a change in pH or chemical modification would be expected to affect the conductance levels shown.

The different effects due to chemical modification of histidine residues of haemocyanin and to variation of pH may reflect the aggregation requirements for channel formation by haemocyanin. Where haemocyanin subunits are capable of the type of aggregation involved in forming the $9 \cdot 10^6$ molecules, but do not undergo end-to-end aggregation of these units, we observe ionic channel formation as previously described [10,11] with a single conductance for each channel under a negatively applied voltage. The chemical modification by diethylpyrocarbonate causes disaggregation of the molecule in solution, and loss of the ability to form channels. Both these effects were reversed when the carbethoxy groups were removed. At low pH values, at which haemocyanin forms higher aggregates in solution [6], multistate behaviour of the haemocyanin ionic channel is seen.

Detailed investigations of the effects of chemical derivatives and change in pH on channel properties have been reported recently for other model membranes. The pH dependency of both the single channel conductance and overall conductance of many channel membranes formed with succinyl and pyromellityl derivatives of gramicidin A indicated that for channel formation the polypeptide must be neutral in order to penetrate the membrane [28,29], and the ability of the dimeric malonyl derivative to form channels confirmed the hypothesis that the N-terminals are associated in the dimer of the gramicidin channel [30].

The capacity of haemocyanin from many molluscan species to form ionic channels in planar lipid bilayers requires further study (cf. Van Holde and Miller [31]). There is no obvious in vivo function that this could serve; indeed, it appears likely that channel formation by haemocyanin is either an artifact, due possibly to the greater thickness of a black lipid membrane that results from incorporated solvent [32], or to increased accessibility of lipid in the model bilayer over that in a cell membrane to the haemocyanin. The channel particles observed by McIntosh et al. [14] in the electron microscope are clearly in a different state of aggregation to the molecules in solution. For insertion into the membrane, therefore, some conformational change to the typical 'inside-out' structure of a channel [33] would be necessary. Membrane particles detected in the presence of haemocyanin conform in appearance to the five or six parallel helical structures required by the model of Edmonds [34] in his proposal of a clathrate structure of ionic channels. Our findings of different conductance states of the haemocyanin channel that can be manipulated chemically, and which may arise from changes in states of aggregation, suggest that haemocyanin is a useful experimental system for examining theoretical models of ionic channels.

Note added in proof: (Received November 19th, 1982)

Since completion of this manuscript we have become aware of complementary experimental work by Menestrina and Antolini [35], from which they have proposed a role for histidine residues in the function of the haemocyanin ionic channel.

Acknowledgements

This work was supported in part by the Medical Research Council project grant No. G977/923/C. The authors are indebted to Dr. E.J. Wood for generously donating several samples of haemocyanin. We thank Dr. W.G. Griffin for helpful discussions throughout this study. Dr. S. Hladky kindly provided advice on the electrical measurements on planar lipid bilayers.

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