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FORMATION OF LARGE, ION-PERMEABLE MEMBRANE CHANNELS BY THE MATRIX PROTEIN (PORIN) OF *ESCHERICHIA COLI*

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Summary

One of the major proteins of the outer membrane of *Escherichia coli*, the matrix protein (porin), has been isolated by detergent solubilisation. When the protein is added in concentrations of the order of 10 ng/cm^3 to the outer phases of a planar lipid bilayer membrane, the membrane conductance increases by many orders of magnitude. At lower protein concentrations the conductance increases in a stepwise fashion, the single conductance increment being about 2 nS (1 nS = 10^{-9} siemens = 10^{-9} Ω^{-1}) in 1 M KCl. The conductance pathway has an ohmic current vs. voltage character and a poor selectivity for chloride and the alkali ions. These findings are consistent with the assumption that the protein forms large aqueous channels in the membrane. From the average value of the single-channel conductance a channel diameter of about 0.9 nm is estimated. This channel size is consistent with the sugar permeability which has been reported for lipid vesicles reconstituted in the presence of the protein.

Introduction

The cell envelope of gram-negative bacteria such as *Salmonella typhimurium* or *Escherichia coli* consists of the cytoplasmic membrane, a rigid peptidoglycan layer and the outer membrane [1,2]. The outer membrane is virtually impermeable to hydrophilic solutes of molecular weight greater than 1000, but allows the passage of sugars and other water-soluble compounds up to a molecular weight of 500—600 [3—8]. The transmembrane diffusion of small sugar molecules presumably occurs through aqueous channels, since both the activation energy and the specificity of the transport are low [2,3]. The structure and composition of the outer membrane have been extensively studied [9—31].

Apart from phospholipids, the outer membrane contains lipopolysaccharide [11] and a class of major proteins. The best characterized of these proteins

is a lipoprotein of molecular weight of about 7000 [17,18]. Recently, evidence has accumulated that the permeability properties of the outer membrane depend on the presence of another major protein, which has been variously described as protein I or 1 [11,13], matrix protein [14] and porin [32].

In *E. coli* B the matrix protein consists of a single polypeptide chain of molecular weight 36 500 [14], whereas the protein from K12 strain can be separated electrophoretically into two bands which correspond to two very closely related polypeptides [23,24,33—35]. The matrix protein is present in about 10^5 copies per cell and covers a substantial fraction of the cell surface [14,29]. Electron microscopic analysis shows that the protein is arranged on a hexagonal lattice whose repeat distance is 7.7 nm [29]. The unit cell of the lattice probably contains three molecules of the matrix protein; in negatively stained specimens the center of the unit cell appears as a pit which is filled with stain and which may represent the opening of a pore. These structural findings are consistent with cross-linking experiments [25,26,31] carried out with whole cells or isolated cell walls (outer membrane plus peptidoglycan layer), in which the matrix protein was specifically cross-linked to itself; from the analysis of the resulting protein complexes it was proposed that the basic unit is a trimer [31].

The matrix protein appears to span the outer membrane. It is tightly associated with the underlying peptidoglycan layer [14,24,29,30] and is at the same time accessible from the outside, acting as a phage receptor [23,36,37]. Evidence that the major proteins of the outer membrane may be involved in passive transmembrane diffusion comes from experiments of Nikaido et al. [38] showing that mutants of *Salmonella typhimurium* which were deficient in the major proteins had reduced permeabilities towards cephaloridin, a hydrophilic solute of molecular weight 415.

Further, incorporation of isolated protein components of the outer membrane into phospholipid vesicles rendered the vesicle membrane permeable to small sugar molecules [32,39,40]. In particular, Nakae [32] was able to demonstrate that phospholipid/lipopolysaccharide vesicles became permeable to hydrophilic solutes up to molecular weight of about 550 when the matrix protein from *E. coli* was incorporated into the vesicle membrane.

If the matrix protein forms aqueous pores of a size large enough to allow the passage of di- and trisaccharides, then these pores should also be permeable to a variety of ions. In the following we show that incorporation of the matrix protein into planar lipid bilyer membranes creates electrically conducting pores of the expected size.

Experimental

(a) Isolation of the matrix protein [32,40]

E. coli K 12, strain pop 1730, was grown for 12 h in L-broth (bacto-tryptone 10 g/l, bacto yeast extract 5 g/l, NaCl 10 g/l) or for 5 h in Di YT medium (bactetrypton 16 g/l, bacta yeast extra 10 g/l, NaCl 5 g/l). Strain pop 1730 has a deletion in *lamB* and lacks the λ-receptor [43]. The cells were harvested, resuspended in 20 vols. of potassium phosphate buffer, pH 7.6, containing 0.1% β-mercaptoethanol, and were passed three times through a French pres-

sure cell at 600 atm at 0°C. The extract was centrifuged for 110 min at 27 000 × *g*. The pellet was washed by resuspension in 10 mM Tris · HCl, pH 7.5, and centrifuged for 60 min at 100 000 × *g*. The material was stored at −70°C until used.

A portion of the pellet containing 400 mg protein was extracted with 50 ml 2% sodium dodecyl sulfate (SDS) (Serva, Heidelberg, reinst.) at 32°C for 30 min and the suspension centrifuged at 100 000 × *g* for 30 min at 15°C. The pellet was washed 5 times by resuspension in distilled water and centrifugation. The suspension was then dialysed against 3 mM NaN_3 at 4°C for 4 days. After centrifugation (100 000 × *g*, 30 min) the pellet was resuspended in 50 cm^3 5 mM Tris · HCl buffer, pH 8, containing 3 mM NaN_3, and was dispersed by sonication (Branson sonifier, 90 s). The suspension was incubated for 2 h at 37°C after addition of 4 mg trypsin (Boehringer, zur Analyse). In this treatment the murein lipoprotein is hydrolysed [17]; the matrix protein, as long as it is associated with the peptidoglycan layer is resistant to trypsin [14,32]. The sonication and addition of trypsin was repeated twice. The insoluble material was spun down and the pellet was resuspended in 40 ml Tris · HCl, pH 8, containing 2% SDS. After incubation at 25°C for 30 min a slightly opaque solution was obtained. This mixture was applied to a Sepharose-4B column (2.5 × 50 cm) equilibrated with 5 mM Tris · HCl, pH 8, containing 0.1% SDS and 3 mM NaN_3, and the column was eluted by the same buffer at a rate of about 20 cm^3/h. The protein content was continuously monitored by measuring the optical density at 254 nm in an Uvicord spectrophotometer (LKB, Stockholm).

In a second preparation, cholate (Merck, zur Analyse) was used throughout instead of SDS, while the rest of the procedure remained the same. The results obtained with both detergents were almost identical (see below), but the yield of matrix protein in the cholate preparation was higher.

A sugar test using the anthrone reaction [52] showed that the protein contained less than 1% (w/w) sugar. From this result one can conclude that the protein contained if anything only traces of the peptidoglycan layer.

The binding of SDS to porin was found not to be very tight [14]. In 1% SDS solution the protein contained about 10% (w/w) SDS. But this amount has been shown to be easily reduced to 0.2% (w/w) by several washing procedures [14].

The Sepharose-4B chromatogram which is shown in Fig. 1 for a cholate preparation is similar to that obtained by Nakae [32], although the relative peak heights are somewhat different. The main peak (I) corresponds to the peak I in Nakae's preparation which was found to be highly active in the permeability test with lipid vesicles [32]. According to Nakae [32] the protein of peak I is an aggregate of a molecular weight of about 800 000.

SDS polyacrylamide gel electrophoresis was carried out after heating the protein in 2% SDS at 100°C for 2 min [41]. Peak I gave a single band corresponding to a molecular weight of about 37 000, in close agreement with the value given by Rosenbusch (36 500); for calibration lactate dehydrogenase (mol. wt. 35 000) and aldolase (mol. wt. 40 000) were used (The two subfractions of the matrix protein present in *E. coli* strain K12 are not separated in gel electrophoresis under the conditions given).

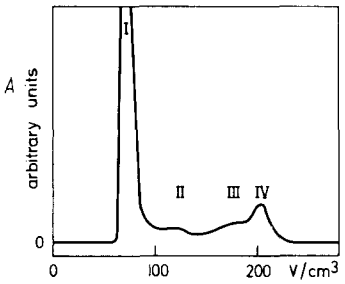

Fig. 1. Gel chromatography (Sepharose-4B) of outer membrane proteins (cholate preparation). The elution medium was 5 mM Tris · HCl, pH 8, containing 3 mM NaN_3 and 0.1% cholate. The SDS preparation gave similar chromatograms, but with a reduced height of peak I relative to peak IV.

A sample of the matrix protein prepared by the method of Rosenbusch [58] (which was kindly given to us by Dr. Rosenbusch) was indistinguishable from our preparation in gel electrophoresis. Protein determinations were carried out according to the method of Lowry et al. [42].

For the membrane experiments, protein of peak I was used. Material from peak IV was also found to be active to some extent; however, the increase in membrane conductance was of an unspecific nature in this case and discrete conductance steps (as seen with peak I) were never observed (see below). The protein was stored either in lyophilized form at −25° C or in 5 mM Tris · HCl buffer, pH 8, containing 0.1% SDS and 3 mM NaN_3. In this solution the protein remained active in membrane experiments for several months. At high ionic strength (1 M NaCl), however, the protein became inactive within 20 h; for this reason fresh solutions for membrane experiments were prepared every day using small aliquots of the stock solution.

(b) Membrane experiments

Optically black lipid membranes were formed in the usual way [44] in a circular hole in a Teflon septum separating two aqueous solutions. The membrane area was $2 \cdot 10^{-2}$ cm^2 for the measurements of macroscopic conductance and about 10^{-4} cm^2 for the conductance-fluctuation experiments. The Teflon cell was contained in a thermostated metal block; the temperature was 25° C throughout. The aqueous solutions bathing the membrane were unbuffered and had a pH between 5.5 and 6. In separate experiments it was established that the results were virtually pH-independent in the range pH 5.5—7. In some experiments the protein was already present prior to the formation of the membrane; in other experiments the protein was added with stirring after the membrane had become completely black.

The following lipids were used for membrane formation: monoolein (NuCheck Prep, Elysian, Minn., U.S.A.); 1,2-dioleoyl-*sn*-glycerol-3-phosphorylcholine (dioleoyl phosphatidylcholine) synthesized as in ref. 45; egg lecithin, egg phosphatidylethanolamine, brain phosphatidylserine, brain phosphatidylinositol (purified by standard methods) [46,47]. Oxidized cholesterol was prepared by boiling a 4% suspension of cholesterol (Eastman, reagent grade) in *n*-octane for 4 h under reflux and bubbling oxygen through the suspension [48,49]. All lipids, except oxidized cholesterol, gave a single spot in the thin-

layer chromatogram. Where not otherwise indicated, the lipids were used as a 2% (w/v) solution in *n*-decane (Merck, standard for gas chromatography).

For the electrical measurements the membrane cell was connected to the external circuit through Ag/AgCl electrodes or (for the determination of the zero-current membrane potential) through agar bridges via Ag/AgCl electrodes. For the conductance-fluctuation experiments a Keithley 427 preamplifier was used in connnection with a Tectronix 5111/5A22 storage oscilloscope. The amplified signal at the output of the oscilloscope was recorded with a strip-chart recorder. In some cases the signal was stored on tape. The bandwidth of the fluctuation measurements was 100 Hz—3 kHz. For the measurement of macroscopic conductance a Keithley 150 B Microvolt Ammeter or a Keithley 610 C electrometer were used. Membrane potentials were measured with the Keithley 610 C Electrometer. For the study of current transients a battery-operated pulse generator was used and the current was measured as a voltage drop across an external resistor with a Tectronix 5115/5A22 storage oscilloscope.

Results

Time behaviour of membrane conductance

When matrix protein from a stock solution in SDS or cholate was added in small quantities to the aqueous solutions bathing the membrane, the membrane conductance started to increase in a stepwise fashion (Fig. 2). The occurrence of these conductance steps is specific to the porin and is not seen when an

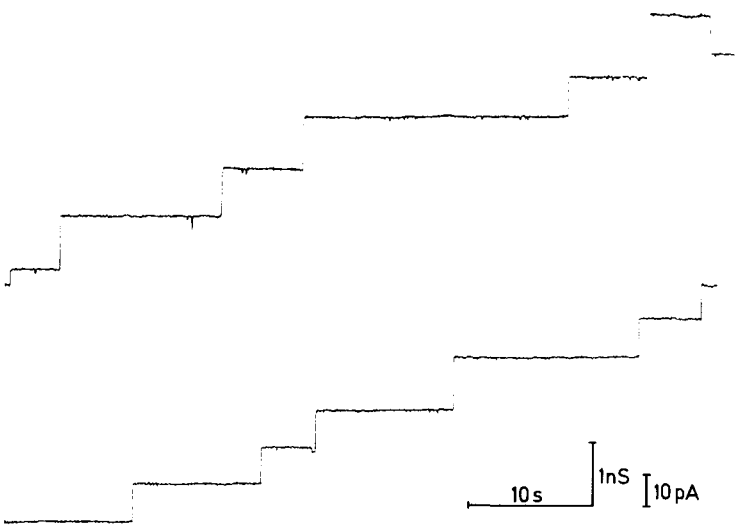

Fig. 2. Stepwise increase of membrane current after addition of matrix protein. A small amount of a stock solution containing 0.2 mg/cm^3 porin and 1 mg/cm^3 SDS was added to aqueous solutions on both sides of the membrane to give a nominal protein concentration of about 0.5 ng/cm^3. The aqueous solution contained 0.3 M KCl and 1 μg/cm^3 SDS; T = 25°C. The membrane was formed from a 2% (w/v) solution of oxidized cholesterol in *n*-decane. The applied voltage was 20 mV, the current prior to the addition of porin was about 0.6 pA. The bandwidth of the measurement was 1 kHz. The record starts at the left of the lower trace and continues in the upper trace.

equivalent amount of detergent alone is added. Furthermore, after heating the stock solution (0.2 mg/cm^3 porin, 1 mg/cm^3 SDS, 5 mM Tris · HCl, 3 mM NaN_3, pH 8) to 100°C for 5 min, the activity of the protein is completely lost. The conductance steps are observed irrespective whether the porin is added to the aqueous solution on one side of the membrane only or to both solutions. As seen from Fig. 2, most steps in the current record are directed upward, whereas terminating events are rarely observed. From records extending over prolonged periods, the average lifetime of the conductive unit may be estimated to be at least 5 min. It was independent of salt concentration. The risetime of the single conductance step was less than 0.5 ms; within this time resolution the conductance rise was always smooth without any indication of smaller intermediate steps.

The conductance increments are not uniform in size but distributed over a certain range. Histograms of the conductance steps observed with matrix protein solubilized in SDS and in cholate are shown in Fig. 3. As can be seen the distribution of the conductance increments is virtually the same for both protein preparations; for protein solubilized in SDS the average conductance increment in (1 M KCl) is $\overline{\Lambda} \simeq 1.9$ nS, and $\overline{\Lambda} \simeq 2.0$ nS for protein solubilized in cholate. A close agreement between $\overline{\Lambda}$ values from the two protein preparations was also observed with other salt solutions (0.1 M KCl or 1 M NaCl). The

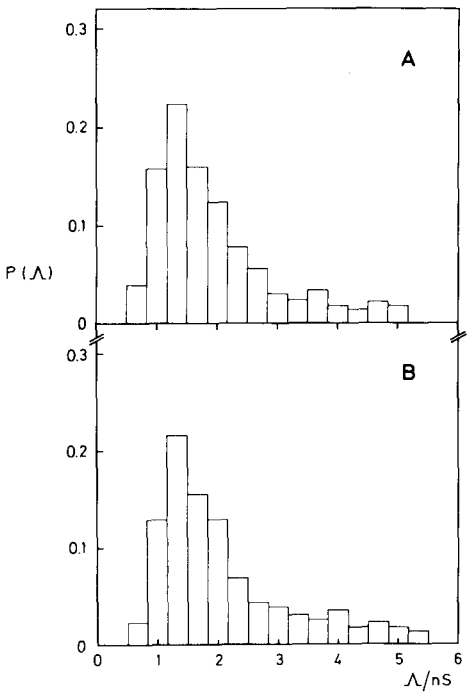

Fig. 3. Probability $P(\Lambda)$ of the occurrence of a conductance step of magnitude Λ. $P(\Lambda)$ is the number of observed steps within an interval of width $\Delta\Lambda = \pm 0.17$ nS centered at Λ, divided by the total number (n) of steps. The membranes were made from 2% (w/v) oxidized cholesterol in n-decane. The aqueous phase contained 1 M KCl; $T = 25$°C. The applied voltage was 20 mV. A: porin solubilized in SDS ($n = 321$); B: porin solubilized in cholate ($n = 231$). The conditions of protein addition were similar to those indicated in the legend of Fig. 2.

average value $\overline{\Lambda}$ of the conductance increment was usually by about 20—30% larger than the most frequently assumed Λ value in the distribution.

When larger concentrations of the porin are present, the conductance quickly rises after formation of the bilayer and reaches values many orders of magnitude above the level of the untreated membrane. The time course of the specific conductance λ after protein addition is shown in Fig. 4. With membranes made of monoolein in *n*-decane the rate of change of λ increased with time in the initial phase of the experiment, whereas the reverse behaviour was found for oxidized-cholesterol/*n*-decane membranes. Under both conditions a steady conductance level was not reached, the membranes usually broke at a conductance of approx. 5—20 mS · cm^{-2} (1 M NaCl).

When the membrane was formed in a solution of salt and detergent of the same concentration, but without porin, only a insignificant conductance increase was observed. (λ^* in Table III). Using valinomycin in 1 M KCl as a probe for the surface potential, it was also tested that 1 μg/cm^3 SDS or 1 μg/cm^3 cholate did not change the surface potential appreciably.

Reducing the concentration of SDS resulted in a steeper conductance rise with time, as Fig. 4 shows. A possible explanation for this phenomenon would be that at a lower detergent concentration the hydrophobic surface of the porin is more exposed to water. This would increase the tendency of the protein to become associated with the lipid membrane.

In order to test whether or not the rate of conductance rise depends on voltage, $\lambda(t)$ was recorded under two different conditions. First the membrane was maintained at zero voltage during most of the time and only brief voltage pulses of 10 mV were applied in order to measure λ. Second a constant voltage of 70 mV was applied. In both cases the values of $\lambda(t)$ were virtually the same.

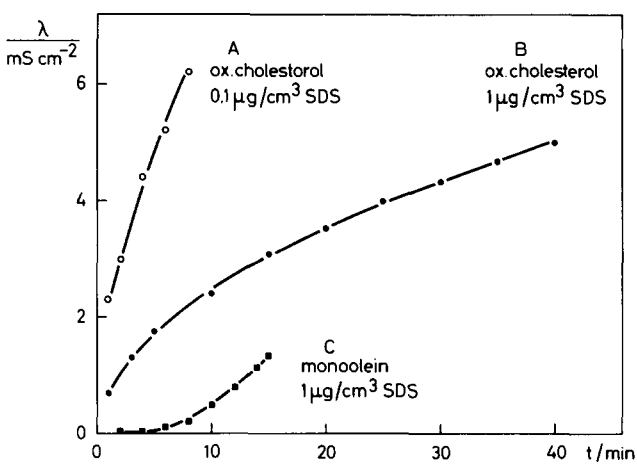

Fig. 4. Specific membrane conductance λ as a function of time t. The membrane was formed at time zero in a 1 M NaCl solution containing 20 ng/cm^3 porin and 0.1 or 1 μg/cm^3 SDS. Curve A: oxidized cholesterol membrane, 0.1 μg/cm^3 SDS; Curve B: oxidized cholesterol membrane, 1 μg/cm^3 SDS; Curve C: monoolein membrane, 1 μg/cm^3 SDS. At time $t \simeq 1$ min the membrane was completely black. The applied voltage was 10 mV. The membrane conductance in a solution containing 1 M NaCl and 1 μg/cm^3 SDS (but no protein) was of the order of $3 \cdot 10^{-8}$ S · cm^{-2}. In a series of experiments with different membranes the reproducibility of $\lambda(t)$ was about ±30%.

As mentioned above, porin could be inactivated by heating the stock solution to 100°C for 5 min. A partial inactivation was observed after maintaining the same stock solution at 70°C for 3 min. In this case about 10 times more protein had to be added in order to obtain a given macroscopic conductance. The appearance of the conductance fluctuations, however, was very similar to those observed with the fully active preparation and the value of the average conductance increment was virtually the same in both cases. The only difference was that the fluctuating current signal became increasingly noisy with time. This noise possibly results from the adsorption of inactivated protein to the membrane.

Conductance as a function of protein and salt concentration

Since a steady conductance level could not be reached (Fig. 4), the dependence of conductance on porin concentration could not be accurately determined. But a meaningful comparison was possible on the basis of $\lambda(t)$ curves which were reproducible to ±30%. In experiments with oxidized cholesterol membranes in 1 M NaCl and 1 $\mu g/cm^3$ SDS $\lambda(t)$ was found to be virtually independent of porin concentration in the range of 2—200 ng/cm^3; below 2 ng/cm^3 the conductance rise became small and was less reproducible. Such a behaviour is reminiscent of systems exhibiting a critical micellar concentration. It could result from the existence of an equilibrium between porin monomers and aggregates in water if only the monomers are able to enter the membrane. Another explanation would be that the membrane surface becomes saturated with adsorbed protein at aqueous protein concentrations above 2 ng/cm^3 and that pore formation occurs from the adsorbed state.

The effect of salt concentration on λ was studied by measuring the conductance at a given time after the formation of the bilayer; this time was chosen to be 30 min in all cases. As can be seen from Fig. 5 that the conductance λ

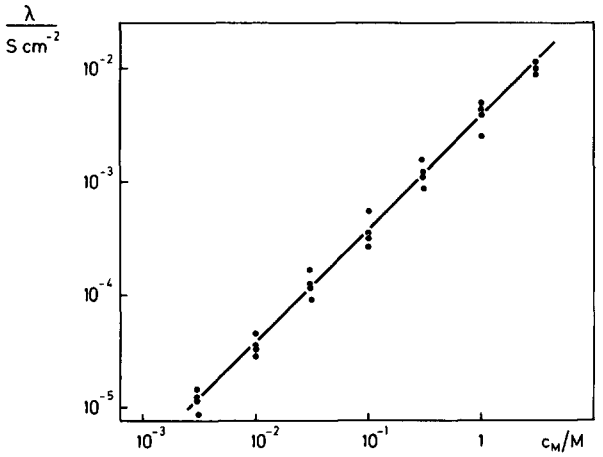

Fig. 5. Specific membrane conductance as a function of NaCl concentration c_M in water. The aqueous phase contained 200 ng/cm^3 porin and 1 $\mu g/cm^3$ SDS, $T = 25°C$. The membrane was formed from 2% (w/v) of oxidized cholesterol in *n*-decane. The conductance was measured 30 min after membrane formation (applied voltage: 10 mV). Each point corresponds to a different membrane.

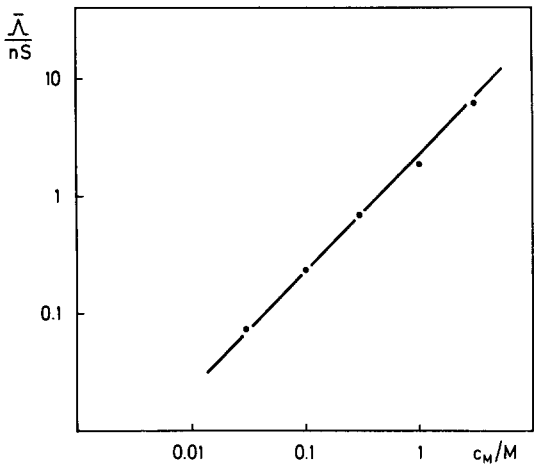

Fig. 6. Average conductance increment $\bar{\Lambda}$ as a function of KCl concentration c_M in water; $T = 25°C$. The applied voltage was 50 mV. Oxidized cholesterol/*n*-decane membranes. The porin was solubilized in SDS. With the exception of $c_M = 0.03$ M ($n = 19$) the number n of events used for the evaluation of Λ ranged between 180 and 320.

is a linear function of salt concentration within the limits of experimental uncertainty. The interpretation of this experiment is complicated by the possibility that the rate of incorporation of the protein into the membrane may depend on aqueous salt concentration so that the observed concentration dependence of λ may not only reflect the intrinsic concentration dependence of the single conductive unit but also an effect of salt concentration on the number of conductive units in the membrane.

In order to study this questions further, the dependence of the average conductance increment $\bar{\Lambda}$ on salt concentration was directly determined. From Fig. 6 it is seen that $\bar{\Lambda}$ is a linear function of KCl concentration between 0.03 and 3 M. This result suggests that in the experiment represented in Fig. 5 the rate of porin incorporation was independent of salt concentration.

Conductance as a function of voltage

The dependence of the average conductance increment $\bar{\Lambda}$ on voltage is represented in Table I. $\bar{\Lambda}$ was found to be virtually constant over the experimental voltage range between 0 and 150 mV. Current and voltage measurements over a larger voltage range were performed by applying short voltage pulses

TABLE I

DEPENDENCE OF THE AVERAGE CONDUCTANCE INCREMENT $\bar{\Lambda}$ ON VOLTAGE

$\bar{\Lambda}$ was obtained from current records such as shown in Fig. 2 by dividing the current increment by the applied voltage V and averaging over a large number n of events. Oxidized cholesterol/*n*-decane membranes in 0.3 M KCl, $T = 25°C$. The porin was solubilized in 1 $\mu g/cm^3$ SDS.

V (mV)	20	40	50	60	100	150
$\bar{\Lambda}$ (nS)	0.72	0.79	0.69	0.70	0.79	0.85
n	192	120	315	183	208	53

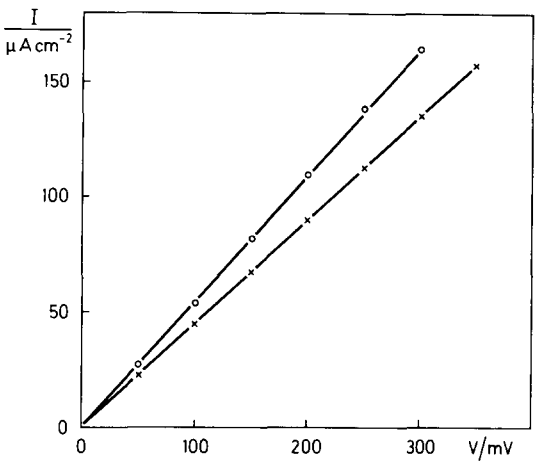

Fig. 7. Current vs. voltage characteristic of an oxidized cholesterol membrane doped with matrix protein. The aqueous phase contained 20 ng/cm^3 porin, 1 μg/cm^3 SDS and 1 M KCl; T = 25°C. Voltage pulses of 1 ms duration were applied and the current measured after the decay of the capacitive transient. The results from two different membranes are shown.

to a membrane in the presence of higher porin concentrations; for voltage pulses of 1 ms duration (or less) the membrane was stable up to several hundred millivolts. The result of such experiments is represented in Fig. 7. As can be seen the membrane current is a linear function of voltage up to at least 350 mV. It was also found that the current remained constant during the pulse length of 1 ms after the decay of the capacitive transient. In separate experiments at lower voltages (100 mV) it was established that the conductance after a voltage jump was nearly constant in the time range between 0.1 ms and several seconds. These experiments show that the properties of the single conductive units are virtually independent of voltage.

Ion specificity

In one series of experiments the ion specificity of the induced conductance was studied by measuring the zero-current membrane potential V_0 in the presence of a salt concentration gradient. In the case of an oxidized cholesterol membrane and for a concentration ratio of 10 : 1 the membrane potential V_0 was about 25 mV for KCl and about 15 mV for NaCl, with the positive side in the dilute solution. For these experiments the membrane conductance had to be kept low in order to avoid effects of unstirred layers in the aqueous solution adjacent to the membrane surface. The observed values of V_0 correspond to permeability ratios $P_K/P_{Cl} \simeq 3.5$ and $P_{Na}/P_{Cl} \simeq 2.1$. This slight preference for the cation may result from negative charges on the porin protein or on the oxidized cholesterol membrane.

In a second series of experiments the average conductance increment $\bar{\Lambda}$ was determined in different electrocytes. As can be seen from Table II that in different 1 M alkali chloride solutions (and NH_4Cl) $\bar{\Lambda}$ varies only within a factor of about 3 (between 0.72 and 2.3 mS). Also given in Table II is the ratio $\bar{\Lambda}/\sigma$ where σ is the specific conductance of a 1 M solution of the corresponding salt in water. This ratio varies by a factor of 2. The low ion specificity

TABLE II

AVERAGE CONDUCTANCE INCREMENT $\overline{\Lambda}$ IN DIFFERENT 1 M ELECTROLYTE SOLUTIONS

Oxidized cholesterol/*n*-decane membranes; $T = 25°C$. The aqueous solutions contained 1 $\mu g/cm^3$ SDS. The applied voltage was 50 mV. n is the number of events used for the evaluation of Λ; σ is the specific conductance of a 1 M aqueous solution of the electrolyte (at 18°C).

Salt	$\overline{\Lambda}$ (nS)	$\overline{\Lambda}/\sigma$ (10^{-10} m)	n
LiCl	0.72	1.0	418
NaCl	1.2	1.4	206
KCl	1.9	1.7	321
RbCl	2.1	1.8	251
CsCl	2.3	2.0	194
NH_4Cl	2.0	1.8	213

of $\overline{\Lambda}$ is consistent with the assumption that the protein forms large water-filled pores in the membrane.

Effect of membrane composition

The magnitude of the conductance increase in the presence of porin depends strongly on lipid composition of the membrane (Table III). With membranes made of oxidized cholesterol or of monoolein the conductance λ in the presence of porin is by several orders of magnitude larger than the conductance λ of the undoped membrane. A much smaller effect (in the given time of the experiment) was observed with phosphatidylserine, phosphatidylinositol, phosphatidylcholine (lecithin) and phosphatidylethanolamine. Discrete conductance steps such as observed with oxidized cholesterol or monoolein membranes were also found with phosphatidylserine and phosphatidylinositol membranes (see below); they could not be detected, however, with lecithin and with phosphatidylethanolamine membranes. For this reason it cannot be excluded that in the case of lecithin membranes the observed conductance

TABLE III

SPECIFIC MEMBRANE CONDUCTANCE λ MEASURED t MIN AFTER FORMATION OF THE BLACK FILM

The membrane was formed from a 2% (w/v) solution of the lipid in *n*-decane. The aqueous phase contained 1 M NaCl, 1 $\mu g/cm^3$ SDS and 20 ng/cm^3 protein; $T = 25°C$. λ^* is the membrane conductance measured at time t in the same solution but without porin. The applied voltage was 10 mV. Monoolein dissolved in *n*-dodecane or *n*-hexadecane gave similar values of λ as monoolein in *n*-decane.

Lipid	t (min)	λ ($S \cdot cm^{-2}$)	λ^* ($S \cdot cm^{-2}$)
Oxidized cholesterol	40	$5 \cdot 10^{-3}$	$3 \cdot 10^{-8}$
Monoolein	12	$8 \cdot 10^{-4}$	$2 \cdot 10^{-7}$
Brain phosphatidylserine	40	$9 \cdot 10^{-6}$	$5 \cdot 10^{-7}$
Brain phosphatidylinositol	30	$6 \cdot 10^{-6}$	$3 \cdot 10^{-7}$
Dioleoyllecithin	50	$4 \cdot 10^{-7}$	$4 \cdot 10^{-8}$
Egg lecithin	30	$7 \cdot 10^{-7}$	$5 \cdot 10^{-8}$
Egg phosphatidylethanolamine	20	$5 \cdot 10^{-7}$	$1 \cdot 10^{-7}$

TABLE IV

AVERAGE CONDUCTANCE INCREMENT $\overline{\Lambda}$ FOR MEMBRANES OF DIFFERENT COMPOSITION IN AQUEOUS KCl SOLUTION (CONCENTRATION, c_M)

$T = 25°C$, $V = 50$ mV. The porin was solubilized in 1 $\mu g/cm^3$ SDS. d is the thickness of the hydrophobic portion of the membrane, as calculated from capacitance measurements using a value of 2.1 for the dielectric constant [45,50,51]. n is the number of events from which $\overline{\Lambda}$ has been calculated.

Lipid/solvent	d (nm)	c_M (M)	$\overline{\Lambda}$ (nS)	n
Oxidized cholesterol/*n*-decane	3.4 *	1	1.9	321
Monoolein/*n*-decane	4.8	1	1.9	142
Monoolein/*n*-hexadecane	3.2	1	1.8	252
Brain phosphatidylserine/*n*-decane	5.3	1	2.5	42
Brain phosphatidylserine/*n*-decane	5.3	0.1	0.30	35
Brain phosphatidylinositol	5.2	0.1	0.29	40

* Benz, R., unpublished results.

increase is of an unspecific nature. An explanation of the striking influence of lipid structure on the protein-induced conductance seems difficult at the moment. As a true equilibrium distribution of porin between water and membrane was not reached under the conditions of the conductance experiments, the observed effects of lipid composition may be mainly of kinetic nature. That is, the activation energy for the incorporation of the porin into the membrane may be much larger for the phospholipids than for oxidized cholesterol or monoolein.

In contrast to the pronounced lipid effects on macroscopic conductance, the magnitude of the discrete conductance steps did not depend much on the composition of membrane. As can be seen from Table IV that the average conductance increment $\overline{\Lambda}$ varies only by a factor of 1.5 in a series of different lipids (oxidized cholesterol, monoolein, phosphatidylserine, phosphatidylinositol). The observation that $\overline{\Lambda}$ is not much different in neutral membranes (monoolein) and in negatively charged membranes (phosphatidylserine, phosphatidylinositol) is consistent with the finding that the conductive unit has a poor cation-to-anion selectivity (see above). Furthermore, $\overline{\Lambda}$ was virtually the same in membranes made from monoolein in *n*-decane and monoolein in *n*-hexadecane, despite a considerable difference in membrane thickness.

Discussion

The experiments described above show that the matrix protein from the outer membrane of *E. coli* is able to increase the ion permeability of lipid bilayer membranes by several orders of magnitude. At low porin concentration the change of membrane conductance occurs in descrete steps, suggesting that the conductive pathways consist of localized structures. These findings may be compared with recent studies of Nakae [32] who showed that the matrix protein renders lipid vesicles permeable to sugars up to a molecular weight of about 550. However, in contrast to Nakae's results [32] no lipopolysaccharide was needed in lipid bilayers to receive channel activity. If the protein forms wide aqueous pores in the bilayer (as Nakae proposed) then the size of the pore may

be estimated from the magnitude of the discrete conductance increment which is about 1.9 nS in 1 M KCl. Assuming that the pore is filled with a solution of the same specific conductivity as the external solution and assuming a pore length of 4 nm, the cross-section of the pore is calculated to be 0.68 nm^2. This corresponds to a diameter of 0.93 nm if the cross-section is circular. The minimum diameter that a circular pore must have in order to allow the passage of sucrose (which was found to be permeable in Nakae's experiments) is about 0.9 nm, as may be estimated from molecular models. Similar minimum pore diameters apply to rod-shaped trisaccharides such as raffinose which also have been used in the permeability studies with vesicles. The average pore size obtained from the conductance experiments is thus of the right magnitude to account for the nonelectrolyte permeability of outer membranes [4] and of reconstituted vesicles [32]. However, at the present time we cannot exclude the possibility that the larger conductance steps in the histogram arise from the formation of aggregates containing more than one pore. More information bearing on this question should be obtained from specificity studies with large organic ions.

The additional results are also consistent with the notion that the porin forms wide water-filled channels in the membrane. Such channels should have a poor cation-to-anion discrimination as well as a low selectivity for small ions in general. Furthermore, the current vs. voltage character of a wide, unselective channel should be ohmic. The observation that the conductance of the single channel does not depend much on the composition and thickness of the membrane (Table IV) suggests that the pore has a fixed length which is determined by the porin molecule itself. In order to accommodate a pore of fixed length, the lipid structure has to be distorted locally to a lesser or greater extent, depending on the membrane thickness [53].

So far, the available experimental results provide only limited information on the structure of the channel. From the histograms of the conductance fluctuations (Fig. 3) it is clear that under the conditions of our experiments the channel can assume a broad range of conductance values. This broad distribution of single-channel conductances may have a number of different origins. The channel may be an oligomeric structure composed of several of the 36 500-dalton subunits of the matrix protein [14], and the number of subunits may vary from one channel to the other. A well studied example of such an oligomeric channel with different conductance states is the alamethicin channel [54—57]. In that case, however, several peaks in the histogram should be found, corresponding to different discrete states. Another possibility would be that the porin is chemically (or conformationally) heterogeneous. The matrix protein of *E. coli* strain K12 in known to consist of at least two subfractions representing two closely related peptides [23,24,33—35].

Furthermore, the possibility that the isolation procedure which includes a trypsin treatment and solubilisation with detergent may lead to a spectrum of peptides with slightly modified structure has been shown to be rather unlikely [32]. In addition, on polyacrylamide gel electrophoresis no heterogeneity of the protein was observed.

Control experiments carried out in the absence of porin have shown that the detergent in the aqueous solution does not interfere with the experiments.

On the other hand, detergent molecules are probably associated with the solubilized protein and thus a certain amount of detergent may be incorporated into the membrane together with the porin. The question therefore arises whether the observed conductance effects are influenced by detergent introduced into the membrane. From the finding that the histogram of conductance fluctuation is virtually the same for cholate and for SDS preparations this possibility seems rather unlikely, although it cannot be completely excluded.

From gel filtration experiments it is known that the solubilized porin is highly aggregated, the molecular weight being of the order of 800 000 [32]. This corresponds to an aggregate of about 20 monomers of molecular weight 36 500. It is not known whether in aqueous solution an equilibrium between monomers and aggregates exists and in what form the porin is incorporated into the membrane. Even if only monomers are able to enter the lipid, the porin may form aggregates of various sizes in the lipid and this may result, as discussed above, in a broadening of the conductance histogram. It is therefore not clear at the moment whether channels are formed singly and the distribution of conductance values results from chemical (or conformational) heterogeneity of the individual channels, or whether each reductance step represents the formation of an aggregate of channels of variable size.

Note added in proof (Received May 29th, 1978)

Schindler and Rosenbusch [59], using a different method, have recently incorporated porin into planar bilayer membranes and observed discrete conductance steps of a similar size as those reported here.

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