Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1071–1075, February 1988
Biophysics

Voltage gating of conductance in lipid bilayers induced by porin from outer membrane of *Neisseria gonorrhoeae*

(bilayer-membrane technique/triplet of channels/voltage-dependent gating)

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Communicated by Emil C. Gotschlich, October 26, 1987 (received for review August 24, 1987)

ABSTRACT **Porins, polypeptides of ≈35 kDa, are present as integral membrane proteins in the outer membranes of a variety of Gram-negative bacteria. As reported previously for a purified porin from *Escherichia coli*, voltage gating of conductance was found to be induced in a lipid bilayer by the solubilized purified porin, protein I, from *Neisseria gonorrhoeae*. The unitary response to an applied potential showed a cascade of current from an initial level through at least three levels, more or less equal, to a persisting lower level. The initial level of current corresponded to 1.0–1.3 nS for 0.2 M NaCl on either side of the bilayer. Briefly reducing the potential to zero restored the current to its initial level. Interpretation of the unitary response is suggested by electron microscopic data obtained on negatively stained outer membranes of *E. coli* indicating the presence of "pores" appearing in triplets. Moreover, low-resolution x-ray and neutron diffraction studies on crystals obtained with an *E. coli* porin show that three polypeptides associate to form a unit. Combining such structural data with the present electrical data lends support for the hypothesis that the unitary response results from three pores acting as a unit in response to an applied potential. Evidence obtained with the patch-clamp technique is mounting for a similar mechanism of many channels operating as a unit in a variety of cell membranes. The porin channel holds promise as a concrete model for the analysis of voltage gating of ionic conductance.**

A discovery of revolutionary significance for membrane biophysics was made some 15 years ago. It established the existence of a "two-state channel" with average open and closed lifetimes that are voltage dependent. This was accomplished by using planar lipid bilayer membranes doped with a chemical substance referred to as EIM (excitability-inducing material) (1). EIM was extracted from a Gram-negative bacterium, *Enterobacter cloacae*, but its chemical nature and location in the cell remain to be elucidated (2). More recently, it has been established that in the outer membrane of a variety of Gram-negative bacteria a characteristic polypeptide of ≈35 kDa is present as a major integral membrane protein that can induce in a bilayer membrane voltage-dependent gating of ionic conductance (3–6). This polypeptide has been purified from at least two bacteria, *Escherichia coli* and *Neisseria gonorrhoeae*, and in the former its amino acid sequence has been determined (7). It has been hypothesized that darkly staining holes, appearing as triplets in electron micrographs of the outer membranes of *E. coli* obtained with negatively stained specimens, are due to this polypeptide spanning the outer membrane as a triplet and thus forming a triplet of "pores" (8). Accordingly, these polypeptides are referred to as porins. The view that three polypeptides associate to form a unit has been reinforced more recently by low resolution x-ray and neutron diffraction studies on crystals obtained with an *E. coli* porin solubilized in a suitable detergent (9, ‡).

The goal of this brief communication is to extend the previous report on the electrical behavior induced by the solubilized porin of *N. gonorrhoeae*, protein I, in a planar lipid bilayer (5) and to emphasize certain features that are not observed in the EIM-doped bilayer. Indeed, in sharp contrast to the EIM-induced two-state gating behavior (10), the unitary response of the protein I-induced conductance to an applied potential cascades through at least three levels, more or less equal, to a persisting lower level, reflecting "inactivation" of the gating process in the face of the applied potential. This response is repeated upon returning the potential to zero for a brief interval (estimated to be several milliseconds). Interpretation of this gating behavior in terms of the triplet of pores will be discussed. The progress being made in determining the composition and sequence of amino acids and their conformations in the polypeptide chain holds promise for providing a well-defined system that can be used to analyze the physical mechanism underlying a specific case of voltage gating of membrane conductance.

MATERIALS

The lipids used (Avanti Polar Lipids) were synthetic 1-palmitoyl-2-oleoyl phosphatidylethanolamine (PtdEtn) and 1-palmitoyl-2-oleoyl phosphatidylcholine (PtdCho); these were stored at −80°C in chloroform (20 mg/ml). The lipid/decane solution to form the bilayers was made by mixing 200 μl of PtdEtn and 50 μl of PtdCho, blowing off the chloroform with a stream of N_2, and dissolving the lipids in 50 μl of *n*-decane (Wiley Organics, Columbus, OH) to give a concentration of 100 mg/ml. The electrolyte was 0.2 M NaCl (except where noted in text) and the temperature was ≈25°C.

Formation of Planar Bilayers and Electrical Measurements. Bilayer studies were carried out by using a variant of the classical cup technique of Rudin and Mueller—namely, a system of two overlapping chambers that consisted of a plastic rectangular block with two cylindrical cavities, one of which permitted insertion of a plastic cup with a 500-μm hole, the other serving as the outer chamber (11). The bilayer separating two 5-ml electrolytic solutions was formed by "brushing" the lipid/decane solution across the hole by means of a 5-μl glass pipette fused at one end. The bilayer could be viewed at all times through a glass wall at one end of the outer chamber via a light source and a monocular dissecting microscope. Silver/silver chloride electrodes were used to measure current flow at constant voltage through the bilayer in conjunction with a Keithley current

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Abbreviations: EIM, excitability-inducing material; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine.

‡Garavito, R. M., 45th Pittsburgh Diffraction Conference, Nov. 18–20, 1987, Charlottesville, VA, abstr.

amplifier (model 427), which served as a "virtual ground" (connected to cup), and an adjustable voltage source (100 mV) provided by a dry cell and a potentiometer (connected to outer chamber). The entire system of electrodes and plastic block was housed in a shielded box, and stirring of the cup solution was accomplished via a magnetic stirrer and a magnet driven by a variable speed motor. Porin was introduced into the cup at a minimal concentration, which was established empirically to obtain single unitary responses. The membrane capacity (600–800 pF) was monitored by applying a 5-mV (peak-to-peak) triangular waveform at 100 Hz and measuring the resulting capacitative current with a cathode ray oscilloscope. The current recordings were obtained with a Kipp and Zonen (BD40) chart recorder.

Purification of Major Outer Membrane Protein, Protein I. The strain RIO used in this study has been cultivated in our laboratory for several years. The bacteria were grown on solid typing medium defined by Swanson (12). All purification steps to obtain protein I were carried out according to the method described (13). Protein I was stored at 4°C in 0.2 M NaCl in the presence of Zwittergent 3-14—i.e., the detergent that was used to solubilize the peptides in the purification process (≈1 mg/ml).

RESULTS

Upon pipetting the porin solution into the cup in the face of stirring, a delay of 1–5 min occurred before an insertion event. Incorporation in the bilayer of solubilized porin was registered by a sudden excursion of the chart recorder pen upward, indicating current flow across the bilayer into the outer chamber. These observations were made usually at a potential of −50 mV (sign of the potential of the outer chamber with respect to the cup). It was observed that using different potentials did not change the number of insertion events, at least not within the range of 100 mV. An insertion event gave an initial value of current (level 1) that corresponded to a conductance of 1.0–1.3 nS. The current varied linearly with the potential over the range of 120 mV. After increasing to level 1, in general the current decreased in steps with time, giving rise to a pattern that shall be referred to as the unitary response.

The characteristic features of the unitary response of current to a step of voltage (starting from 0 mV) are shown in Fig. 1. Upon application of −70 mV, the current increases suddenly to level 1 and is steady for several seconds (the cusp is due to the capacitative current), whereupon it falls successively to a second, third, and fourth level (levels 2, 3, and 4, respectively). The time the current resides at the first, second, and third levels is variable and relatively short (order of seconds), while at the fourth level—the "residual current"—the residence time can be very long (order of minutes). Indeed, this level of current was recorded in some unitary responses for as long as an hour. It should be noted that at the first, second, and third levels, the current can drop momentarily and then recover as is shown in Fig. 1 (levels 2 and 3). The second unitary response of the same conducting unit shows that the current amplitude and residence time at levels 1, 2, and 3 can vary from response to response. To indicate as clearly as possible the occurrence in the unitary response of four current levels and variations in each, the current data obtained at −50 mV were chosen as being most suitable for analysis. The results of experiments are shown in Fig. 2 as histograms of five conductance parameters—namely, the conductance at level 1, the first, second, and third decrements in conductance (given by the difference between levels 1 and 2, levels 2 and 3, and levels 3 and 4, respectively) and the residual conductance. It should be noted that although such an event is relatively

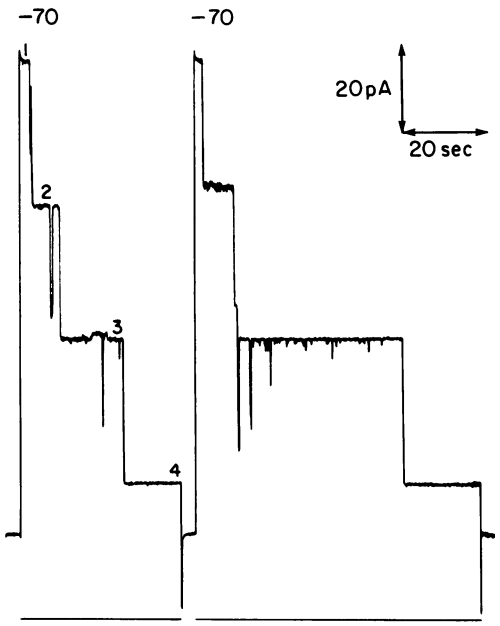

FIG. 1. Unitary response of a protein I-doped lipid bilayer to a step of potential. The cascade of current through four levels and the possibility of a momentary drop and recovery of current at a given level are shown. The bars below each recording indicate duration of the step of potential of −70 mV (polarity of outer chamber). The capacitative current transient is shown at the beginning and end of the applied potential. The variations in residence time at each level are also shown for two successive responses. Another striking feature displayed is the recovery from inactivation of the response brought about by interrupting the potential. Bilayer was formed with PtdEtn/PtdCho (4:1) in *n*-decane. The volume of electrolytic solution in the cup and outer chamber was 5 ml of 0.2 M NaCl. Stock solution of purified porin was diluted 1:40 and added to the cup in units of 10 μl to obtain a unitary response.

rare, the residual current (level 4) can fall to a very low value, approaching zero (Fig. 3, arrow).

The cascade of current through a series of levels is the reflection of a process of "inactivation" that takes place in the conduction mechanism. This term was used in 1949 to describe a striking feature of the sodium current observed in biophysical measurements on the membrane of the squid giant axon—namely, decline of the current after its rise (activation) upon depolarization of the membrane (14). It should be emphasized that the same term is adopted here only in reference to the cascading of the current in the unitary response in the face of an applied potential. What is most intriguing is that a brief reduction in the applied potential (to zero) will bring about restoration of the conduction process—i.e., restoration of the current response, as is clearly shown in the second response of Fig. 1.

Several other striking features of the unitary response should be pointed out. As the potential is increased, the overall time for the cascade of current is shortened (Fig. 4*A*). Indeed, at potentials of about −100 mV the levels are not recorded unless oscillographic techniques are used to resolve the steps of current. And starting from low values of potential—e.g., 10–20 mV—the time required to observe the steps of current increases markedly; that is, the residence time at different levels can be as long as 20–30 sec. In the limited number of experiments carried out in this range of potentials, only levels 1, 2, and 3 were observed; the fourth level was absent (see Fig. 4*B*).

The recordings in Figs. 2 and 3 show evidence for smaller excursions of the current (substates) at various levels of the unitary response as is reflected in thickening of the tracing at the relatively slow speed of the chart recorder used to obtain

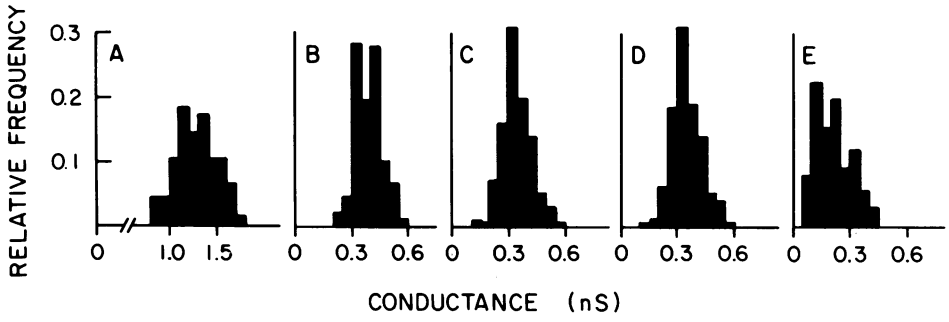

FIG. 2. Histograms of the relative frequency of occurrence of the conductance components in the unitary response. (*A*) Level 1. (*B*, *C*, and *D*) First, second, and third decrements in conductance (given by difference in levels 1 and 2, levels 2 and 3, and levels 3 and 4, respectively). (*E*) Residual conductance. The histograms were constructed from 88 recordings of the unitary response obtained at an applied potential of −50 mV.

most of the recordings. These substates could be easily examined with an oscilloscope at suitable sweep speeds. In Fig. 3 (*Inset*) a short tracing is shown of substates at a higher recording speed. It is clear that in addition to the large scale excursions of the current—i.e., levels 1, 2, 3, and 4—the current at any level can also show a two-state behavior of small amplitude. It should be noted that in a few experiments two-state excursions have been observed between two levels, which persisted for extended periods of time (order of minutes).

The data reported here have been obtained with negative values of potential, the polarity used for the majority of experiments. With the opposite polarity (i.e., positive), the responses have, in essence, the same features as described.

DISCUSSION

The introduction of the lipid bilayer as a model of the cell membrane, and the ensuing discovery that bilayers treated with a bacterial extract, EIM, showed ionic conductance that was voltage dependent and time variant, were crucial for the study of voltage gating of ionic conductance, a basic mechanism in cell membranes (15). Bilayer studies that followed yielded evidence for discrete fluctuations in the macroscopic ("smooth") variations of the current with time observed in EIM-doped bilayers (16). Indeed, these fluctuations were resolved clearly to be unitary two-state events (1). Subsequently, data were reported confirming that two-state behavior was induced by EIM and, in addition, described two new features in the current response (17, 18). First, it was observed that two-state current tracings showed during the open states relatively small two-state responses (referred to in the present communication as substates). Second, when lipids other than oxidized cholesterol were used, it was found that in general the current responses did not show two-states but rather "an initial, standard-size formation step followed by on–off fluctuations to a lower level and to the base level" (ref. 17, p. 21). Indeed, recordings showed clearly responses that were described as consisting of "three transitions" (figure 6 in ref. 17) and "triple fluctuation units" (figure 10 in ref. 18), features that have been emphasized here and referred to as the unitary response consisting of a cascade of at least three current levels.

FIG. 3. Unitary response with a relatively low residual current (arrow) and pronounced substates. (In a few recordings, the residual current was found to be very nearly zero.) As depicted clearly in a subsequent recording of a segment during level 3 taken at a higher paper speed (*Inset*), the substate component shows two-state behavior. Bar indicates duration of applied potential of −60 mV.

It is very instructive to read the review cited (18), especially the section concerning the hypotheses that may account for multilevel behavior of the EIM conducting unit, since they are relevant to interpreting the protein I-induced responses described here. The author hypothesizes that EIM forms either a channel with "three states" (hypothesis 1) or "three semi-independent fluctuation units" (hypothesis 2). Throughout, he is constrained by one important experimental fact from his research: namely, EIM induces "an initial standard-size formation step [of current]," which does not depend significantly on the lipid used to form the bilayer (table 1 in ref. 18). The lipid does, however, influence whether the initial step is followed by a sequence of on–off transitions (two-state behavior) or by multilevel transitions. The arguments for and against each hypothesis are lengthy and need not be repeated here. It will suffice to note that hypothesis 1 is favored—namely, "a single ion path in a macromolecular complex that can take on several different configurations to affect conductance." The absence at the time of the review of either electron microscopic or x-ray diffraction data to support the idea that a channel-forming substance extracted from bacteria could form a complex of channels as a unit upon inserting into a bilayer may have played a role in the choice of hypothesis 1. In the case of porins, however, such data do exist, and thus hypothesis 2 is worthy of serious consideration in discussing multilevel currents observed in porin-doped bilayer membranes. With regard to a complex of channels acting as a unit, it should be noted that a unit consisting of two channels was postulated to interpret current data obtained on chloride channels

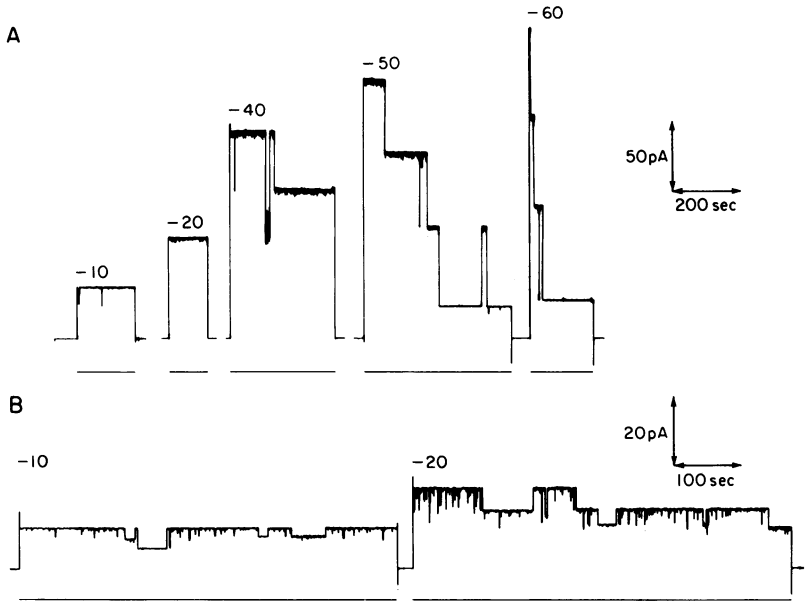

FIG. 4. Sequence of unitary responses obtained at different applied potentials. (*A*) Responses obtained successively at −10, −20, −40, −50, and −60 mV. Lipid bilayer was formed with only PtdEtn in *n*-decane and the electrolyte concentration was 1 M NaCl. (*B*) In another preparation, the recording at −10 mV and at −20 mV was extended to show that switching between current levels was observed at relatively low potentials when sufficient time was allowed for the transitions to occur. The lipids and electrolyte concentration were the same as in previous figures. Duration of applied potential is indicated by the bar under each recording.

extracted from *Torpedo californica* electroplax inserted into the planar lipid bilayers (19). Recently, in cell membranes, evidence has been obtained with the voltage patch-clamp method for multilevel currents in mouse pulmonary alveolar epithelial cells (20), molluscan neurons (21, 22), and renal tubules (23). In each case, two alternative hypotheses are considered to explain the patch current recordings: the current arises from either a single channel or a "cluster" of channels acting as a unit.

By electron microscopy and image processing of *E. coli* envelopes prepared by negative staining or by freeze drying and shadowing, images were obtained in 1977 showing "triplet indentations" extending over the entire surface (8). More recently, purified porin trimers from *E. coli* have been incorporated in lipid vesicles and, by using essentially similar electron microscopic techniques on negatively stained preparations, three-dimensional images have been obtained of pores forming a "tripod" structure—that is, each pore extending from the outer surface to a single pore at the inner surface of the vesicle membrane (24). [However, subsequent studies indicate that the three pores may not converge to a single pore (D. L. Dorset, personal communication); this issue remains to be resolved.] It is relevant to note here that evidence for the pores being aqueous filled have been obtained from osmotic and diffusion measurements with solutes of graded sizes (25). Additional data that provide evidence for a trimeric unit of porin polypeptides have been acquired from circular dichroism, intrinsic fluorescence, light-scattering, and sedimentation studies in micellar detergent solutions (26, 27).

Related to these studies, the detergent *n*-octyl glucoside has made possible the growth of porin crystals that are suitable for x-ray diffraction analysis (9). The high-resolution analysis carried out thus far is not complete, but model building at low resolution has provided clear evidence for a unit consisting of trimers.‡ The investigators carrying out these studies are now obtaining data at higher resolution and are most sanguine about having the full structure in the near future.‡

From the studies herein cited, it is reasonable to conclude that *E. coli* porin appears as a trimer both in outer cell membranes and in reconstituted bilayer membranes. And thus, until direct evidence is available, it will be assumed that the same trimeric structural unit holds for other Gram-negative bacteria such as *N. gonorrhoeae*.

The data reported here for protein I-doped bilayers agree qualitatively with similar experimental data obtained previously with *E. coli* porin (4, 5), especially with regard to the cascade of current through three downward steps. However, there is one difference: our experiments thus far have not shown, as in the case of the *E. coli* porin (4, 5), that an "initiation voltage" of ≈150 mV is required to activate the voltage-gating process. The requirement of a potential to activate gating may depend on the experimental procedure followed to obtain insertion of porin—e.g., detergent-solubilized porin with a preformed solvent-containing bilayer vs. fusion of lipid vesicles whose membranes contain porin to form a solvent-free bilayer (4, 5). On the other hand, two other groups of investigators who also have used lipid bilayers formed with a solvent (*n*-decane) have reported no evidence for voltage gating with other porins (28, 29). Thus, it may be that voltage gating in porin-doped bilayers depends on both the lipid and the porin; this matter should be settled by future research. In any event, for the results reported here with protein I, the qualitative features of the unitary response were the same for PtdEtn/PtdCho, PtdEtn, and oxidized cholesterol bilayers.

The studies of voltage gating of unitary currents in bilayers treated with an integral membrane protein extracted from the outer membrane of mitochondria—predating those with bacterial porins—have provided data that may be relevant here (30), particularly in the case of the gating protein obtained from mitochondria of the fungus *Neurospora crassa* (31). By extraction with Triton X-100 from suspensions of outer membranes of *N. crassa* and using low concentrations of the resulting solubilized protein, the insertion of triplets and the decay of current in steps have been recorded in bilayer experiments (potential of 40 mV). Moreover, recovery of the response upon interrupting the potential briefly has also been observed (30). It would appear that gating proteins with properties in common are present in the outer membranes of both mitochondria and Gram-negative bacteria.

By way of summarizing our interpretation of protein I-induced gating, let us attempt to describe an idealized "porin channel" by invoking the following properties: (*i*) A trimer forms three identical channels. (*ii*) Each channel is open at zero potential. (*iii*) Upon applying a potential, each channel closes to zero conductance with a variable (random) delay such that, on average, the higher the potential, the shorter the delay. (*iv*) Each channel is reactivated (opens) upon the

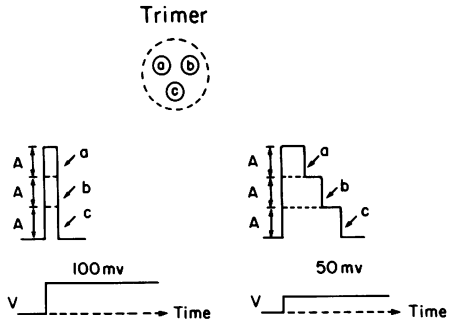

FIG. 5. Diagram illustrating generation of unitary response in idealized "porin channel." Each trimer provides a unit of three identical channels: a, b, and c, each giving current A with an applied potential V. The unitary response results from the summation of the three currents. For a low potential (e.g., 50 mV; *Right*) the unitary response shows three levels of current owing to three currents having different lifetimes; for a high potential (e.g., 100 mV; *Left*) the three currents have shorter and virtually the same lifetimes, whereupon the unitary response does not show a cascade of current levels (unless high-speed recordings are used). Note, scale of current on the left is reduced by one-half.

potential dropping to zero. (*v*) Channels are independent. (Analysis of residence times may very well show that interaction occurs.)

The diagrams in Fig. 5 depict the three separate currents a, b, and c with amplitude A generated by a step of potential and the addition of the currents (currents act in parallel), which gives the unitary response resulting from the three channels closing. In the diagram on the right, the general case is shown with the three currents having different lifetimes. It is important to be aware that in subsequent responses, the lifetime of each channel can change, either becoming longer or shorter than the previous one. Thus, in general, the summation of the currents with time will result in the recorded unitary response with the cascade of three current levels. Note that in the idealized model the fourth level of current—the residual current—is absent since each channel closes to zero conductance. In the diagram on the left, the case for a relatively high potential (100 mV) is shown with the three currents having shorter and virtually the same lifetimes, whereupon the summation of currents produces a unitary response in which the cascade of current levels is not observed unless high-speed recordings are used.

To understand more fully the mechanism underlying voltage gating induced by porins, it is clear that every effort must be made to determine the chemical nature and structure of the active component in EIM. It is tempting to ask: Is it porin-like or some other molecule? To resolve this question our goal will be (*i*) study the gating behavior induced by the porin from the outer membrane of *E. cloacae*, obtained by the standard procedure for isolation and purification used for protein 1; (*ii*) extract EIM from *E. cloacae*, following the prescribed procedure (16), and isolate the fraction that gives the classical two-state behavior in oxidized cholesterol membranes. The outcome of these studies should determine whether the substances are the same or different.

The amino acid composition and sequence of one of the porin polypeptides are known and determination of the three-dimensional structure appears imminent. A combination of such knowledge with that of the electrical properties of porin-doped bilayers should make the porin channel a concrete model that holds promise for understanding the physics of voltage-gating of conductance—a fundamental property of cell membranes.

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