Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2302–2306, April 1981
Biophysics

Matrix protein in planar membranes: Clusters of channels in a native environment and their functional reassembly

(criteria for reconstitution/porin trimers/lipid–protein interactions/channel stoichiometry/*Escherichia coli* outer membranes)

HANSGEORG SCHINDLER AND JÜRG P. ROSENBUSCH

Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Communicated by Saul Roseman, December 29, 1981

ABSTRACT **Planar bilayers formed from *Escherichia coli* outer membrane vesicles exhibit conductance properties similar to those previously observed in bilayers reconstituted from aggregates of matrix protein, the major outer membrane protein. Discrete conductance steps are observed, reflecting voltage-dependent transmembrane channels. These exist in clusters which are activated by voltage. After activation, channels close with increasing potentials and reopen reversibly at lower voltage. Depending on the sign of the potential, two distinct closed states of the pores are observed. Cooperative interactions, hysteresis effects, relaxation times, and values of channel conductance depend on cluster size. These properties provide the reference data for the reconstitution of membrane function from individual components. Planar bilayers were formed from vesicles containing either solubilized matrix protein in a homogeneous trimeric state or bacterial glycolipid (lipopolysaccharide), or both. Activation of channel conductance required the presence of glycolipid and the formation of channel clusters, leading to conductance properties of the channels closely resembling those observed in native outer membranes. At very low concentrations of trimers, irreversible association to clusters by lateral diffusion was observed. Nearly quantitative recoveries of channels allowed the assignment of three pores per trimer.**

Outer membranes of Gram-negative bacteria are highly organized structures with defined permeability properties. A group of transmembrane proteins, the porins, forms channels which convey to these membranes what appear to be molecular sieve-like properties (for a review see ref. 1). In strain B^E of *Escherichia coli*, a single gene codes for porin. Its product, matrix protein, is well suited for the detailed study of its structure (2–4) and its function (1).

The recent incorporation of matrix protein into planar lipid bilayers has indeed confirmed that it forms uniform, water-filled, transmembrane channels with a diameter of about 1 nm (5, 6). These channels were found to exist in a dynamic equilibrium of two states, open and closed. High transmembrane potentials cause the pores to close reversibly (6). If this were to occur also *in vivo*, it could significantly affect the electrochemical potential across the plasma membrane. The number of pores per matrix protein trimer (3, 6–9) also remained uncertain. Stoichiometries of 1 channel (9) and of 3 channels (3, 6) per trimer, each containing three polypeptide chains of molecular weight 36,500 (2), have been proposed.

A molecular understanding of porin function requires the reconstitution of activity from solubilized individual components in a manner allowing high resolution and high recovery. To do this, we have applied a novel concept (10) to the characterization of matrix protein channels based on the transformation of vesicular to planar membranes. This procedure may be used at any level of reconstitution. In the study reported here, it is applied to a range from undissociated membrane vesicles through intermediate stages of purification to vesicles reassembled *de novo* from individual membrane components. Comparison of the results obtained with these different preparations indicates the physiological significance of the observed effects and provides insight in their molecular basis. This approach has previously been shown to reproduce *in vitro* the electrical properties of acetylcholine receptor (11), which are well established by *in vivo* electrophysiological techniques, and thus demonstrates its validity for reconstitution. The study reported here establishes its usefulness in a system that is inaccessible to direct electrophysiological methods.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Strategy. Planar membranes were transformed from vesicles at three different levels of reconstitution. The following preparations were used: (*i*) undissociated outer membrane vesicles, (*ii*) matrix protein in an aggregated state (6), and (*iii*) individual trimers, incorporated into vesicles with or without glycolipids after solubilization and purification. (Lipopolysaccharides are the predominant components of bacterial glycolipids; the latter term will be used throughout this paper.) The electrical properties of the resulting planar membranes were characterized and compared.

Protein. Dextran-free matrix protein aggregates were prepared as described (6), except that gel filtration was performed on controlled pore glass (CPG-10/113, Serva, Heidelberg). This avoids the massive binding of dextran (about 1:1 by weight) which occurred inadvertently in the original preparations (6). Trimers of matrix protein were solubilized from cell envelopes (2) by repeated extractions with 3% β-octylglucoside (Sigma) at 37°C for 1 hr in 20 mM $NaPO_4$/0.1 M NaCl/3 mM NaN_3, pH 7.0. Insoluble material was removed by centrifugation (20 min, 20,000 × *g*). The supernatant, enriched in matrix protein, was subjected to gel filtration on CPG-10 in the same buffer containing 1% β-octylglucoside. The major protein peak in the excluded volume contained matrix protein in homogeneous trimeric form (12), as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis and analytical centrifugation (2). Glycolipids were quantitated by labeling growing cells with $^{32}P_i$ and monitoring the radioactivity by autoradiography of dodecyl sulfate gels. Matrix protein in the excluded peak fraction (II) contained no detectable phospholipids and substoichiometric amounts (6) of glycolipids. Leading edge and trailing edge (fractions I and III) were pooled separately. Fraction I contained matrix protein only (no detectable glycolipids); fraction III contained the glycolipid peak with trace amounts of protein only.

Vesicle Preparation. Outer membrane vesicles (13) were incorporated into bilayers without dilution or were mixed with liposomes prepared (11) from partially purified (14) soybean lec-

ithin (Sigma). Matrix protein trimers were reconstituted to vesicles by extensive dialysis. Appropriate amounts were added to a lecithin solution in buffer A (20 mM Hepes/1 M NaCl/3 mM NaN_3, pH 7.0), containing 1% β-octylglucoside, to final phospholipid concentration of 1 mg/ml, and protein concentration of 0.1 μg/ml, corresponding to 1 trimer per 1.6×10^6 lipid molecules. Solutions were dialyzed extensively against buffer A. Removal of detergents was quantitative after 30 hr, as judged by β-[^{14}C]octylglucoside (New England Nuclear). The resulting vesicles were predominantly unilamellar, with an average diameter of 80 nm as judged by electron microscopy after uranyl acetate staining (3). Complete insertion of proteins into vesicles (6) yields 1 trimer per 25 vesicles, if random incorporation is assumed. Lower protein concentrations were obtained by mixing the above vesicle suspension with protein-free liposomes.

Formation of Bilayers and Electrical Measurements. Transformation of vesicles to planar membranes (15) via monolayer self-assembly (16) was used for all three types of vesicle preparations. Vesicle concentrations were 1 mg/ml. Bilayers were formed over circular apertures (2.1×10^{-4} cm^2) by apposition of two vesicle-derived monolayers, in analogy to that of solvent-spread monolayers (17). Their borders, showing structural irregularities ≤1 μm, were pretreated with 2% hexadecane in chloroform. Asymmetric monolayers, one of which was free of protein, were combined to bilayers within 1 min after monolayer formation. All results were obtained with membranes successfully formed at the first attempt. The criteria for bilayer formation (15) and the electrical measurements (6) were as described. Experiments were performed at 20°C.

RESULTS

Planar Bilayers from Outer Membrane Vesicles. Application of an electrical potential across a bilayer formed from *E. coli* outer membrane vesicles and pure lipid vesicles caused initiation of conductance which occurred in large steps and corresponded to an irreversible activation. It was followed by the relaxation to a steady state in distinct, regular steps (Fig. 1*a*) that are assigned to closing and opening events of individual membrane channels (6). Current relaxation by channel closing is a consequence of the applied voltage because activated channels reversibly reopened at zero voltage (Fig. 1*a*, center tracing). Reversal of the sign of the potential not only caused current

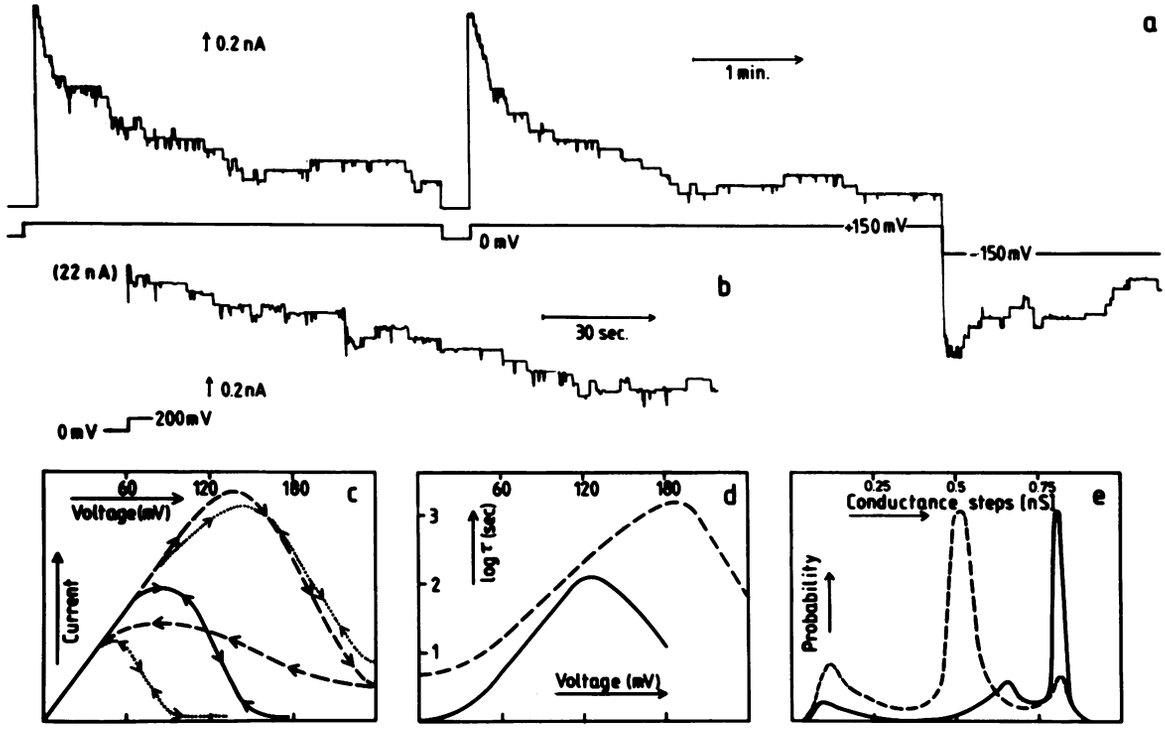

FIG. 1. Properties of conductance levels observed with bilayers derived from native, undissociated, membrane vesicles diluted with lipid vesicles 1:100 by weight. (*a*) Initiation of current corresponding to 18 channels occurred seconds after application of 150 mV. Individual conductance steps (Λ = 0.8 nS) were resolved during relaxation to a steady state (τ = 1 min). A brief interruption of the potential (cf. voltage trace) resulted in the reopening of channels which reversibly closed at 150 mV. Changing the sign of the potential caused a negative peak representing a temporary increase in conductance, followed by relaxation to the steady state. (*b*) A membrane containing 220 channels exhibited slow relaxation (τ = 20 min) even at 200 mV, and a unit step size of Λ = 0.5 nS. The activation (not shown) occurred in steps of 12, 27, 27, 48, and 105 channels at 250 mV. (*c–e*) Comparison of membranes after activation at different voltages. A potential of 150 mV resulted in initiation steps of <30 channels (defined as small channel clusters; solid lines); 250 mV caused initiation steps of >30 channels (large clusters, broken lines). (*c*) Small clusters exhibited reversible current–voltage dependences with a midpoint of negative resistance (V_m) at about 125 mV. Conductance levels of the various curves were normalized to allow comparison. Reversibility was as obtained with membranes derived from matrix protein isolated in an aggregated state (dotted line at right, redrawn from ref. 6). Similar aggregates without bound dextran yielded current–voltage curves shifted to lower voltage (dotted line at left). Large clusters in bilayers derived from outer membranes showed hysteresis loops (broken lines). In the example shown, the membrane (described in the legend to Fig. 1*b*) was subjected to increments of 20 mV at 15-min intervals. (*d*) Dependence of relaxation time on voltage. The maximal value (τ_m at V_m) is about 100-fold higher than the relaxation time at 0 voltage. τ_m shifted to higher values in large clusters. Relaxation times were estimated by assuming single exponential decay after voltage increments from 0 mV to values within the region of negative resistance, or voltage decrements from V_m to smaller values. (*e*) Probabilities of step sizes (in arbitrary units) reached a sharp maximum at 0.8 nS for small channel clusters and a broader maximum at 0.5 nS for large clusters. Notice the sublevels at 0.1 nS for both types of membranes. Upon activation at intermediate voltages, both types of relaxation rates and channel conductances were observed concomitantly. The values of channel conductances are not unique; they were found to vary with the lipids used. All experiments shown were performed in buffer A.

reversal but also reopened channels that were closed at the steady state before the voltage reversal. Subsequently, the pores closed again until a level corresponding to the steady state of the potential at the opposite polarity was reached. This transition via an open channel state provides evidence that two distinct states exist at opposite polarities. The value of the steady-state conductance is independent of the voltage sign.

Fig. 1*b* shows the relaxation of current after activation of about 220 channels. Even at such high currents, discrete steps were resolved during relaxation. The fluctuation of the number of active channels at steady state was small. This observation, together with the simultaneous activation of large numbers of pores, reflects high cooperativity and strongly suggests the occurrence of channel clusters. The steady-state distribution of the channel states and the transition times between them depended on the applied potential (Fig. 1 *c* and *d*) but not on its polarity (not shown). The relationship between current and voltage exhibited, as its most prominent property, negative (differential) resistance which is a consequence of channel closing by voltage (6). The current–voltage dependence of individual channels in the open state was linear (data not shown). Relaxation times were longest (τ_m) at voltages (V_m) corresponding to the midpoint of negative resistance. These observations are consistent with the interpretation that the closing of channels by potentials of either sign represents conformational changes of the pores which, after removal of the applied field, relax to their original, open configuration.

When the initiation voltage was increased, larger channel clusters were activated (Fig. 1) in analogy to our previous observations with matrix protein aggregates (6). These large clusters exhibited a current–voltage relationship with a hysteresis loop that persisted for up to 6 hr (Fig. 1*c*), comparatively longer relaxation times even at zero voltage (Fig. 1*d*), and distinctly lower values of channel conductance (Fig. 1*e*). These observations indicate a noncontinuous change of channel properties with increasing cluster size.

Whereas the results described above were highly reproducible, the levels of the inducible conductances were not. Variations within a factor of 10 occurred, and even the highest levels observed (4×10^7 channels per cm^2) were orders of magnitude lower than expected if all channels present were open. Dilution of outer membrane vesicles with various concentrations of liposomes prior to monolayer formation did not affect the cooperative interactions observed, indicating that channel clusters do not dissociate in lipid membranes. Thus, all of the described electrical properties closely resemble those previously observed with matrix protein isolated in an aggregated state upon incorporation into planar membranes (6). The high values of V_m (Fig. 1*c*) and the relatively slow relaxation times may be due to the large size of the aggregates obtained with isolated matrix protein. Their voltage-dependent characteristics indeed are virtually superimposable on those observed now for the activation of large channel clusters in bilayers from outer membranes (except for the absence of hysteresis). Avoidance of the considerable binding of dextran to matrix protein aggregates caused a shift of V_m to lower voltages (Fig. 1*c*, dotted line at left).

Membrane Reconstitution from Single Matrix Protein Trimers. Planar membranes formed from vesicles containing single matrix protein trimers (concentration $\leq$1 trimer per 25 vesicles) exhibited characteristics comparable to those derived from outer membranes. Fig. 2*a* shows initiation and relaxation to a steady state of a membrane with 66 active channels. Analysis of the dependence of current on voltage showed properties corresponding to those presented in Fig. 1 *c–e*, including hysteresis loops and the shift to smaller conductance steps at the highest protein concentration used (data not shown). The role of glycolipids in channel activation was examined by comparing membranes containing glycolipids and matrix protein in various ratios. Bilayers reconstituted from protein without detectable amounts of glycolipids (fraction I) and others containing glycolipids with trace amounts of matrix protein only (fraction III)

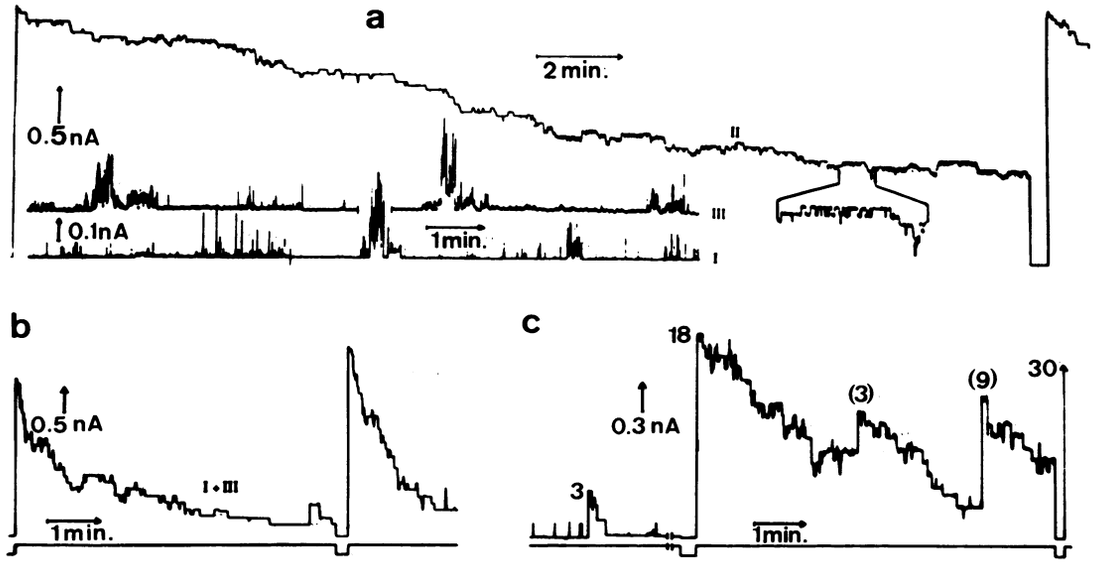

FIG. 2. Properties of bilayers derived from vesicles containing either single trimeric units of matrix protein or glycolipids, or both. Solutions containing proteoliposomes with 1 trimer per 25 vesicles were diluted 1:40 (by weight) with lipid vesicles before formation of planar membranes (*a* and *b*) or 1:1000 (*c*). (*a*) Current relaxation of a bilayer containing the peak fraction (II) of the elution profile at 100 mV. The expanded area shows the rectangular shape of individual steps. Reopening of the channels at 0 mV is shown on the right. The two lower traces were observed with fractions I and III at 150 mV. Both exhibit irregular, rapid events (notice the expanded current scale). (*b*) Concomitant incorporation of fractions I and III in amounts identical to those used for the lower traces of *a* yield membranes which, at 150 mV, exhibited initiation and relaxation properties observed in fraction II. Relaxation was faster, as expected (Fig. 1*d*) for smaller current levels. (*c*) Channel activation at 150 mV in a membrane containing about 20 trimers. The numbers of activated channels are indicated on the graph. Notice the initiation of the first triplet $\approx$2 min after membrane formation. Upon removal of the potential, the renewed voltage caused an instantaneous current reflecting all 18 channels induced previously (not shown). Additional initiation events (values in parentheses) led to a total of 30 channels.

both exhibited short-lived irregular events (Fig. 2*a*). The main tracing in Fig. 2*a* was obtained with protein from peak fractions (II) which contained small amounts of glycolipids. These are therefore required for channel activation. This conclusion is supported by the result that combining fraction I and III yielded a tracing (Fig. 2*b*) that showed all of the characteristic properties mentioned.

The stoichiometry of glycolipids relative to protein was not evaluated. Heterogeneity of glycolipids render ambiguous such determinations (18). The previously reported stoichiometry of 3 mol of glycolipids per mol of matrix protein monomer (6) is not pertinent in this context because aggregates have not been disassembled into their component parts then. In previous reports on glycolipid requirements (19), the state of the protein remained undefined. Subsequently, glycolipids were added routinely to trimeric solutions (7). Finally, a physically meaningful interpretation of the results observed in reconstitutions of two-dimensional sheets (20) appears to be difficult.

Association of trimers was investigated by studying the kinetics of channel activation with vesicles containing single trimers (fraction II) at very low concentrations (1 trimer per 2.5 $\times 10^4$ vesicles, or 20 trimers per bilayer). Only spikes in the millisecond range appeared initially. Even at high potentials (200 mV), the first stable conductance levels occurred only after several minutes, with levels usually corresponding to the simultaneous activation of triplets (three channels) and consecutive closing of single channels (Fig. 2*c*, left). Their number slowly increased over a period of about 1 hr, almost exclusively in triplet steps (or small multiples thereof). Subsequently, the number of activated channels remained constant, and their properties were identical to those described for membranes reconstituted at higher concentrations. We therefore conclude that the slow appearance of stable channel conductance is due to the apparently irreversible association of trimers via lateral diffusion.

The diffusion coefficient of trimers within the bilayer was estimated from 10 independent experiments. With the assumption of binary collisions upon random walk with a jump length of 8 Å and a collision diameter of 70 Å, a minimal estimate of 10^{-10} cm^2/sec is obtained. This value is clearly different from the undetectable diffusion of matrix protein aggregates (21) but is in the same range as that observed with other membrane proteins (22). Due to the higher diffusion coefficient of glycolipids (1.5×10^{-9} cm^2/sec; cf. ref. 21), this estimate is insensitive to dissociation of trimers and glycolipids because, with the conditions used, the two components remain in proximity also after monolayer formation. Twenty trimers were expected within the bilayer area in the diffusion experiments cited. This expectation is based on a molar stoichiometry of 6×10^{-10} trimers per phospholipid in vesicles. It depends on the following points. (*i*) Deviation of the protein-to-lipid ratios in vesicles and monolayers is $\leq$10%, as determined by a method described previously (11). (*ii*) The molar stoichiometry can be assumed to be preserved because only bilayers successfully formed at the first attempt were used. (*iii*) Fusion of vesicles into the bilayer does not occur. This was assessed by using planar membranes consisting of pure phospholipid with vesicles in solution which contained matrix protein. (*iv*) Protein replenishment from the membrane frame is unlikely because the protein is insoluble in the hexadecane torus and adheres strongly to Teflon. Because the number of eventually stabilized channels ranged from 27 to 60 (average, 40), the recovery amounts to 45–100%, provided there are three pores per trimer. If they contained a single channel, this value would rise to 135–300%. We therefore conclude that more than one channel exists per trimer, most likely three.

DISCUSSION

The studies reported here on the channel properties of matrix protein extend previous ones (6) in two directions. First, we transformed undissociated outer membrane vesicles into planar bilayers. This allowed us to gain insight in the properties of the pores in an environment resembling that in the cell and to compare channel activity in a native membrane environment to that in bilayers containing matrix protein extracted in an aggregated state (6). Second, we reconstituted vesicles reassembled from isolated matrix protein trimers and bacterial glycolipids. These experiments identified the basic requirements of channel activation. Because the techniques used were identical to those used in the study of undissociated outer membranes, the results obtained with the latter provide the reference data with which the validity of reconstitution experiments with isolated components can be evaluated. Such a strategy (10) is critical in a system such as this which is inaccessible to electrophysiological techniques.

The similarity of the conductance properties, observed at three levels of reconstitution, show that the ability of matrix protein to form channels is preserved during the isolation procedures used. It also shows that the properties observed in bilayers derived from outer membranes reflect those of matrix protein rather than of more specific pores which also occur in outer membranes (1). Although the pore properties in isolated outer membranes cannot actually be equated to those in the cell, our results demonstrate that the observed characteristics are not qualitatively affected by membrane dissociation.

Independent of the level of reconstitution, several configurations of channels can be distinguished. In addition to an open state, voltage reversal experiments show that two distinct closed states exist, analogous to observations with excitability-inducing material (23). The occurrence of negative resistance and the coincidence (23) of the longest relaxation times with the midpoint of negative resistance indicate that the equilibrium between channel conformations is governed by the contribution of the electrical field to the free energy of the states. The cooperative interactions among pores, as they were observed with matrix protein isolated in an aggregated state (6), demonstrate their arrangement in clusters. The appearance of hysteresis in the relationship of current to voltage beyond a threshold size of these clusters and the slower relaxation times are predicted for a system that exhibits two-dimensional cooperativity (24).

On a molecular level, the reassembly of pores from solubilized matrix protein trimers has revealed that restoration of channel activity requires meeting two conditions: the presence of bacterial glycolipids in addition to matrix protein, and association of the components into stable clusters. This result shows that channel function is not uniquely determined by the properties of the protein alone but rests also on its interaction with other components. The observed number of pores at low concentrations of protein excludes a single channel per trimer, rendering an assignment of three pores per trimer the most likely.

In view of this nearly quantitative recovery *in vitro*, an earlier observation that most channels are closed *in vivo* (25) is surprising. Yet, we have also shown that the conductance properties of channels appear to be highly sensitive to the size of clusters. Thus, changes in the state of association could provide a basis for a subtle regulation of outer membrane permeability which could account for the differences observed *in vivo* and *in vitro*. Potential modulators of channel function have not yet been identified, but the apparent interaction (2) with the substratum of outer membranes, peptidoglycan, could affect channel clustering. Our present observation that the binding of dextran, another crosslinked polysaccharide, significantly increases

the potential at which negative resistance occurs, might reflect such an effect. Potential modulation by smaller effectors either at the outside or at the inside (the periplasmic space) of outer membranes should be investigated also, particularly because the method is now easily available.

We thank M. Regenass and A. Frank for their expert technical assistance. Support by the Swiss National Science Foundation (Grants 3.390.78, 3.152.77, and 3.656.80) is gratefully acknowledged.

1. Nikaido, H. & Nakae, T. (1979) *Adv. Microbial Physiol.* **20,** 163–250.
2. Rosenbusch, J. P. (1974) *J. Biol. Chem.* **249,** 8018–8029.
3. Steven, A. C., ten Heggeler, B., Müller, R., Kistler, J. & Rosenbusch, J. P. (1977) *J. Cell Biol.* **72,** 292–301.
4. Garavito, R. M. & Rosenbusch, J. P. (1980) *J. Cell Biol.* **86,** 327–329.
5. Benz, R., Janko, K., Boos, W. & Läuger, P. (1978) *Biochim. Biophys. Acta* **511,** 305–319.
6. Schindler, H. & Rosenbusch, J. P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3751–3755.
7. Nakae, T., Ishi, J. & Tokunaga, M. (1979) *J. Biol. Chem.* **254,** 1457–1461.
8. Ueki, T., Mitsui, T. & Nikaido, H. (1979) *J. Biochem.* **85,** 173–182.
9. Benz, R. & Nakae, T. (1979) *Annu. Meeting Deutsche Ges. Biophysik*, eds. Adam, G. & Stark, G. (Springer, Berlin), p. 63.
10. Schindler, H., Rosenbusch, J. P. & Quast, U. (1980) *Neurochem. Intl.* **2,** 291–298.
11. Schindler, H. & Quast, U. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3052–3056.
12. Rosenbusch, J. P., Steven, A. C., Alkan, M. & Regenass, M. (1980) *Electron Microscopy at Molecular Dimensions*, eds. Baumeister, W. & Vogell, W. (Springer, Berlin), pp. 1–10.
13. Osborn, M. J., Gander, J. E., Parisi, E. & Carson, J. (1972) *J. Biol. Chem.* **247,** 3962–3972.
14. Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem.* **246,** 5477–5487.
15. Schindler, H. (1980) *FEBS Lett.* **122,** 77–79.
16. Schindler, H. (1979) *Biochim. Biophys. Acta* **555,** 316–336.
17. Montal, M. & Mueller, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3561–3566.
18. Fried, V. A. & Rothfield, L. I. (1978) *Biochim. Biophys. Acta* **514,** 69–82.
19. Nakae, T. (1975) *Biochem. Biophys. Res. Commun.* **64,** 1224–1230.
20. Yamada, H. & Mizushima, S. (1980) *Eur. J. Biochem.* **103,** 209–218.
21. Schindler, M., Osborn, M. J. & Koppel, D. E. (1980) *Nature (London)* **283,** 346–350.
22. Cherry, R. (1979) *Biochim. Biophys. Acta* **559,** 289–327.
23. Ehrenstein, G. & Lecar, H. (1977) *Q. Rev. Biophys.* **10,** 1–34.
24. Stankowski, S. & Grunewald, B. (1980) *Biophys. Chem.* **12,** 167–176.
25. Bavoil, P., Nikaido, H. & Meyenburg, K. (1971) *Mol. Gen. Genet.* **158,** 23–33.