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Properties of the major outer membrane protein from *Neisseria gonorrhoeae* incorporated into model lipid membranes

(planar bilayer/detergent dilution/anion selectivity/voltage-dependent conductance)

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ABSTRACT **The major outer membrane protein from *Neisseria gonorrhoeae* was incorporated into artificial planar bilayer membranes by a detergent-dilution procedure. The integrated protein forms voltage-dependent aqueous pores with a minimal pore diameter estimated to be 11 Å. A pore of this size suggests a role for this protein in macromolecular sieving at the level of the outer membrane. This protein self-associates preferentially in triplets of three equal unit conductance steps of 130 pS (in 0.1 M NaCl) each. The two-state model may be applied to explain the voltage-dependent conductance. The average lifetime of the open state of single channels is strongly dependent on the applied voltage, the channels shifting to the closed state at higher voltages. The pore is anion selective, differing from porins of other Gram-negative bacteria studied so far but resembling the voltage-dependent anion-selective channel of the outer membrane of mitochondria.**

The outer membranes of a number of Gram-negative bacteria contain highly organized passive diffusion pathways for macromolecules between 600 and 3,000 daltons (Da) (1). Porin, the protein responsible for these molecular sieving properties, has been characterized for several different types of bacteria (1–14). Porin from *Escherichia coli* is available now in crystalline forms amenable to ultrastructural studies (15). Porins from *E. coli* and *Salmonella typhimurium* have been incorporated into model lipid membranes (1, 3, 11–14). Membranes containing porin develop aqueous channels with pore sizes large enough to account for the observed permeability of the bacteria to certain nonelectrolytes (1). Crosslinking (4, 5), ultrastructural (6, 7), physicochemical (8, 9), and electrophysiological (10–14) studies have indicated that porin molecules preferentially aggregate as stable trimers.

Similar to that of other Gram-negative bacteria, the outer membrane of *Neisseria gonorrhoeae* contains a major protein (or protein I) that by itself accounts for more than 60% by weight of the protein in the outer membrane (16, 17). The molecular mass of this protein is strain dependent and lies between 32 and 39 kDa (17). Previous studies have shown that the outer membranes of *N. gonorrhoeae* are more permeable to macromolecules than are those of *E. coli* and other wild-type Gram-negative bacteria (18, 19). The relationship of protein I to the permeability of the gonococcal outer membrane is not clear.

In this report, we have incorporated the purified gonococcal protein I into high-resistance planar lipid bilayer membranes by a simple procedure that may be applicable to other integral membrane proteins. The high resolution of electrical measurements achieved on planar bilayers allows the dissection of molecular properties associated with permeability changes conferred by channel-forming proteins. We present evidence here that the major outer membrane protein from *N. gonorrhoeae* is a pore-forming protein that may be responsible for the permeability properties of the outer membrane of this bacterium. A preliminary report on the permeability properties of protein I has been presented elsewhere (20).

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (PtdCho; type VI-E), cholesterol (Chol; grade 1), and phosphatidylethanolamine (PtdEtn; type III) were obtained from Sigma. Octyl β-D-glucoside was obtained from Calbiochem–Behring. ^{3}H-Labeled tetraphenylphosphonium ([^{3}H]Ph_4P^+) (0.125 Ci/mmol, as bromide salt; 1 Ci = 3.7 × 10^{10} Bq) was a generous gift from H. R. Kaback of the Roche Institute of Molecular Biology. Buffer A consisted of 0.1 M NaCl/10 mM Hepes, pH 7.0; buffer B, 0.25 M sucrose/10 mM Hepes, adjusted to pH 7.0 with Tris base; and buffer C, 0.05 M NaCl/0.165 M sucrose/10 mM Hepes–Tris, pH 7.0.

Purification of Major Outer Membrane Protein. Protein I was purified by using methods previously described (21). On sodium dodecyl sulfate/polyacrylamide gel electrophoresis the results of such purification appeared as one band migrating in approximately the 34-kDa region. Contamination with lipopolysaccharide was ruled out by gas/liquid chromatography for carbohydrates and lipids and by phosphorus analysis.

Formation of Planar Bilayer Membranes and Electrical Measurements. Membranes were made by a modified procedure of Schindler's method (22). Ten microliters of a lipid suspension of PtdEtn/PtdCho/Chol, 3:2:1 (wt/wt), at 150 mg of lipid per ml and 20 mM octyl glucoside in buffer A was sonicated to clarity in a water-bath sonicator and deposited on each side of two Teflon chambers (surface area 3.5 cm^2) separated by a Teflon partition (0.125 mm thick). A 0.2-mm-diameter hole spanned the Teflon membrane. To reconstitute the outer membrane protein, the gonococcal protein was added to the lipid/detergent mixture, sonicated for an additional 5 min, and similarly layered on one side of the Teflon partition. Monolayers were formed by a one-step dilution process by adding 3–4 ml of buffer A to each side, below the level of the hole contained in the Teflon partition. Formation of monolayers was ascertained in preliminary experiments by a change in surface tension measured with a Langmuir balance (23) (data not shown). This procedure allowed immediate incorporation of protein into the monolayer during each experiment. Bilayers were then formed 5 min after the detergent-dilution step by raising the level of the monolayers above the level of the hole. Membrane formation was monitored electrically by a rise in capacitance. Electrical measurements were made as described (24). The potential of the rear or *trans* side of the membrane was defined as virtual ground. Positive current flowed from *cis* to *trans* side.

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Abbreviations: Da, dalton(s); PtdCho, phosphatidylcholine; PtdEtn; phosphatidylethanolamine; Chol, cholesterol; Ph_4P^+, tetraphenylphosphonium.

Protein I was always added to the *cis* chamber.

[^{3}H]Ph_4P^+ Uptake by Proteoliposomes. Proteoliposomes were prepared from a mixture of PtdEtn/PtdCho/Chol, detergent, and protein by a 1:30 dilution with buffer B. The uptake of [^{3}H]-Ph_4P^+ into lipid vesicles was quantitated by filtration, as described (24). Briefly, the reaction was initiated by diluting 0.1 ml of lipid vesicles, at protein/lipid concentrations specified in the text, into 0.9 ml of buffer C, containing 50 μM [^{3}H]Ph_4P^+. At intervals aliquots of the reaction mixture were filtered and washed, and radioactive material remaining on the filter was determined (24).

RESULTS

Bilayers Formed by One-Step Detergent Dilution. Bilayer membranes were assembled by the one-step detergent dilution protocol outlined in *Materials and Methods*. Protein-free bilayers formed by this procedure had baseline conductances that typically did not exceed 20 nS/cm^2 and a mean capacitance of 0.72 ± 0.06 μF/cm^2, which are comparable to the values obtained by others using different procedures (11, 25). Bilayers were often stable for at least 3 hr at room temperature. The presence of residual amounts of detergent did not seem to affect membrane stability.

Transmembrane Channels Formed by Porins. Addition of the gonococcal protein I to the lipid/detergent mixture on the *cis* side of the Teflon membrane prior to bilayer assembly resulted in bilayers showing high membrane conductance values that responded to the electrical field in a characteristic way (Fig. 1). An initial activation voltage was not necessary to turn the conductance "on." The instantaneous current flowing through the membrane responded linearly to the applied voltage and was symmetrical with respect to the polarity of the electrical field (Figs. 1 and 2). Up to 80 mV, the membrane conductance remained at high levels, without decay, and was not inactivated by transiently turning off the voltage (Fig. 1).

However, at voltages over 80 mV the instantaneous current values relaxed to lower steady-state levels (Fig. 1). The extent of the relaxation was a function of the applied voltage (Figs. 1 and 2). This relaxation occurred in defined discrete steps, indicative of closing of individual ion channels (Fig. 1). The magnitude of each individual channel was proportional to the ionic strength of the bathing buffer (not shown). Even at a time resolution of 1 msec, we could not resolve the risetime of each step. The pattern of relaxation gave characteristic negative-resistance regions between −140 and −80 mV and between 80 and 140 mV in the many-channel current–voltage plot (Fig. 2), with some asymmetry for the two different polarities of the electrical field. Thus, in the region of negative resistance, the membrane current was driven to a lower level by increasing the

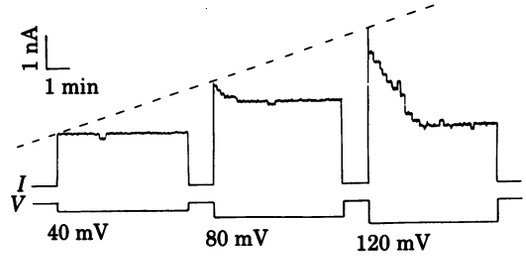

FIG. 1. Current relaxation of a membrane doped with outer membrane protein. Voltage steps were applied as indicated (lower tracing) to a membrane (buffer A, with 2 μg of protein) 15 min after bilayer formation. The voltage was turned off for 1 min between two steps. The final current level (after 5 min) was defined as the steady-state current, and it was used to generate Fig. 2. *I*, current; *V*, voltage.

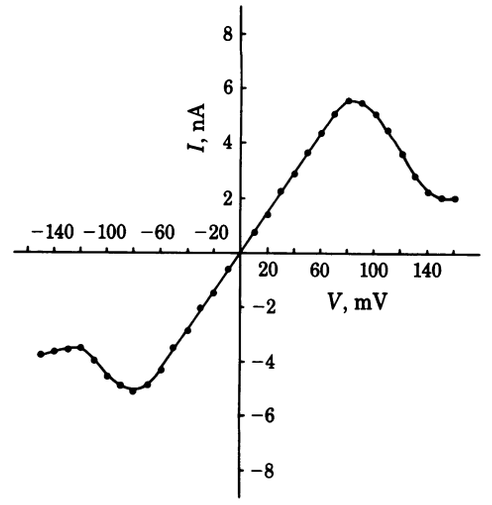

FIG. 2. Steady-state *I–V* relationship for a membrane containing outer membrane protein (2 μg). Voltage steps were applied from −150 to +150 mV, at 10-mV intervals. Values for current were determined as shown in Fig. 1.

electrical field, the effect being more pronounced for positive voltages.

Single-Channel Fluctuations. When nanogram amounts of protein I were incorporated into the lipid bilayer, discrete conductance steps of 390 pS in 0.1 M NaCl could be observed (Fig. 3*A*). The channels corresponding to this 390-pS step remained open for several minutes before the voltage was turned off. At voltages above 80 mV, the 390-pS step could be observed to relax to lower levels (Fig. 3*A*). The smallest unit step attained by increasing the voltage was 130 pS, and this conductance remained unchanged with further increases in voltage. When the voltage was returned to values below 80 mV, the 390-pS steps reassembled and remained fully activated, thus demonstrating that the effects of voltage on channel kinetics were reversible. Multiples of the 390-pS step were also observed. Fig. 3*B* shows three 390-pS steps conducting simultaneously in the membrane. These multiple steps responded to higher voltages in a similar fashion. In all instances, individual steps of 130 pS oc-

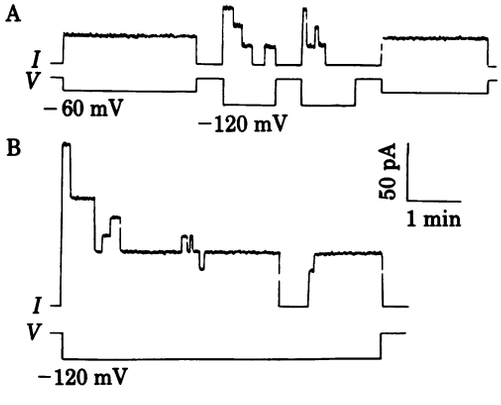

FIG. 3. Single-channel fluctuations in bilayer induced by low amounts of outer membrane protein. (*A*) The amount of protein incorporated into the bilayer membrane was 0.05 ng. Voltage steps were applied as shown in the lower tracing. The upward deflections represent opening of ion channels. At −120 mV, the initial current step is twice that seen at −60 mV, but the channels that constitute this step have a shorter open-state lifetime at the higher voltage. (*B*) Similar experiment with 0.1 ng of protein. Three triplets can be seen closing down in sequence when a voltage of −120 mV was applied. The capacitative transient due to the voltage drop across the membrane was deleted from both recordings.

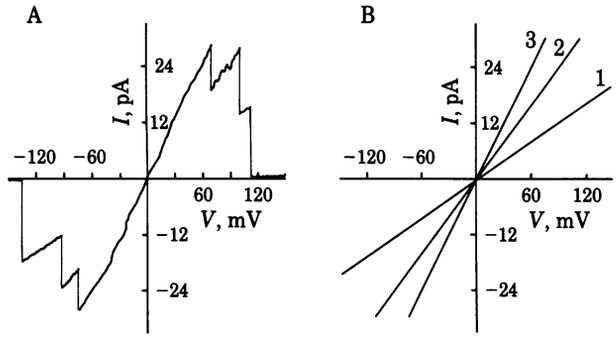

FIG. 4. Single-channel *I–V* relationship obtained with a continuous ramp. Bilayer in buffer A was formed with 0.1 ng of protein. It was conducting at 390 pS, when a ramp lasting 1 min was applied from −150 to +150 mV. (*A*) Continuous current recording (duration, 1 min) of the bilayer when the voltage ramp was applied. The background or voltage-insensitive conductances were subtracted from the recording by skewing the two axes so that the lowest levels of current observed at high voltages were zeroed. (*B*) *I–V* relationships for 1, 2, and 3 channels reconstituted from recordings of the type shown in *A*.

curred as intermediates during the transition between open and closed states.

Nature of the Voltage Dependence. It is interesting to note that the transition between steps of 390 pS and 130 pS occurred only at voltages that paralleled the region of the negative resistance for a many-channel-containing membrane—i.e., for a membrane showing macroscopic conductance (Figs. 2 and 3). The application of a continuous triangular pulse to a 390-pS step illustrates this transition at the single-channel level. In Fig. 4*A*, a ramp from −150 mV to +150 mV was performed on a membrane conducting at 390 pS. An open-channel *I–V* relationship can be obtained by using such a voltage-clamping ramp. The current increased proportionally with voltage until transition regions above 80 mV were reached. At voltages greater than 80 mV (in both polarities), a region of instability was reached where the 390-pS step switched to lower conductance values (130 and 260 pS) until channels closed completely (Fig. 4*A*). The *I–V* plot for a single channel continued to behave linearly even in the transition region (Fig. 4*B*). That is, although the current driven through each channel increased proportionally with higher voltage, the average lifetime of the open channel decreased in the same direction, thus explaining the nonlinear *I–V* plot of Fig. 2. This observation is in accord with the two-state model proposed for channel kinetics (26–28). Thus, the voltage dependence of macroscopic conductances may be adequately explained by the behavior of single channel fluctuations. Taken together, these data indicate that the protein preferentially self-associates as triplets and multiples thereof that may functionally assemble from individually conducting monomers.

The two-state model (27, 28) further predicts that an energy activation barrier must be overcome for the transition of an open to closed channel to occur. The relative number of open (n_o) and closed (n_c) channels may then be given by the Boltzmann distribution (27),

$$\frac{n_o}{n_c} = e^{-E(V)/kT}, \qquad [1]$$

in which $E(V)$ is the voltage-dependent energy barrier associated with the open–closed transition, k is the Boltzmann constant, and T is the absolute temperature. By expressing the total voltage-dependent conductance in the membrane (G_o) as a function of the open channel conductance (g_o)—i.e., $G_o = n_o g_o$—the number of transmembrane charges n required for the open to closed state transition per channel can be given by

$$\frac{n_o}{n_c} = \frac{G_o}{G_c} = e^{-nq(V - V_o)/kT}, \qquad [2]$$

in which $E(V)$ has been replaced by $nq(V - V_o)$, $G_c = (G_{o(max)} - G_o)$, q is the electronic charge, and V_o corresponds to the V at which $n_o = n_c$. A plot of $\ln[G_o/G_c]$ vs. V gives a straight line, the slope and intercept of which yield n and V_o (Fig. 5). For the gonococcal protein, V_o is 110 mV and $n = 2.5$. From the slope, it can also be seen that the ratio of open to closed channels changes e-fold for every 10.5 mV. In units of energy, the barrier that must be overcome in the transition region represents 4.4×10^{-20} J per channel.

Dose Dependence and Insertion of Protein from the Aqueous Phase. The channel-forming activity of protein I could also be assayed by adding it directly from a detergent-containing solution to the aqueous phase of preformed bilayers. Adding protein I, in 0.5% Triton X-100 or 25 mM octyl glucoside, to such bilayers resulted in conductance jumps indicative of direct insertion of the protein into the bilayer membrane (Fig. 6). However, incorporation of the detergent-treated protein into preformed bilayer resulted in changes in the shape of the *I–V* plot with a small shift of the negative resistance region towards lower voltages (data not shown). With similar amounts of protein, membrane conductance was higher when the protein was added

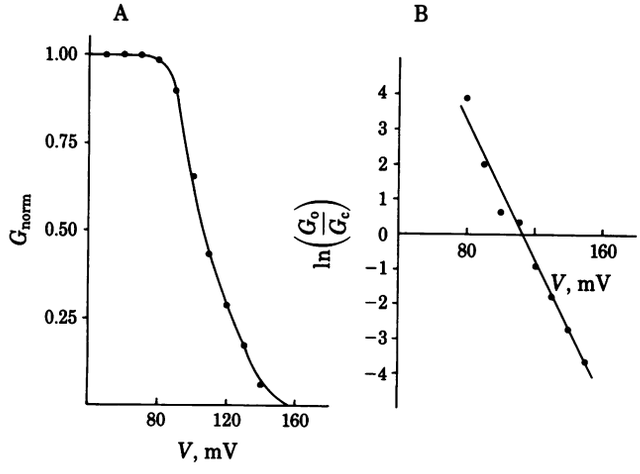

FIG. 5. (*A*) Normalized steady-state voltage-dependent *G–V* plot. Values for conductance (*G*) were obtained from Fig. 2. The voltage-insensitive conductances (at large *V*) were subtracted from each point and normalized with $G = 1$ near $V = 0$. Only values for *G* at positive voltages were plotted. (*B*) Replot of *A* with $G_c = (G_{o(max)} - G_o)$, in which G_o is the value for conductance in *A* prior to normalization and $G_{o(max)}$ is *G* obtained near $V = 0$. The intercept with the *V* axis gives V_o (or the switching voltage, at which $G_{norm} = 0.5$). The number of equivalent charges n is given by the slope (see text).

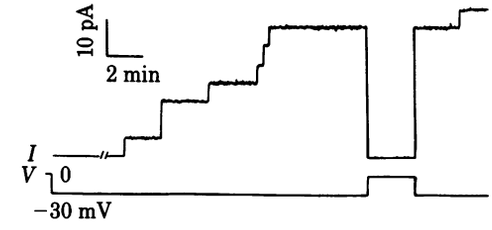

FIG. 6. Insertion of outer membrane protein into a preformed bilayer. Protein (1 μg) was added to a preformed bilayer (in buffer A) through the aqueous phase, followed by immediate stirring (noise deleted from the recording, as indicated by the parallel bars). The upward deflections represent insertion and opening of single or groups of channels in the bilayer. The applied voltage (−30 mV) is shown in the lower tracing. The voltage was turned off for 3 min, as indicated, to demonstrate that inactivation in the absence of an electrical field did not occur.

through the aqueous phase of the membrane than when it was added by the procedure described above (Fig. 7).

The dose–response curve showed a linear relationship for protein reconstituted by both protocols (Fig. 7). Such a relationship leads to two tentative interpretations: (*i*) preferential oligomerization into multiples of triplets occurs but is not required for an ion-conducting state, with monomers being fully functional, or (*ii*) trimers are stable in both the aqueous and lipid phases and remain undissociated as such, therefore giving rise to the simple linear relationship of membrane conductance as a function of trimer concentration used.

Ion Selectivity. The ion selectivity was determined from the reversal potential necessary to null current flow from a 10-fold higher KCl or NaCl concentration, with the *cis* compartment being more concentrated. The results showed a reversal potential (E_r) of +28 mV for KCl and +31 mV for NaCl, which can be inserted into the Nernst–Planck equation,

$$E_r = \frac{(u_{anion} - u_{cation})}{(u_{anion} + u_{cation})} \frac{kT}{F} \ln\left(\frac{A_{cis}}{A_{trans}}\right), \quad [3]$$

in which u represents the mobility of the ionic species, F is the Faraday constant, and A is ion activity. It gives a ratio of $Cl^-:Na^+:K^+:Ca^{2+}$ of 1.00:0.28:0.30:0.04. This indicates that the major outer membrane protein from *N. gonorrhoeae*, unlike porins from other types of bacteria (11, 14, 15), is more anion selective.

These results were further validated by the $[^3H]Ph_4P^+$ experiment described in Fig. 8. Proteoliposomes were formed by the one-step detergent dilution procedure in the presence of sucrose and diluted into buffer containing equiosmolar amounts of NaCl and $[^3H]Ph_4P^+$. The influx of Cl^-, which occurs at a faster rate than influx of Na^+, generates a transient membrane potential, which can be measured by the uptake of $[^3H]Ph_4P^+$. The membrane potential is dissipated after a few minutes, indicating that the proteoliposomes also show some permeability for Na^+, albeit lower. The magnitude of anion flow measured by this technique is also dependent on the amount of protein used during reconstitution (Fig. 8).

Lipid Requirements. Changing the lipid composition of the bilayer from PtdEtn/PtdCho/Chol to pure PtdEtn, PtdCho,

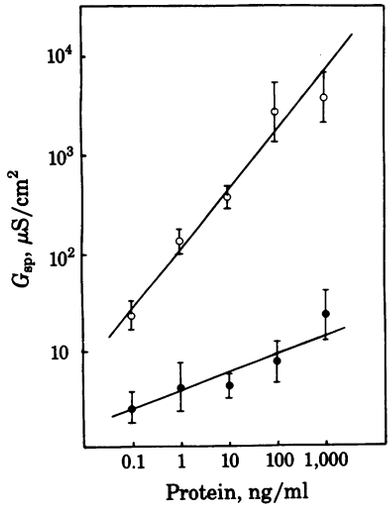

FIG. 7. Double-logarithmic plot of membrane conductance as a function of protein concentration at a constant voltage (+30 mV). The protein was either incorporated into the bilayer during membrane formation (●) or added to preformed bilayers through the aqueous phase (○). The conductance was measured 10 min after membrane formation in the former experiment and 30 min after addition of protein in the latter. Points represent means ± SD of four or five experiments. G_{sp}, specific conductance.

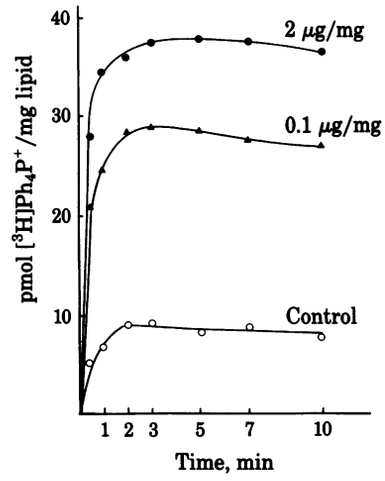

FIG. 8. Permeability of proteoliposomes containing outer membrane protein by $[^3H]Ph_4P^+$ uptake. Proteoliposomes containing gonococcal protein were reconstituted by detergent dilution at two protein concentrations: 2 μg of protein per mg of lipid (●) and 0.1 μg/mg (▲). The lipid concentration was 50 mg/ml. Control experiments were done in the absence of gonococcal material (○). Points refer to means of four experiments.

or Chol membranes did not change the overall characteristics of the channel. However, in PtdEtn membranes, the *I–V* plot was displaced, with the negative resistance region shifted to higher voltages.

DISCUSSION

The experiments described here show that the major outer membrane protein (or protein I) from *N. gonorrhoeae* functions as a hydrophilic channel in model membranes, resembling porin from other Gram-negative bacteria (1). We describe a simple and reproducible procedure for incorporation of membrane proteins into artificial planar bilayers. The procedure is a modification of Schindler's method (22) in that a single detergent-dilution step is used for both proteoliposome and monolayer formation. This procedure is reproducible and allows reconstitution of membrane proteins into planar bilayers amenable to electrical studies. Preliminary experiments using vesicle suspensions formed by sonication or detergent dialysis for monolayer formation (22) were often unsuccessful in our hands. This may have been due to the relatively smaller size of sonicated (500 Å) and detergent-dialyzed (200–500 Å) vesicles (29, 30), in contrast to vesicles formed by detergent dilution, which may exceed 2,000 Å (ref. 29; unpublished observations). The spontaneous formation of monolayers from vesicles is dependent on the size of the vesicles and appears to favor larger vesicles (22). In addition to the lipid exchange of vesicles in solution with the air/water interphase (22, 23), the lipid that is diluted out in the solution may directly appose to and rapidly collapse at the interphase to form lipid monolayers. Bilayers formed by this detergent-dilution protocol have all the electrical properties described for high-resistance planar bilayers (22, 25). The presence of residual detergent, after a 300- to 400-vol dilution, apparently does not affect membrane stability.

The major outer membrane protein from *N. gonorrhoeae* shows remarkable voltage dependence in the planar bilayer (Figs. 1 and 2). The macroscopic conductance is driven to lower levels by increasing the voltages above 80 mV in both polarities. This variable conductance can be explained by the voltage-dependent lifetime of single channels. Thus, increasing the voltage above 80 mV reduces the open-state lifetime of the individual channels (Figs. 3 and 4). The two-state model (26–28), even though being an oversimplified picture of the mechanics of

channel gating, may be applied here to account for most of the details related to the voltage-dependent conductance. This model also implies in an energy activation barrier that must be overcome for the transition of open to closed states to occur. We obtained a value of three equivalent charges that must move across the membrane to switch the channel from the open to the closed state.

The voltage dependence displayed by the gonococcal porin suggests a way in which the permeability to ions, nonelectrolytes, or both might be regulated *in vivo*. For *E. coli*, both voltage-dependent (11, 12) and voltage-independent (13–15) conductances have been described. This divergence in data probably reflects the use of differing protocols for porin incorporation into the lipid bilayer. The asymmetry that we observe in the macroscopic *I–V* plot for gonococcal protein I, as a function of the polarity of the applied voltage (Fig. 2), suggests a vectorial insertion of the protein into the lipid bilayer.

By assuming that the protein is a cylindrical structure filled with an aqueous solution of the same specific conductance as the external solution (14), the size of the pore can be estimated from the magnitude of each discrete conductance step by the relationship

$$g_o = \sigma\pi r^2/l, \qquad [4]$$

in which σ is the specific conductance of the aqueous phase and r and l are the radius and length of the pore, respectively. In 1 M NaCl, g_o = 1.4 nS; σ = 84 mS/cm, and an average length l of 60 Å can be assumed, which would correspond to the thickness of the outer membrane [this value has been reported as 40–75 Å in previous studies (11, 13–15)]. This calculation yields a minimal pore diameter of 11 Å and a cross section of 100 Å^2, which would be of the right magnitude to account for the nonelectrolyte permeability properties of the outer membrane (1, 19). For instance, sucrose, which requires an aperture of 9 Å to pass through, would be predicted to pass through this pore. However, it is important to note that the magnitude of the conductance step per channel may not reflect the actual size of the pore, and channel-forming substances with small conductance steps have been associated with rather large pores (31, 32).

It is yet unclear how many channels are associated with each triplet (1). Both our biochemical data (21) and the data presented here would support the view that porin self-associates as trimeric structures and that the smallest conductance step can be dissociated into three equal unit steps by applying voltage in the negative resistance region (Figs. 3 and 4). Thus, from our observations, the gonococcal porin appears to be arranged in triplets, with each monomer contributing a single pore.

The porin from *N. gonorrhoeae* is anion selective.* This clearly contrasts with porins from *E. coli* (11–14) and *S. typhimurium* (15). However, it resembles the voltage-dependent anion-selective channel (VDAC) extracted from the outer mitochondrial membrane of a variety of eukaryotic organisms (28, 35). The detergent-solubilized VDAC inserts into planar bilayers preferentially in triplets and multiples of triplets and electron images also reveal the pores arranged in highly organized arrays of triplets in patches of the mitochondrial membrane (36, 37). This resemblance raises the intriguing possibility of evolutionary conservation of a protein found abundantly in the outer membranes of both bacteria and mitochondria.

Besides mediating permeability to ions and macromolecules, it is also possible that the gonococcal outer membrane porin plays a role in the pathogenicity of this organism. Recent experiments in this laboratory have shown that viable *N. gonorrhoeae* bacteria can transfer porins directly to the planar lipid bilayer (33, 34). The ionophoric properties of this protein may contribute to the activation of phagocytosis of this bacterium by the host epithelial cells as shown by Ward *et al.* (38) and McGee and Horn (39).

* This is in accord with preliminary data obtained by Lynch, using a different protocol for porin reconstitution (33); the cationic nature of porin previously reported by Greco *et al.* (20, 34) could not be confirmed.

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