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PROBING THE PORE SIZE OF THE HEMOCYANIN CHANNEL

XIMENA CECCHI [a], RICARDO BULL [b], ROBERTO FRANZOY [a], ROBERTO CORONADO [a] and OSVALDO ALVAREZ [a]

[a] *Departamento de Biología, Facultad de Ciencias Básicas y Farmacéuticas and* [b] *Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Casilla 653, Santiago (Chile)*

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We have studied single-channel conductance for different monovalent cations and streaming potentials caused by osmotic gradients of non-electrolytes in hemocyanin-treated membranes. We have found that the smaller ion, which cannot pass through the channel, is tetramethylammonium and that acetamide is the smaller non-electrolyte excluded from the pore. From the streaming potentials measured, we calculated that no more than three water molecules can accompany the ion through the channel in a row. From these results we conclude that the hemocyanin channel has in its structure a narrow portion which can be represented as a cylinder 6 Å long and 5 Å in diameter.

Introduction

Keyhole limpet hemocyanin (hereafter: hemocyanin) interacts with black lipid membranes [1] to form ionic channels [2]. These channels present several conductance states and the distribution among these states is voltage-dependent [3]. In membranes containing hemocyanin in only one side, the channel can be held in the open state if the membrane potential is negative at the *trans* side. In this condition it is possible to compare the conductance of the open channel in different cation solutions by measuring the amplitude of the conductance increase associated with the formation of the single channel [4].

Antolini and Menestrina [4] have measured single-channel conductance using Rb, K, Na, Li, Ca, Ba, Mg, NH_4, tetramethylammonium and tetraethylammonium chloride salts. They found a good correlation of channel conductance with cation equivalent conductivity, with the exception of lithium. They concluded that the channel structure is that of a wide aqueous pore with a diameter of 30 Å to accomodate freely the large organic cations such as tetramethylammonium and tetraethylammonium. Ion transport inside the pore is seen as no different from that in free solution.

This picture of the hemocyanin channel conflicts with the results reported by Cecchi et al. [5] on the voltage-dependent conductance of the open channel, ion competition and channel saturation. To explain these results, a channel model involving single ion occupancy and conduction by jumping over three energy barriers was proposed.

To solve these conflicting pictures of the open hemocyanin channel we have revised the single-channel conductance concept using sulfate salts, since hemocyanin is ideally cation-selective when sulfate salts are used [5]. We have also measured streaming potentials caused by a concentration gradient of non-electrolytes. Streaming potentials arise when a concentration gradient of an osmotically active non-electrolyte exists across a cation or anion selective membrane. Comparing the streaming potential caused by any non-electrolyte in a hemocyanin-treated membrane to that measured for an impermeant molecule we can discern between those molecules which can pass through the

channel and those which cannot.

From our measurement we can conclude that the pore size is small and excludes urea and tetramethylammonium ions. The large conductance of the channel is explained by the length of the narrow pathway, which is only three water molecules long.

Materials and Methods

Membranes were formed by apposition of two monolayers in the presence of decane [6]. Monolayers were spread from a solution of soybean lipids (10 mg/ml) and cholesterol (5 mg/ml) in a mixture of hexane and decane 20 : 1 (v/v). Soybean lipids, cholesterol and all organic solvents were from Sigma Chemical Co. (St. Louis, MO). Electric measurement equipment was the same described by Alvarez et al. [7].

Single channel conductance was measured forming the membrane under a 100 mequiv./l solution of sulfate or chloride salt of the cation under study, buffered with 5 mM Tris-HCl (pH 7). Hemocyanin (Calbiochem-Behring Corp., San Diego, CA) was added to one side (*cis* side) of the membrane and the *trans* side was held at −50 mV. Current jumps associated with the formation of hemocyanin channels were measured. In some experiments, concentrated potassium sulfate solution was added after 10–20 jumps had been recorded to compare the conductance jump size to that of potassium.

Streaming potentials were measured by forming the membrane under asymmetric solutions, the *cis* side containing 50 mM K_2SO_4/5 mM Tris-HCl (pH 7) and the *trans* side containing the same solution plus 1.3 to 1.8 M of non-electrolyte. Hemocyanin was added to the *cis* side only and membrane conductance was continuously monitored. When it had reached at least 0.1 μS, the open-circuit transmembrane potential was measured by means of a hight input impedance electrometer (Keithley Instruments, Cleveland, OH, model 602). The membrane was then broken by tapping the chamber and the potential in the absence of the membrane was recorded. The potential due to hemocyanin for a given non-electrolyte is the difference between the potential in the presence and in the absence of the membrane, which corrects for any contribution of the electrodes to the measured potential [8].

To correct for dilution potential and liquid junction potential, we calculated the streaming potential by subtracting from the hemocyanin potential the potential measured in a valinomycin- (Calbiochem, La Jolla, CA) treated membrane [8]. The streaming potentials were normalized to an osmolality difference of 1 osmolal/kg by directly measuring the solutions' osmolalities after the membrane had been broken by means of a vapor pressure osmometer (Wescor Inc., UT, model 5100 B).

Results

Single channel conductance

Table I is a summary of the single-channel conductance measurements in sulfate salts. Channel conductance is compared with ion equivalent conductivity in water. K, Rb, NH_4 and Na ion conductances appear to be dependent on the respective water conductivities. Cs and Li conductances are lower than those expected from their water conductivities. Tetramethylammonium, ethanolamine and Tris ions appear to be excluded from the channel, since channel conductances are near zero. Hydroxylammonium is a special case, since it hydrolyzes at pH 7; therefore, it was measured at pH 5 and the channel conductance is found to be dependent on pH [9]. Hydroxylammonium can pass through the channel and the channel conductance is comparable to that of potassium at the same pH (hydroxylammonium, 30 pS; K, 34 pS). From single-channel conductance, we conclude that the channel bore is small enough to exclude tetramethylammonium and ethanolamine ions but large enough to allow hydroxylammonium ions to pass through.

When single-channel conductance is measured in chloride salts, we obtain significantly larger values, indicating that chloride ions do pass through the channel. We measured 51.8 ± 1.6 pS for Tris, 53.8 ± 1.4 pS for tetraethylammonium and 43.6 ± 1.5 pS for tetramethylammonium (results expressed as the mean $\pm$ S.E. for at least 17 determinations).

Single-channel conductance measurements in 25 mM K_2SO_4 in the presence of 100 mequiv./l of

TABLE I

SINGLE-CHANNEL CONDUCTANCE OF HEMOCYANIN TO DIFFERENT CATIONS IN LIPID BILAYER MEMBRANES

Membranes were formed in 50 mM sulfate salts of the indicated cations, 5 mM Tris-HCl (pH 7.0) at room temperature. Conductance values are corrected for buffer conductance. Ionic radii for alkaline metal ions are from Ref. 12. Other ionic radii were estimated using the atomic increments method [13]. The normalized conductance for cation conductivity relative to that of potassium is given in the second column. The last column shows the channel conductance after adding K_2SO_4 up to 25 mM to both sides of the membrane. Conductance values are expressed in pS and represent the mean ± S.E. for at least ten determinations. TMA, tetramethylammonium.

Cation	Ionic radius (Å)	Channel conductance (100 mequiv./l cation) (pS)	Relative normalized conductance	Channel conductance (100 mequiv./l cation) plus 50 mequiv./l K^+ (pS)
Cs	1.69	112.0 ± 1.4	0.68	
Rb	1.48	152.8 ± 4.0	0.92	
K	1.33	157.4 ± 1.4	1.00	
Na	0.95	103.8 ± 1.0	0.97	
Li	0.60	26.4 ± 0.4	0.34	69.0 ± 0.3
NH_4	1.5	148.2 ± 2.4	0.94	
Ethanolamine	2.4	0.6 ± 0.1	0.01	62.0 ± 2.8
TMA	2.8	1.8 ± 0.2	0.02	90.0 ± 2.0
Tris	2.9	1.0 ± 0.1	0.02	78.0 ± 4.2
None				134.0 ± 4.2

TABLE II

STREAMING POTENTIALS CAUSED BY NONELECTROLYTE CONCENTRATION GRADIENTS ACROSS LIPID BILAYER MEMBRANES TREATED WITH HEMOCYANIN

Membranes were made under assymetric solutions, the cis side containing 50 mM K_2SO_4/5 mM Tris-HCl (pH 7.0) and the trans side the same solution plus 1.3–1.8 M of the indicated non-electrolyte, at room temperature. Valinomycin was added to both sides of the membrane. Hemocyanin was added only to the *cis* side. Molecular raddi were estimated using the atomic increments method [13]. Potentials are the difference between the potential measured with hemocyanin minus the potential measured with valinomycin for each non-electrolyte. Osmolality of the solutions was measured at the end of the experiments. The potentials are expressed in mV/1 osmol per kg of osmolality gradient and represent the mean and the standard error of the mean of at least five determinations.

Non-electrolyte	Molecular radius (Å)	Streaming potential ($mV \cdot kg \cdot osmol^{-1}$)
Glucose	3.3	1.18 ± 0.08
Urea	2.4	1.24 ± 0.12
Acetamide	2.4	1.23 ± 0.22
Formamide	2.1	0.16 ± 0.11

Li, tetramethylammonium, Tris or ethanolamine ions are smaller than for potassium alone. This result indicates that the organic ions can enter part of the channel structure and partially block the channel.

Streaming potentials

We used streaming potential measurements as an alternative method to estimate pore size. Table II is a summary of the determinations. We assumed that glucose is an impermeant non-electrolyte because it is larger than Tris, which is excluded from the channel. Glucose, urea and acetamide streaming potentials are equal, which indicates that neither urea nor acetamide can cross the channel. The formamide streaming potential is significantly smaller; therefore it does pass through the channel.

Discussion

Our results on single-channel conductance and streaming potential measurements indicate that the channel formed by hemocyanin is narrow. Tris ion being the largest cation excluded from the channel,

we chose glucose, which is bigger than Tris, as an impermeant non-electrolyte. Comparing the glucose streaming potential to those of the other non-electrolytes tested, it can be concluded that acetamide is the smallest one which is excluded from the channel. This sets the pore diameter near 5 Å. A pore of such a diameter should exclude tetramethylammonium ions and let hydroxylammonium ions pass, which is consistent with the experimental single channel conductances.

This pore width result does not agree with the 30 Å diameter channel proposed by Antolini and Menestrina [4]. It must be noted that their experiments were made with chloride salts and chloride may have an important contribution to their measured tetramethyl- and tetraethylammonium conductances. In addition, they worked with oxidized cholesterol membranes, in which the hemocyanin channel may have different properties.

Once a 5 Å pore is accepted, a new problem arises: why is the single channel conductance so high? A possible answer to this question is to imagine that there exists only a small portion of the channel which is narrow, an hour-glass shape. Streaming potentials can be used to estimate the length of the channel, assuming single-file movements of ions and water in the channel [8,10]. If the salt osmolality is the same at both sides of the membrane, it can be shown that the following relation exists between the streaming potential and the number of water molecules (N) inside the channel [8,10]:

$$V_{\text{streaming}} = N\frac{RT}{F}\varphi_s\frac{n_s(2)}{N_w(2)} \quad (1)$$

were R, T and F have their usual meaning, φ_s is the molal osmotic coefficient for the non-electrolyte, $n_s(2)$ and $n_w(2)$ are the number of moles of non-electrolyte and water, respectively, at the side containing the non-electrolyte. At room temperature and with a 1 osmolal solution, this equation reduces to:

$$V_{\text{streaming}} = N \times 0.46 \quad (2)$$

For a streaming potential of 1.2 mV per osmol per kg and applying Eqn. 2, 2.6 molecules of water interact with a potassium ion in the channel. Three molecules of water and one potassium ion placed in a row made a 6-Å-long channel, assuming single-ion occupancy [5].

Channels as short as we are proposing for the narrow part of the hemocyanin pore have also been described. Miller [11] demonstrated that the K^+ channel isolated from rabbit sarcoplasmic reticulum has a narrow portion and that 65% of the applied potential drops in a distance not longer than 6–7 Å.

The results taken altogether support that an hour-glass-shaped pathway is a good model for the hemocyanin channel. On the one hand, there has to be a wide entrance to the channel to account for the blocking effect of large organic ions which can reach this wide part but cannot cross the channel. On the other hand, there has to be a narrow part to account for the fact that molecules as small as urea and acetamide are excluded from the channel.

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