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Electrical Properties of Ionic Channels Formed by *Helix pomatia* Hemocyanin in Planar Lipid Bilayers

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Abstract. *Helix pomatia* hemocyanin forms ion-conducting channels in planar lipid bilayer membranes when added at mg/ml concentration. These channels have several original features. They fluctuate between one conducting and some poorly conducting states and fluctuations can be grouped in bursts. Different channels can have widely different conductance amplitudes. Both channel conductance and burst lifetime are dependent on the applied voltage. Fluctuations within a burst show a complex kinetic behaviour which has been explained developing a multistate model. The model calls for one single open state and six different closed states. Transitions are allowed only between one of the closed states and the open one and obey first oder kinetics. This model is able to fit all our experimental curves obtained in single channel experiments.

Key words: Hemocyanin channel – Planar lipid membrane – Conductance – Current fluctuations – Voltage dependence

Introduction

Hemocyanins are oxygen-carrying copper proteins of large molecular weight which are freely dissolved in the hemolymph of a number of invertebrates [20, 21]. In 1972 Pant and Conran [17] could show that the hemocyanin extracted from the blood of the Giant Keyhole Limpet *Megatura crenulata* was able to increase the conductance of black lipid membranes by several orders. In 1975 Alvarez et al. [1] proved that the mechanism underlying this increase is the formation of ionic channels. Recently McIntosh et al. [9] examined the interaction of hemocyanin with different lipid complexes by means of electron microscopy. With the hemocyanin of the Keyhole Limpet, they correlated the formation of the ionic channel with the appearance of a proteic ring shaped structure associated with the lipid bilayer. This structure, called "annulus", has a diameter of 7 nm, protrudes about 3 nm into the bathing solution and presents a central pool of stain, about 2 nm in diameter, which might be related to the ionic

pathway. With four other hemocyanins, including that of *Helix pomatia,* they observed neither conductance increase in BLM nor appearance of the annulus at the electron microscope. However we have shown that two other hemocyanins those extracted from *Aplysia* and *Paludina vivipara* are able to form channels in BLM [2, 10]. Furthermore we have now consistently observed the formation of single ionic channel in planar bilayers after the interaction with *Helix pomatia* hemocyanin. To accomplish this we used a concentration of protein 3 orders of magnitude greater than that used by McIntosh et al. [9]. These data have led us to believe that many hemocyanins, besides Keyhole Limpet, can form ionic pores in planar bilayers but the probabilities of their interaction with the lipid film are very different. In this respect Keyhole Limpet hemocyanin would have the higher probability, while *Helix pomatia* hemocyanin would have the lower probability of interaction. *Helix pomatia* hemocyanin channels have many interesting features. Different conductance amplitudes which seem to be multiples of one single value, have been observed. Each channel fluctuates between one conducting (open) state and some poorly conducting (closed) states; the conductance of the open channel is slightly dependent on applied potential. Fluctuations can be grouped in intervals, called bursts, separated by relatively large interbursts periods during which the channel is silent. The time distribution between burst and interburst intervals is also voltage dependent. Open to close fluctuations within one burst show a complex kinetic behavior which is compatible with a multistate model which employs one single open state and six different closed states. All transitions, which may be grouped in two classes, fast and slow, are voltage dependent in the sense that both high positive and high negative voltages tend to close the channel. A much similar behaviour has been recently described in detail for the channels of the gap junctions between amphibian blastomers [19, 7]. Since these were many channel experiments we think that our is the first description of the steady state and kinetic properties of a channel gated by voltages of either polarities at a single channel level.

The occurrence of fluctuations collected in bursts is strongly reminiscent of the behaviour of the acetylcholine-receptor channel as shown by Sakmann et al. [18] who used the patch-clamp method. Furthermore a complex kinetic which exhibits fast and slow transitions is common to this hemocyanin channel, to the K^+ channel in the Squid axon [5] and to both the acetylcholine-receptor [4] and the glutamate-receptor [6] channels. An even more similar behaviour has been recently found with the Ca^{2+} dependent K^+ channel and described by Methfessel and Boheim [14] in terms of a statistical model which calls for the existence of five different states, one open and four closed. For these reasons *Helix Pomatia* hemocyanin channels can also be considered as models of naturally occurring ionic channels.

Materials and Methods

Membranes were formed by the apposition of two monolayers with the Montal and Mueller technique [16]. Monolayers were formed by placing 8 μl of a 9 : 1

mixture of phosphatidylcholine (PC) and phosphatidylserine (PS), dissolved in *n*-pentane. Total lipid concentration was 12.5 mg/ml. The hole was pretreated with *n*-hexadecane diluted 1 : 30 in *n*-pentane; *n*-pentane was allowed to evaporate from both the monolayers and the hole.

PC was from P.L. Biochemicals, more than 99% pure, and PS was a kind gift of Dr. A. Gorio (Fidia Research Lab. Abano Terme I). The bathing solution, 4 ml on each membrane side, was, in all experiments, 100 mM KCl (Carlo Erba RPE), 10 mM Bistris (Calbiochem), and 1 mM EDTA (Merck). Its pH was adjusted to 7.0 by HCl. *Helix pomatia* hemocyanin, which was a generous gift of Prof. B. Salvato (Center for the study of physiology and biochemistry of hemocyanin, Padova, I), at a concentration of about 40 mg/ml in a 180 mg/ml sucrose solution, was stored at −20° C. Small amounts of this solution were added to only one (*cis*-side), of the two bathing solutions to a final concentration around 1 mg/ml at the time of the experiments. The solution was then vigorously stirred until the appearence of one current step which is representative of a single ionic channel formation. Stopping the stirring was generally sufficient to prevent the appearance of other steps that is the formation of new channels. Although the hemocyanin concentration was high we rarely obtained a real many-channel membrane. As a result in this work we present data only from single channel experiments.

Current was detected through Ag-AgCl electrodes and amplified by means of a virtual grounded operational amplifier (Analog Devices 515 K) with a 10^8 Ω resistor and a 20 pF capacitor in the feed-back loop (time constant 2 ms). Electric signals were recorded simultaneously on a strip chart recorder and on a frequency modulation magnetic tape recorder (Hewlett-Packard 3964A). The kinetic analysis of single channel experiments have been performed by hand on traces reproduced from the magnetic tape at a speed ten times slower than that used during the experiment. Closing fluctuations which do not depart from the mean values of the open state for more than 20% were not taken into account. Potentials refer to the potential of the compartment with protein.

Results

Channel Formation

After the addition of *Helix pomatia* hemocyanin to the *cis*-side of the membrane, which is held in voltage-clamp conditions, current pulses occasionally appear from the bare membrane level to a well defined current value which may be attributed to the formation of an ionic channel. Pulses follow one another quickly indicating open-closed fluctuations of the channel, however the current sometimes remains at the bare membrane level for a long time indicating no activity in the channel, then fluctuations again begin (Fig. 1a). This pattern of fluctuations could last more than 3 h during which the conductance of the open channel did not change. Because of the similarity of this complex behaviour to that of the acethylcholine-receptor channel [18] we have decided to call each group of square pulses between long intervals of apparent inactivation a burst.

The amplitude of the square pulses is not fixed and even applying the same voltage individual channels can display different values of conductance in different membranes. Also on the same membrane, when this contains more than one channel at the same time, current jumps of different amplitudes can be observed. The frequency distribution of the conductance jumps in different experiments is shown in Fig. 1b.

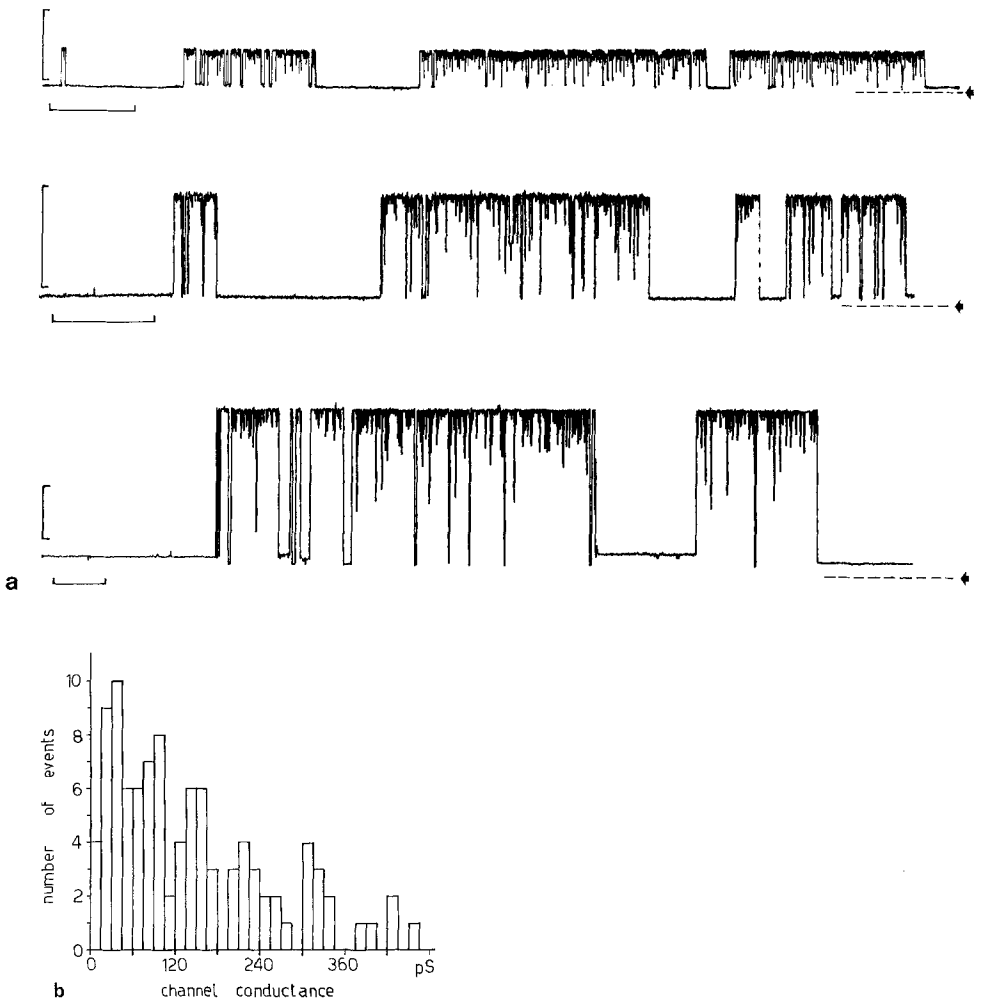

Fig. 1. a Current fluctuations due to the opening and closing of single *Helix pomatia* hemocyanin channels. Three traces have been reproduced showing three channels of different conductance but similar kinetic behaviour. Vertical bar is 100 pS and horizontal bar is 30 s in all the traces. The applied potential was 40, 43, and 47 mV respectively from up to down. On off fluctuations are grouped in "bursts" interrupted by long intervals during which the channel is completely closed. Other experimental conditions here and in the rest: KCl 0.1 M, pH 7.0, room temperature. Zero current levels are indicated by dashed lines pointed by arrows. **b** Histogram of the frequency distribution of the appearence of a *Helix pomatia* hemocyanin channel of a given amplitude. 100 channels have been considered at an applied voltage ranging from 40 to 50 mV

Apparently conductance values collect into a number of broad peaks whose height decreases as conductance increases. The spacing between the centers of the peaks, which represent the most probable conductance values, is almost regular at least within the first four peaks and may be regarded to be about 60 pS. As it is apparent from Fig. 1a the bursting behaviour is common to channels of different conductance amplitude. Furthermore in three cases it was possible to see a channel which changed its conductance during the experiment without apparently changing its kinetic properties. Channels with similar properties, that is on-off fluctuations and different conductance amplitudes, have been observed also in solvent containing bilayers painted with a 50 mg/ml solution of the same PC/PS mixture in n-decane (Menestrina G, unpublished observation).

Voltage Dependence

The applied membrane potential has two clear effects on the channel. First the conductance of the open channel decreases as the potential is increased. This decrease, though not very large, is quite reproducible and is independent of the amplitude of conductance. In Fig. 2 conductance values from different channels have been normalized by dividing by the conductance at −20 mV and plotted together. Qualitatively the behaviour of different channels is similar. However data obtained from a single membrane show a much higher correlation, therefore the scatter of the points of Fig. 2 seems to arise entirely from differences between different membranes.

The second effect of the applied voltage is a strong change in the duration of both bursts and inter-bursts intervals. Increasing the potential, irrespective of the polarity, increases the inter-bursts and decreases the bursts' duration. In Fig. 3a we reproduce some traces lasting about 3 min suitable to observe the division in bursts. The strips are obtained from one channel at five voltages

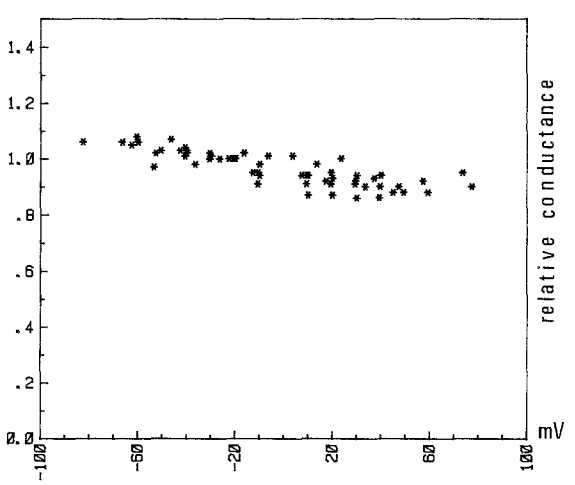

Fig. 2. Voltage dependence of the relative conductance. Values obtained from six different channels, at different potentials, have been plotted together after normalizing them by dividing by the conductance at −20 mV. The conductance decreases slightly as the potential is increased

ranging from about −60 to +60 mV. In part b we report the fraction of time that a burst is on as a function of applied potential, that is, for each voltage, the total bursts duration divided by the total duration of the record, that was typically ten minutes. Due to the large duration of the burst and interburst intervals only a limited number of these could be evaluated in each experiment, for this reason in order to improve the statistic in Fig. 3b we report the average of 10 different experiments with different channels. The effect of the potential is strong and nearly symmetrical and also in this case does not seem to depend on the conductance amplitude of the channel, in fact data from channels of different conductance could be plotted together with a good correlation.

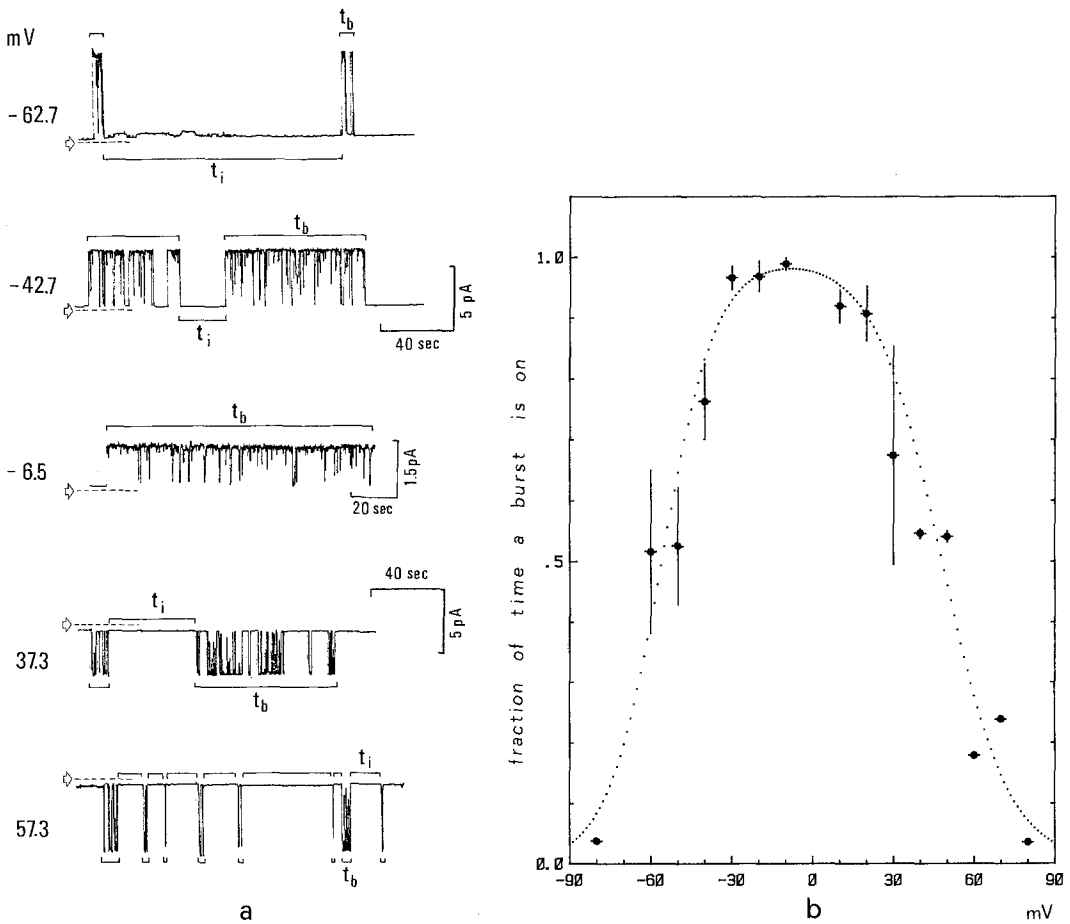

Fig. 3a and b. Voltage dependence of the duration of the bursts and inter-bursts intervals. **a** Direct reproduction of the traces recorded at different potentials with one single channel, zero current is indicated as in Fig. 1 for each trace. **b** Plot of the fraction of time a burst is on. Data obtained from one to 10 different membranes have been averaged, vertical bars are standard deviations, points without error bar are single determinations. The dotted line is drawn according to (6.1) with the parameters listed in Table 1. A mean of 20 ± 13 intervals have been used to calculate the experimental points

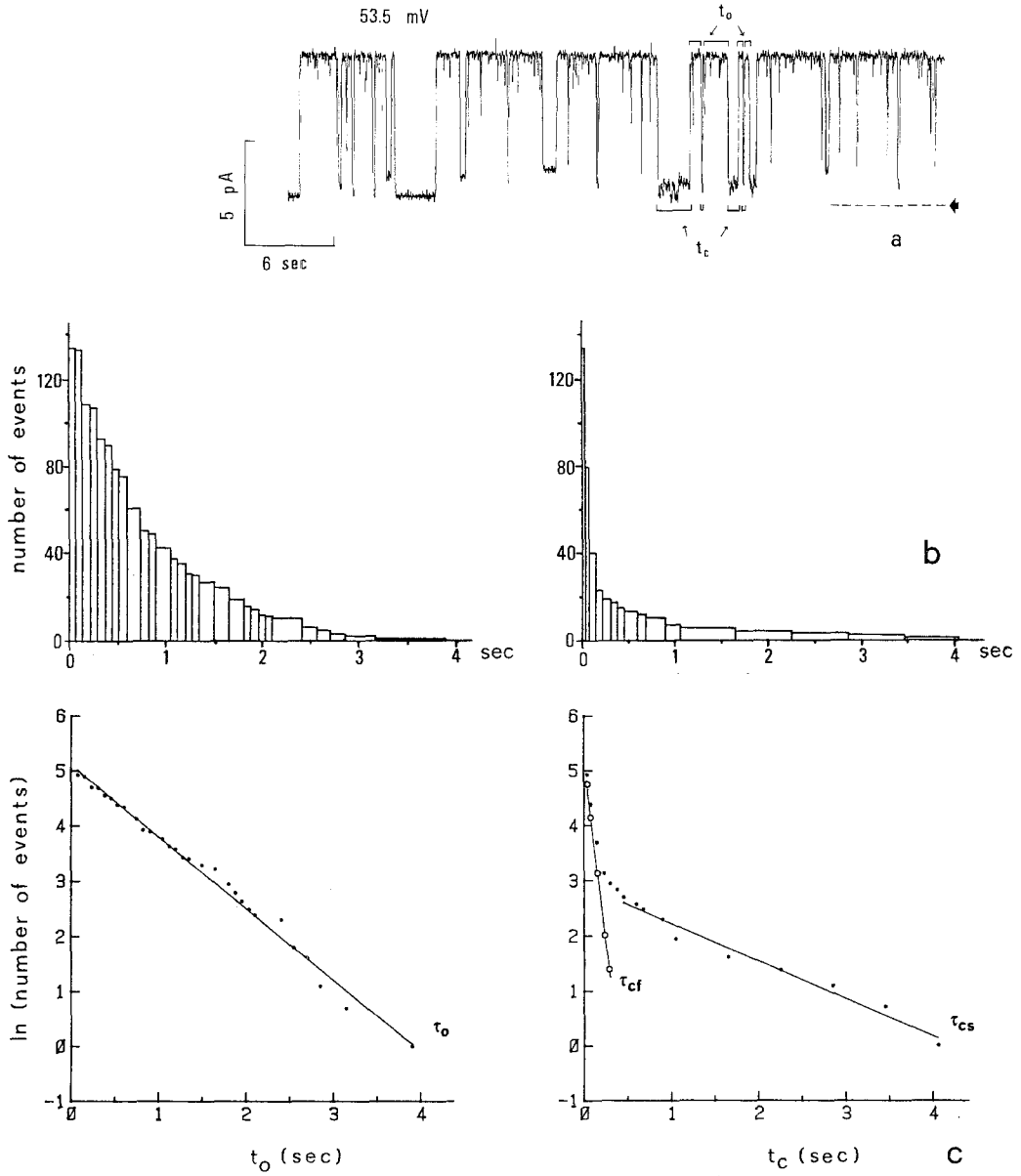

Fig. 4a–c. Statistical treatment of the time intervals spent on the open and closed states within a burst. **a** Direct reproduction of one recorded trace, some closed and open intervals have been indicated. A limited number of fast fluctuations remained unresolved and were not taken into account in the duration histograms. Zero current level is indicated as in Fig. 1. **b** Cumulative histograms, relative to the above trace, where the number of events longer than t is plotted against t. For each trace two histograms, one for the open times, left, and one for the closed times, right, were built up. **c** Semilogarithmic plot of the same duration histograms. The open times histogram was least square fitted with a single exponential of time constant $\tau_0 = 0.77$ s. The closed times histogram was least square fitted with the sum of two exponentials. The slower exponential has a time constant $\tau_{CS} = 1.49$ s, and the faster one, obtained after subtraction of the slower, a time constant $\tau_{CF} = 92$ ms. 26 pair of histograms from three different experiments buildt up of 150 ± 24 (mean $\pm$ SD) events have been fitted. The mean correlation coefficients of the exponentials of time constant τ_0, τ_{CS}, and τ_{CF} were respectively 0.988 ± 0.0003, 0.976 ± 0.004, and 0.991 ± 0.002

Kinetic Properties

We have tried to characterize the kinetics of the fluctuations which occur within a burst. Single channel experiments have been recorded on a magnetic tape and this allowed their reproduction with a greater resolution in time. One example is presented in Fig. 4a. From this trace it is apparent that not all the fluctuations lead to the same low level of conductance but for the sake of simplicity we decided to call all these low conductance states (their conductance does not exceed 20% of that of the open pore) closed states. Using this method we measured the duration of both open, t_0, and closed, t_C, intervals as indicated in the figure and we derived histograms in which the number of events longer than t is plotted against t as shown in Fig. 4b. The analysis has been limited to three channels of conductance around 100 pS, that is belonging to the second peak, from left to right, of Fig. 1b.

We found that the histograms for the open times could always be fitted, with good correlation, by one single exponential, Fig. 4c, which means that one single time constant, τ_0, is sufficient to describe this state. On the contrary the histograms for the closed states were more complicated. We fitted these histograms with the sum of two exponentials, the faster being obtained after subtraction of the slower one as shown in the figure. The identification and

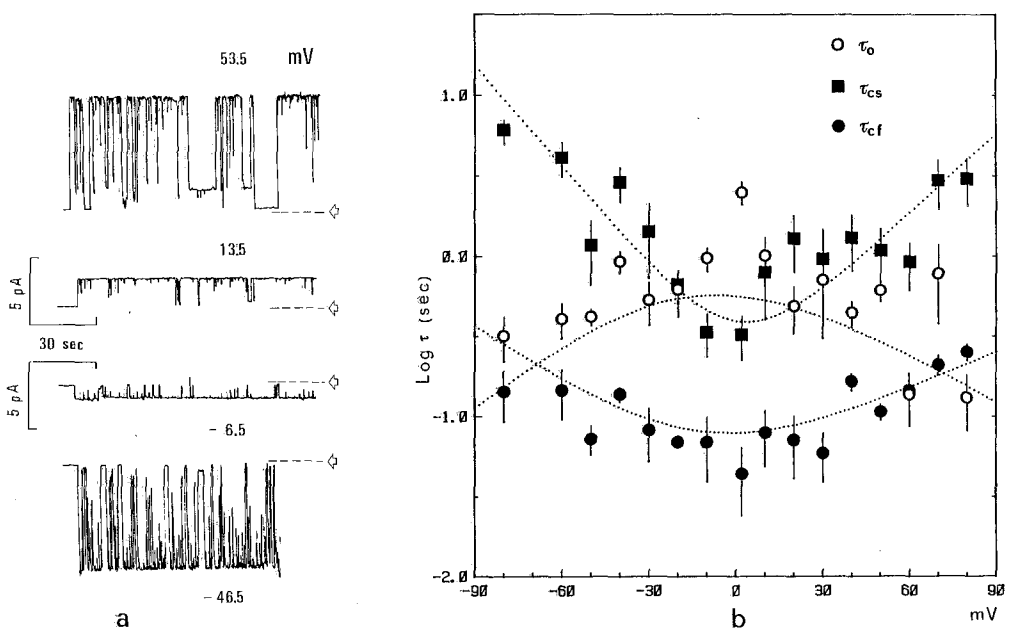

Fig. 5 a and b. Voltage dependence of the three time constants for the open and the two closed states. **a** Direct reproduction of burst traces at different voltages. Zero current levels indicated as in the other figures. **b** Semilogarithmic plot of the three time constants τ_0, τ_{CF}, τ_{CS} as a function of applied potential. Dotted lines are theoretical predictions of (2.1, 2.2, 2.3) of the appendix with the parameters listed in Table 1

separation of two time constants, one fast, τ_{CF}, and one slow, τ_{CS}, was particularly easy because these differed always by about one order of magnitude. Two time constants were always necessary, and sufficient, to fit the closed time histograms; only a few, and much longer, intervals were discarded and these were used to define the interbursts intervals which have been presented in the preceding section.

All the three time constants, τ_0, τ_{CF}, and τ_{CS}, are functions of the applied voltage and these data are shown in Fig. 5. In part a bursts' fluctuations from one single channel at different potentials ranging from about −50 to +50 mV have been reproduced. In Fig. 5b the three time constants have been plotted on a semilogarithmic scale against the applied voltage. As for the probability of a burst to be on, also in this case the effect of the potential on all three constants is

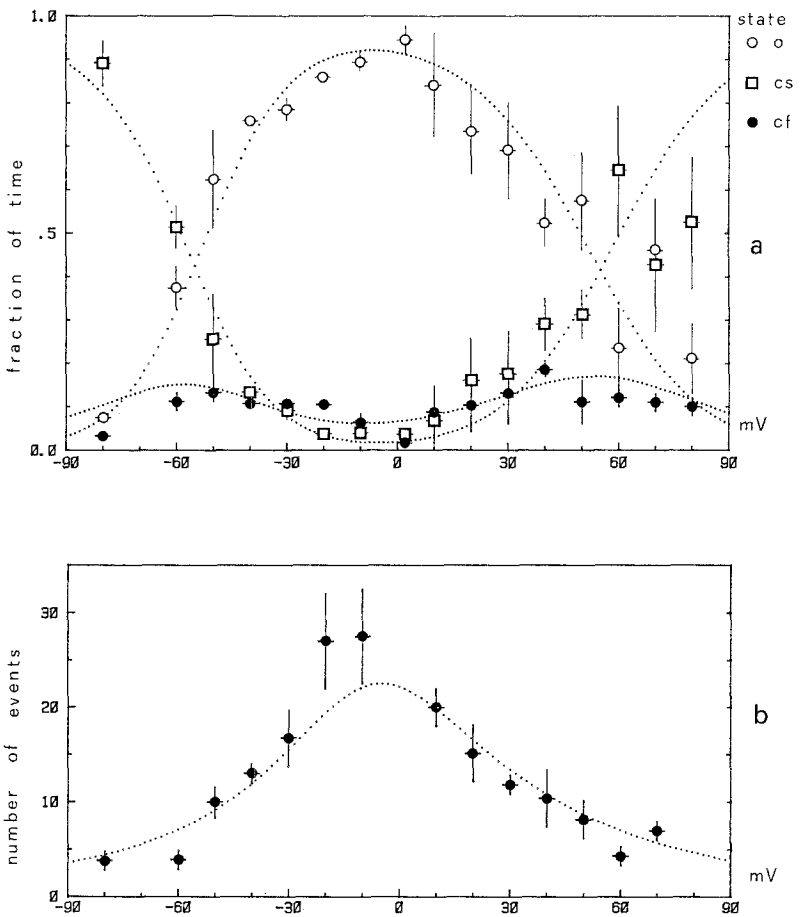

Fig. 6. a Voltage dependence of the fraction of time spent by the channel in the three states, 0, *CF* and *CS* which occur within a burst. **b** Voltage dependence of the mean number of fast fluctuations between two slow fluctuations. Dotted lines of both parts have been drawn according to (4.1, 4.2, 4.3) and (5.1) of the appendix, with the parameters listed in Table 1

symmetrical around roughly 0 mV. The mean open time decreases when increasing the potential either in the positive or in the negative direction while the two mean closed times both increase.

The effect of the potential is illustrated in a greater detail in Fig. 6. In part a we present the fraction of time spent by the channel in the three states open, closed fast and closed slow, as a function of applied voltage. The time spent in each state has been calculated by multiplying the mean dwell time of that state, from the slope of the relevant regression line in plot like those in Fig. 3c, by the number of intervals observed on the state, measured from the interception of the same line with the ordinate axis. The fractional probability then was computed, for each voltage value, by dividing the total time spent on one of the three states by the total time during which a burst was on. In Fig. 6b is shown the number of fast fluctuations (transitions $0 \rightarrow CF$) for each slow fluctuation (transitions $0 \rightarrow CS$), computed dividing the total number of fast fluctuations observed at a given voltage by the total number of slow fluctuations at the same voltage.

Discussion

General Features

The "bursting" behaviour seems now to be a quite general one, showed by at least three natural channels, those activated by acetylcholine, glutamate and calcium [4, 6, 14]. The *Helix pomatia* hemocyanin channel is similar to these even if it seems to be activated only by the applied voltage. A peculiar feature of this channel is the appearance of pores of different conductances. As shown by the frequency histogram of Fig. 1b these amplitudes seem to be equally spaced, that is one can be obtained by the other only by adding or subtracting a multiple of a fundamental unit which results about 60 pS. From the same histogram it is also evident that the probability of observing a channel of a given conductance decreases increasing its size. These facts can be explained at least in two ways. Either the channel can have different open states and it can appear in one of these states with a probability which decreases increasing the conductance of the state. Or the channel is formed by an aggregate of few pores whose conductance is about 60 pS and which are compelled to fluctuate together between an open state and some closed states near the bare membrane level, larger aggregates being less probable than smaller ones. This model has been recently proposed and called a "Gatling gun" structure for a natural Cl^- channel of the *Torpedo* electroplax [15]. The fact that transitions between different open states have been seen only very seldom would be more difficult to understand with the first model than with the second, anyway we feel that the problem is still open.

All the channels, irrespectively of their conductance value, are influenced by the applied potential.

The first effect is a small decrease in the conductance of the open channel as applied voltage is increased (Fig. 2) in the positive direction. This response has

also been observed in membranes treated with hemocyanins from the Keyhole Limpet and *Aplysia* [2]. In the case of Keyhole Limpet two recent papers have discussed this effect in detail [3, 11] and we send to them the interested reader. As in that case the fact that the absolute value and the voltage dependence of the conductance of the open state remains constant within one experiment can be considered a proof that *Helix pomatia* hemocyanin channels are irreversibly inserted and oriented into the planar bilayer.

The scond effect is a strong dependence of the partition between bursts and inter-bursts intervals on applied voltage. As reported in Fig. 3 the channel occupies the activated state (burst on) less frequently as the potential is increased, in either the positive or the negative direction from 0 mV. This dependence of conductance on the magnitude of the potential regardless of its sign is similar to that of EIM[1] [8] and has been recently found also for the Keyhole Limpet channel under appropriate conditions [11]. An even more striking similarity is found with the channels of the gap junction [19] which are also closed by potentials of either polarity. As in that case our data has been interpreted by a scheme in which the transitions between the activated and the inactivated states (burst on and off) are due to the movement of two gating charges under the applied field, see Appendix. The gating charge in our case, equivalent to 2–2.5 electronic charges, is about one half of that of the junction channel.

Kinetic Properties

The analysis of the kinetic properties of the fluctuations occurring within a burst has been shown to be complex. To us the simplest way to interprete these data is to assume that four different closed states and one single open state do exist. Transitions are permitted only between each of the closed states and the open state, and obey first order kinetics.

The model, developed in the appendix, is an extension of that used for example for the glutamate-receptor channel [6]. In our case four steps are needed because not only fast and slow transition are observed but also a double dependence on applied voltage, since both high positive and high negative voltages tend to close the channel.

We assume that each transition depends on applied voltage as in the simple scheme of the two state gating mechanism, see for example [8]. In this way a total of 12 parameters, three for each transition, are introduced and must be obtained fitting five independent experimental curves that are the three time constants: τ_0, τ_{CF}, τ_{CS} and two of the three fractions of time spent on each state: f_0, f_{CF}, f_{CS}. The fitting has been done by visual matching of theoretical lines drawn according to Eqs. 2.1, 2.2, 2.3, and 4.1, 4.2, 4.3 of the appendix respectively, with experimental points. First we fitted the three curves of Fig. 6, and we could evaluate eight from the 12 parameters, then we fitted the three curves of Fig. 5 and we adjusted the last four free parameters. The values of the

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Table 1. Parameters used in Eqs. (2.1, 2.2, 2.3, 4.1, 4.2, 4.3, 5.1) and (6.1) of the appendix to fit the experimental points of Figs. 3, 5, 6. In the upper part are the parameters relative to the fluctuations within a burst. For each transition the parameter A_α represents the gating charge; to obtain the value expressed in electronic units one must multiply it for $2\ kT/e_0 \sim 50$ mV at room temperature, where kT has its usual meaning and e_0 is the electronic charge. So the values range from 0.9 to 2.3 e.u. One should notice that two of the values are negative and two are positive reflecting the closing action of both positive and negative potentials. The parameter η_α gives a measure of the rate constant, the first two raws correspond to slow transitions, while the other two correspond to fast transitions. V_α gives a measure of the conformational energy difference between the closed states and the open one; multiplying it for minus twice the corresponding gating parameter A_α one obtains this energie expressed in units of kT.

In the lower part are the parameters relative to the fitting of the fraction of time a burst is on. A_α and V_α have the same meaning as above

State	V_α [mV]	A_α [mV^{-1}]	η_α [Hz]
CS1	− 55	0.048	0.35
CS2	55	− 0.038	0.65
CF1	− 74	0.028	4.35
CF2	80	− 0.018	4.75
I1	− 56	0.05	
I2	48	− 0.04	

parameters used are listed in Table 1. These values and the whole model are only indicative of a possible mechanism underlying the observed phenomena, so we did not consider a least square fitting to the points as necessary.

One parameter that is generally believed useful [6] is the mean number of fast closings between two slow closing, for this reason we report these data and the theoretical prediction, Eq. 5.1, in Fig. 6, though they are not independent from those previously presented. It may be seen that this number goes through a maximum in a region which correspond to the maximum probability of finding the channel open.

Also in the case of Keyhole Limpet hemocyanin channel fast and slow transitions between different conformational states can be observed [13], so we may conclude that though the different hemocyanin channels have quite different properties at a first sight, nevertheless some general features begin to appear to be common to this class of pore forming proteins.

Finally we should like to point out that the differences that we have found between the *Helix pomatia* and the *Megatura crenulata* hemocyanin channel are not likely to be due to the absence of solvent in the planar bilayers we used in this work. Indeed also solvent containing bilayers gave similar results and furthermore *Megatura crenulata* hemocyanin has been tested by us also on solvent free bilayers [13, 12] without noticing major differences from the solvent containing system.

Appendix

The Multistate Model

Literature data [4, 6, 14] suggest that an ionic channel even when fluctuating between an open and a closed state usually has more than two configurations. Indeed our experimental findings indicate that while the open state of the *Helix pomatia* hemocyanin channels is described by one single time constant three distinct time constants are needed for the closed state. Furthermore each of the three different closed state time constants has a double dependence on the applied voltage that is both high positive and high negative potentials increase it. So we think that the simplest way to represent the channel is to assume that it has one open state, indicated with 0, and six different closed states: CF1, CF2, CS1, CS2, I1, and I2. CF indicates the fast closing and CS the slow closing within a burst, whereas I indicates the long interbursts intervals. The numbers 1 and 2 indicate that the state is favoured at negative and positive voltages respectively. A complete description of the channel should take into account all these seven states but probably would be too cumbersome. Indeed it has been pointed out [14] that it is possible to group together any two or more adjacent states into one group state provided that transitions rates within the group are much larger than those outside the group. If this is the case the group state can be considered like a true channel state. We decided to group together all the states within a burst, that is 0, CS1, CS2, CF1, and CF2, in a "burst" state called B which is in equilibrium with the two interburst states I1 and I2. An inspection on Figs. 5a and 6b insures that the condition on the rate constants is fulfilled; indeed the inactivated states always last more than 20 s whereas the states within a burst do not exceed 1 s of lifetime.

In this way we can divide our model in two parts writing an equilibrium expression between burst and interburst states:

$$B \underset{\beta_{Ii}}{\overset{\alpha_{Ii}}{\rightleftarrows}} I_i \qquad (i = 1,2)$$

and an equilibrium expression for the states within a burst:

$$CS_i \underset{\beta_{CSi}}{\overset{\alpha_{CSi}}{\leftrightarrows}} 0 \underset{\beta_{CFi}}{\overset{\alpha_{CFi}}{\rightleftarrows}} CF_i \qquad (i = 1,2)$$

We have here assumed that each one on the closed state is in equilibrium with the open or with the burst state and that transitions between the different closed states do not occur.

Noting that all the rate constants proved to be voltage dependent we choose to use for each transition the simple two state voltage-gating scheme [8], so the rate constants have the general form:

$$\alpha_{Xi} = \eta_{Xi} \exp\left[-A_{Xi}(V - V_{Xi})\right] \qquad (1.1)$$

$$\beta_{Xi} = \eta_{Xi} \exp [A_{Xi}(V - V_{Xi})] \tag{1.2}$$

with $X = I$, CF or CS
and $i = 1,2$,

where V is the applied potential and the significance of the other constants is discussed in the legend of Table 1.

One can express the concepts underlying Eqs. 1.1–1.2 qualitatively by stating that each transition is due to the movement of one charged gating group over a free energy barrier separating two energy minima. These energy levels are most probably properties of two different configurations of the protein, and their absolute values are changed by the applied electric field of equal and opposite amounts [8].

Using this model the three time constants τ_o, τ_{CS} and τ_{CF} may be expressed by means of the rate constants as follows:

$$\tau_0 = \left(\sum_i \alpha_{CFi} + \sum_i \alpha_{CSi}\right)^{-1} = \left\{\sum_i \eta_{CFi} \exp [-A_{CFi}(V - V_{CFi})] + \sum_i \eta_{CSi} \exp [-A_{CSi}(V - V_{CSi})]\right\}^{-1} \tag{2.1}$$

$$\tau_{CF} = \sum_i \tau_{CFi} = \sum_i (\beta_{CFi})^{-1} = \sum_i \{\eta_{CFi} \exp [A_{CFi}(V - V_{CFi})]\}^{-1} \tag{2.2}$$

$$\tau_{CS} = \sum_i \tau_{CSi} = \sum_i (\beta_{CSi})^{-1} = \sum_i \{\eta_{CSi} \exp [A_{CSi}(V - V_{CSi})]\}^{-1} . \tag{2.3}$$

Here and in the following in all the summations the index i can have the values 1 and 2.

To calculate the fraction of time spent by the channel in each state one way is to note that this is equivalent to calculate, in a fictitious many-channel experiment, the number of channels in each state at equilibrium for a given voltage [8], so we may write:

$$f_0 = n_0/n_{TOT} \tag{3.1}$$

$$f_{CF} = n_{CF}/n_{TOT} = (n_{CF1} + n_{CF2})/n_{TOT} \tag{3.2}$$

$$f_{CS} = n_{CS}/n_{TOT} = (n_{CS1} + n_{CS2})/n_{TOT} \tag{3.3}$$

where f_X means fraction of time and n_X number of channels in state X, and $n_{TOT} = \Sigma_X n_X$. Hence:

$$\sum_X f_X = f_0 + f_{CF} + f_{CS} = 1 . \tag{3.4}$$

Applying the principle of detailed balance to each one of the transitions and substituting for the rate constants 1.1–1.2 we obtain:

$$f_0 = \left\{1 + \sum_i \exp\left[-2A_{CSi}(V - V_{CSi})\right] + \sum_i \exp\left[-2A_{CFi}(V - V_{CFi})\right]\right\}^{-1} \quad (4.1)$$

$$\begin{aligned} f_{CF} = &\left\{1 + \exp\left(2A_{CF1}(V - V_{CF1})\right)\left[1 + \exp\left(-2A_{CF2}(V - V_{CF2})\right)\right.\right. \\ &\left.\left. + \sum_i \exp\left(-2A_{CSi}(V - V_{CSi})\right)\right]\right\}^{-1} \\ &+ \left\{1 + \exp\left(2A_{CF2}(V - V_{CF2})\right)\left[1 + \exp\left(-2A_{CF1}(V - V_{CF1})\right)\right.\right. \\ &\left.\left. + \sum_i \exp\left(-2A_{CSi}(V - V_{CSi})\right)\right]\right\}^{-1} \end{aligned} \quad (4.2)$$

$$\begin{aligned} f_{CS} = &\left\{1 + \exp\left(2A_{CS1}(V - V_{CS1})\right)\left[1 + \exp\left(-2A_{CS2}(V - V_{CS2})\right)\right.\right. \\ &\left.\left. + \sum_i \exp\left(-2A_{CFi}(V - V_{CFi})\right)\right]\right\}^{-1} \\ &+ \left\{1 + \exp\left(2A_{CS2}(V - V_{CS2})\right)\left[1 + \exp\left(-2A_{CS1}(V - V_{CS1})\right)\right.\right. \\ &\left.\left. + \sum_i \exp\left(-2A_{CFi}(V - V_{CFi})\right)\right]\right\}^{-1}. \end{aligned} \quad (4.3)$$

The last parameter we want to calculate is the mean number of fast closing between two slow closings, that is:

$$\begin{aligned} \frac{n(0 \to CF)}{n(0 \to CS)} = \sum_i \alpha_{CFi} \Big/ \sum_i \alpha_{CSi} = &\left\{\sum_i \eta_{CFi} \exp\left[-A_{CFi}(V - V_{CFi})\right]\right\} \Big/ \\ &\left\{\sum_i \eta_{CSi} \exp\left[-A_{CSi}(V - V_{CSi})\right]\right\}. \end{aligned} \quad (5.1)$$

The same arguments exposed above to calculate the fraction of time spent by the channel in each of the interburst states can also be applied to the burst-interburst equilibrium. Rewriting Eq. (4.1) for this simpler three states case we obtain:

$$f_B = \left\{1 + \sum_i \exp\left[-2A_{Ii}(V - V_{Ii})\right]\right\}^{-1} \quad (6.1)$$

which is the expression for the fraction of time a burst is on used in Fig. 3b.

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