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A DIFFERENT KIND OF HEMOCYANIN CHANNEL IN OXIDIZED CHOLESTEROL MEMBRANES

G. Menestrina and R. Antolini

Dipartimento di Fisica, Università di Trento, 38050 - Povo (Trento) Italy

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SUMMARY

A new kind of hemocyanin channel in oxidized cholesterol black lipid membranes has been characterized using the protein extracted from the mollusc Paludina Vivipara. The channel has constant conductance versus applied voltage and is subjected to open-closed fluctuations that follow fairly well a binomial distribution, independent from the applied potential. Single channel electrical properties are in good agreement with those of many channels membranes. This channel is completely different from that already studied formed by Keyhole Limpet Hemocyanin and for some aspects reminds other channels particularly those obtained with Excitability Inducing Material and Alamethicin.

Several works (1, 2, 3) have described the ability of Hemocyanin to open channels through black lipid membranes. Their main purpose was to point out the interesting analogies between the electrical properties of hemocyanin doped membranes and excitable tissues. Hemocyanin is an oxigen transporting copper protein of great molecular weight, which occurs freely dissolved in the hemolymph of a number of invertebrates. Despite of this abundance of different hemocyanins all experiments so far reported were performed with the protein obtained from the blood of the Giant Keyhole Limpet Megatura Crenulata and purchased, in liophilized form, by Calbiochem.

In this work we present some results obtained studying the interaction of another hemocyanin, purified from the blood of the mollusc Paludina Vivipara, which is for many aspects structurally similar to that of Keyhole Limpet; in fact, around pH 7, they are both in the aggregation state known as 100S (4), which means that they have a molecular weight of about eight million, and a hollow cylindrical shape with 30 nm of external diameter and 35 nm of length. We found that also this protein interacts with bilayer

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membranes giving rise to the formation of channels, but the nature of the channel is completely different in the two cases.

The ability to interact with artificial membranes appears now to be a quite general property of hemocyanins of gastropod in the 100S aggregation state, we could observe it also with Busycon Canaliculatum and β-Helix Pomatia Hemocyanin, each channel yet shows individual electrical characteristics.

MATERIALS AND METHODS

Black lipid membranes were formed between two electrolytic solutions with the usual technique (5) and were comprised of oxidized cholesterol prepared following the procedure of Tien (6) with cholesterin and n-octane Fluka puriss. p.a.

Paludina Vivipara Hemocyanin, directly purified from the blood of the animal, at a final concentration of about 30 mg/ml, buffered at pH 7 and stored at -20°C with 180 mg/ml of Sucrose, was a generous gift of Prof. B. Salvato (Center for the study of Physiology and Biochemistry of Hemocyanins, Padova). Before use, it was dialized overnight at 4°C against buffer TRIS, pH 7.2; after dilution the state of aggregation, the degree of oxigenation and the concentration of the protein was determined by means of an U.V. - vis. spectra.
Small amounts of a 3 mg/ml stock solution were added to one side of the membrane, to get a final concentration of about 20 μg/ml.

The electrolytic solution, 0.2 M KCl buffered with TRIS, at pH 7.2, was then magnetically stirred for about one minute. In these conditions we could observe few channels for long periods.

The electrical set-up was composed of a D.C. battery power supply, two Ag-AgCl electrodes and an electrometer (Keithley mod. 602). The output signal was controlled on an x-t chart recorder and through a low noise amplifier (P.A.R. mod. 113) it was recorded on a magnetic F.M. recorder (Tandberg series 115).

The statistical analysis of current fluctuations was carried out subsequently on the recorded traces. To this purpose the signal was sampled through a linear switch (Siliconix DG 201) at a typical frequency of 100 Hz and analyzed by a multichannel (Silena system 27). A scheme of the set-up is given in fig. 1. The statistical analysis gave Gaussian curves (n + 1, when n channels were present in the membrane) corresponding to the possible conductance states of the system. These curves were equally spaced, each space reflecting the single channel conductance, and had an area proportional to the time the system had spent in that conductance state.

In the text positive voltages mean that the protein free compartment is positive with respect to the compartment containing the protein.

RESULTS AND DISCUSSION

Adding small amounts of Paludina Hemocyanin to one of the bathing solution of a black lipid membrane one can observe, keeping membrane voltage

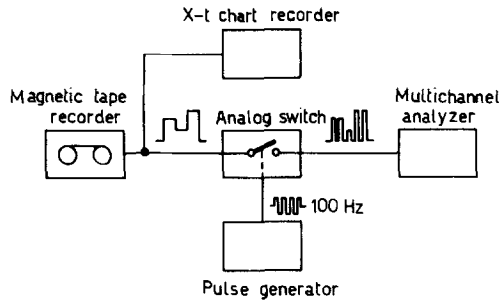

fig. 1 Block diagram of the set up used for the statistical analysis of current fluctuations in membranes with few hemocyanin channels.

constant, a step wise increase of current. These steps which are of equal amplitude, correspond to an increase in the membrane conductance of about 70 pS. Such increase in conductance may be interpreted as due to the formation of a channel. Soon after their appearance these current jumps are subjected to fluctuations, indicating opening and closing of channels. These fluctuations, presented in fig. 2, are quite similar to those already observed with Excitability Inducing Material (7), except for a little difference in the time scale. For analogy we shall call them ON-OFF fluctuations. The single channel conductance as a function of voltage can be measured by these formation jumps at different membrane potentials. As an example, fig. 3 shows some traces at various membrane potentials. The discrete conductance changes are quite evident and allow us to calculate (for the method see (8)) a mean channel conductance of 68 ± 7 pS, independent of the potential.

Informations on the probability of a single channel to be open can be drown by the statistical distribution of the number of channels open at a given time. In fact if few channels are present, say N, the probability that n channels are open at a given time is:

$$P(n) = \frac{N!}{n!\ (N-n)!}\ f^{n}(1-f)^{N-n} \qquad (7)$$

Where f is the probability of each channel to be open. We have analyzed a number of different current traces, in the presence of few channels (2 or 3 typically) at various potentials in order to test whether f, also called

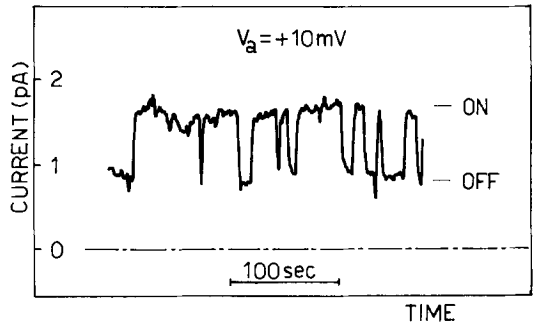

fig. 2 Formation and subsequent ON-OFF fluctuations of a single channel of Paludina Hemocyanin. The discrete conductance jump corresponds to about 73 pS. Protein concentration was 20 μg/ml and salt solution was 0.2 M KCl.

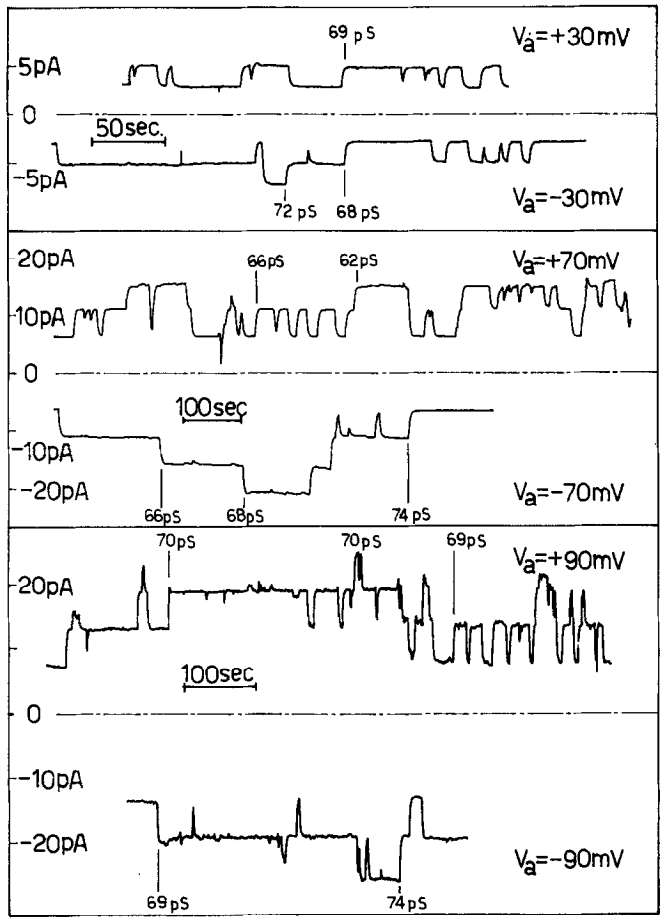

fig. 3 Current fluctuations in oxidized cholesterol membranes modified with Paludina hemocyanin. One can observe the current jumps resulting from the formation of some channels at different membrane potentials. Applied voltage and changes in membrane conductance are reported. Protein concentration was 20 μg/ml and salt solution was 0.2 M KCl.

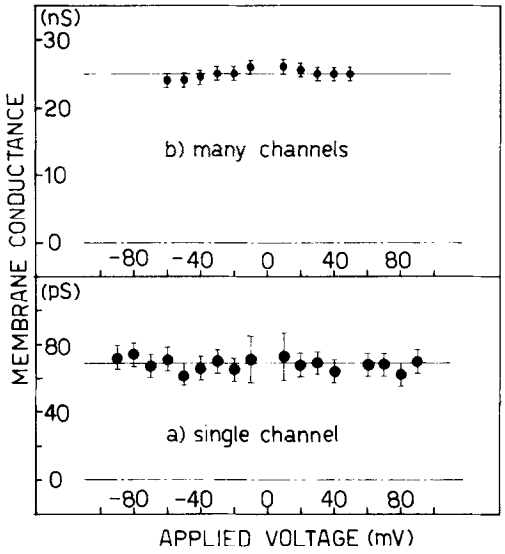

fig. 4 Conductance versus applied voltage in the two cases:
a) single channel (protein concentration 20 μg/ml)
b) many channels membrane (protein concentration 200 μg/ml).
Salt solution was 0.2 M KCl.

T A B L E I

Comparison between the electrical properties of the channel formed by two different kinds of hemocyanins: Keyhole Limpet and Paludina Vivipara.

Keyhole Limpet	Paludina
Single channel conductance (0.2 M KCl) 200 pS	Single channel conductance (0.2 M KCl) 70 pS
Single channel exhibits many conductance levels	Single channel fluctuates between open and closed state
Statistical distribution of conductance levels voltage dependent	Fraction of time open not voltage dependent
Many channels membranes have not linear I-V characteristic	Many channels membrane have linear I-V characteristic

fraction of time open, is a function of the applied voltage. Histograms built up in this way (see an example in fig. 5) showed that the distribution is a pure binomial one, which implies that the channels act independently and that $f = 1/2$. The values of f resulted always between 0.50 ± 0.15 with a random deviation not related to the applied potential.

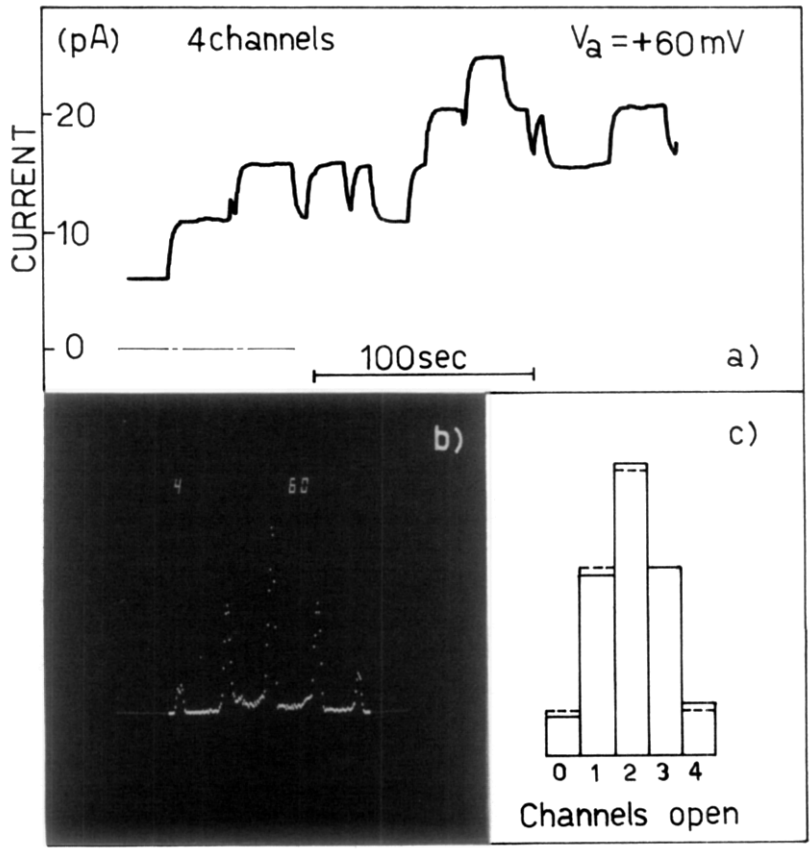

fig. 5 Statistical analysis of the time spent by the system in each conductance state:
a) current trace analyzed
b) photograph of the screen of the multichannel analyzer displaying 5 Gaussian curves corresponding to any possible number of open channels, when 4 channels are present
c) histogram obtained normalizing the areas of the curves of part b (solid line) compared with a binomial distribution (dotted line).

The fact that both single channel conductance and fraction of time open are not dependent on the applied voltage, allows us to foresee that also many channels membranes should have a linear I-V characteristic. Actually membranes with about $4x10^2$ channels (obtained by adding ten times more protein and waiting longer) exhibited a constant conductance versus applied potential as is shown in fig. 4.

CONCLUSIONS

The channel formed by Paludina Vivipara Hemocyanin is completely different from that formed by Keyhole Limpet Hemocyanin (2, 8). Table I stresses the differences in the electrical properties between the two systems. Its new characteristics put it in an intermediate position between the different channels already known:

i) ON-OFF fluctuations remind the Excitability Inducing Material channel in oxidized cholesterol membranes (7)

ii) f, fraction of time open, independent of applied potential reminds Alamethicin channel (9)

As in the case of all studied channels, single channel properties give a perfect account of many channels membrane properties.

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