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Determination of Ion Permeability through the Channels made of Porins from the Outer Membrane of *Salmonella typhimurium* in Lipid Bilayer Membranes

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Summary. The three types of porin (matrix-proteins) from *Salmonella typhimurium* with molecular weights of 38,000, 39,000 and 40,000 were reconstituted with lipid bilayer membranes either as a trimer or as an oligomer (complex I). The specific conductance of the membranes increased several orders of magnitude after the addition of the porins into the aqueous phase bathing the membranes. A linear relationship between protein concentration in the aqueous phase and membrane conductance was found. In the case of lower protein concentrations (10^{-12} M), the conductance increased in a stepwise fashion with a single conductance increment of 2.3 nS in 1 M KCl. For a given salt the conductance increment was found to be largely independent of the particular porin (38 K, 39 K or 40 K) and on the state of aggregation, although porin oligomers showed an up to 10 times smaller conductance increase in macroscopic conductance measurements. The conductance pathway has an ohmic current voltage characteristic and a poor selectivity for different alkali ions. Further information on the structure of the pores formed by the different porins from *Salmonella* was obtained from the selectivity for various ions. From the permeability of the pore for large ions ($Tris^+$, $glucosamine^+$, $Hepes^-$) a minimum pore diameter of 0.8 nm is estimated. This value is in agreement with the size of the pore as calculated from the conductance data for 1 M KCl (1.4 nm for a pore length of 7.5 nm). The pore diameter may well account for the sugar permeability which has been found in reconstituted vesicles. The findings reported here are consistent with the assumption that the different porins form large aqueous channels in the lipid bilayer membranes and that the single conductance unit is a trimer. In addition, it is suggested that one trimer contains only one pore rather than a bundle of pores.

The cell envelope of gram-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* consists of the inner membrane, the peptidoglycan layer, and the outer membrane [10, 21]. The inner membrane contains a large variety of transport systems [12], the respiration chain, and the energy-transducing ATPase.

The outer membrane acts as a molecular filter [8, 13, 20, 21] for hydrophilic solutes up to a molecular weight of about 700. This passive permeability is dependent to a large extent on a class of major outer membrane proteins [16, 17] called matrix proteins [26], protein 1, protein I [11, 30], or porins [16]. The porins have been isolated from the outer membrane of *Escherichia coli, Salmonella typhimurium,* and *Proteus mirabilis.* They were shown to form permeability channels in vesicles reconstituted from phospholipids and lipopolysaccharide [16, 17, 23]. It was suggested that the active unit of porin aggregates is a trimer in *E. coli* and *S. typhimurium* [35, 36]. Recently it has been shown that the incorporation of porins from *E. coli* into planar lipid bilayer membranes resulted in the formation of large ion-permeable pores with a high electrical conductance (in the order of 0.2 nS for 0.1 M alkali chloride solution) [3, 28]. In addition, detergent-free porin from the shock-fluid and detergent solubilized λ-receptor of *E. coli* have also been found to form ion conductive pores in membrane experiments [2, 6]. Although the magnitude of the unit conductance increment, the voltage-independent behavior and the permeability of large organic ions such as $glucosamine^+$ and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid ($Hepes^-$) [4] are consistent with the assumption that porin forms large water-filled pores in lipid bilayer membranes, the structure of these porin pores is largely unknown.

The outer membrane of *S. typhimurium* contains three species of porins of 38,000, 39,000 and 40,000 daltons [17, 34]. These porins (previously

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called 34K, 35K and 36K [1]) also seem to span the outer membrane and are tightly associated with the peptidoglycan layer [1, 17]. Evidence that the porins are involved in formation of permeability channels in the outer membranes comes from the finding that mutants of *S. typhimurium,* producing reduced amounts of porins, had a reduced permeability towards cephaloridin, a hydrophilic solute of molecular weight 415 [22]. The three different porin species of *S. typhimurium* seem to form the permeability channels independently [18]. We have shown [15, 17, 18] that phospholipid lipopolysaccharide vesicles become permeable to hydrophilic solutes up to molecular weights of about 700 when the porins from *Salmonella* were incorporated into the vesicle membranes.

In the present communication we describe lipid bilayer experiments in the presence of porin trimers or oligomers purified from the mutant strains of *S. typhimurium* producing only single species of porin. A large number of experiments are described in which the selectivity was studied by measuring the pore conductance as a function of different electrolytes. The results of these experiments argue against the concept of a bundle of parallel pores, they are consistent however, with the assumption that the single conductance unit is a trimer with one pore of large diameter.

Materials and Methods

Bacterial Strains and Culture Conditions

The strains used throughout this study were derivatives of *S. typhimurium* LT2. Mutant strains producing a particular porin (either 38K, 39K, or 40K) are described previously [18, 24]. Strain TA 1014 was used for identification of three different porins in *S. typhimurium* [17].

The culture media and the growth conditions were described [34]. Strain HN 407 producing only 39K porin was grown in the medium containing 1% tryptone/0.5% yeast-extract/0.4% glucose.

Purification of Porin

Procedure for preparation of porin oligomers was described previously [34]. The SDS-insoluble peptidoglycan-associated porin was treated with lysozyme and complex I was isolated by gel filtration in the presence of SDS as described [17]. Complex I isolated from strains producing a single-species of porin appeared to contain homogeneous porin polypeptide as examined by SDS-slab gel electrophoresis.

The porin trimers were isolated as reported [35].

Porins purified from strains SH 5551 (40K) and SH 6017 (38K) appeared to be homogeneous as examined by gel electrophoresis in SDS.

Porin prepared from the strain HA 417 producing 39K polypeptide were contaminated with a trace amount of unidentified polypeptide of about 48,000 daltons.

All three porins were produced by strain TA 1014 under conditions similar to the previous publication [17]. Roughly equal amounts of these three porins were present in T 1014.

Membrane Experiments

Optical black lipid bilayer membranes were obtained in the usual way [3, 5] from a 1–2% (wt/vol) solution of different lipids in *n*-decane (Fluka, Buchs, Switzerland, purum). The chamber used for bilayer formation was made from Teflon. The circular holes in the wall between the two aqueous compartments had an area of either 2 mm^2 (for the macroscopic conductance measurements) or about 0.1 mm^2 (for the single channel experiments). The temperature was kept at 25 °C, unless indicated otherwise.

All salts, besides N-2-hydroxyethlpiperazine-N′-2-ethanesulfonic acid (Hepes, Sigma, analytical grade), tetramethylammoniumhydroxide and tetraethylammoniumhydroxide (Fluka, purum) were obtained from Merck (Darmstadt, F.R.G., analytical grade). The pH of the aqueous salt solutions was adjusted to the values given in the text by adding the corresponding hydroxide or acid. So in experiments with large organic ions no small ions like Na^+ or Cl^- were present. In the experiments on the pH-dependence of the single channel conductance, the salt solutions were buffered with 10^{-3} M citrate or 10^{-3} M Tris. Tris and citrate did not have any influence on the results in these concentrations. Aqueous solution with a pH around 6 were used without buffering. The protein was added to the aqueous phase from the stock solutions either prior to membrane formation or after the membrane had turned black. In the aqueous salt of high ionic strength all proteins investigated here were found to be completely inactivated overnight.

A variety of different lipids were used for membrane formation: Monoolein (Nu Check Prep., Elysian, Minn.; brain-phosphatidylserine (brain-PS), egg-phosphatidylethanolamine (egg-PE) and egg-phosphatidylcholine (egg-PC) were isolated and purified according to standard methods [27, 31]; oxidized cholesterol was prepared as described earlier [3]. All lipids (besides oxidized cholesterol) were analyzed by thin-layer chromatography and found to be pure. For the electrical measurements Ag/AgCl or platinized platinum electrodes (if no chloride was present) were inserted in the aqueous compartments on both sides of the membranes. The stationary current measurements were performed with a Keithley 610 C electrometer. For the conductance-fluctuation experiments, a Keithley 427 preamplifier was used. The amplified signal was monitored with a Tektronix 5115/5A22 storage oscilloscope and recorded with a stripchart recorder. The rise time of the single fluctuations (steps) in the fluctuation measurements was limited by the bandwidth of the preamplifier and was between 0.3 and 3 msec, depending on the amplification.

Results

Macroscopic Conductance Measurements

A strong conductance increase of lipid bilayer membranes was observed when one of the three porins were added to the aqueous phase prior to membrane formation or after the membranes had turned black. An example of the first case is given in Fig. 1. A membrane from oxidized cholesterol/*n*-decane was formed in a solution of 1 M KCl and 4.7×10^{-10} M porin trimers from strain SH 5551 (40 K). After the membrane had turned completely black (arrow in Fig. 1), a strong conductance increase with time was observed. In these experiments a stationary conductance level could not be reached and the membrane conductance increased continuously until membrane

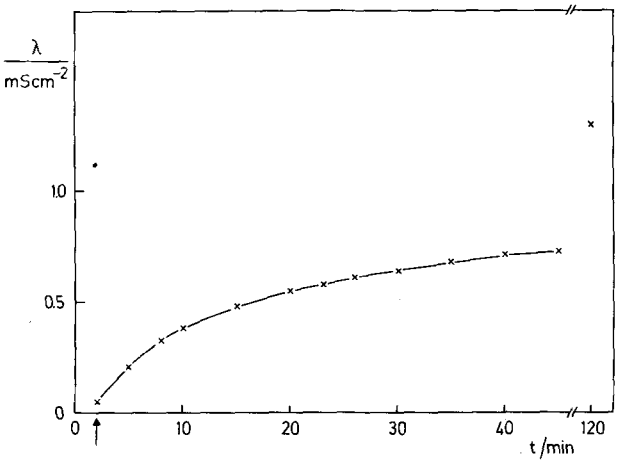

Fig. 1. Specific membrane conductance λ as a function of time t after the formation of the membrane from oxidized cholesterol in *n*-decane. The arrow indicates when the membrane was completely black. The aqueous phase contained 4.7×10^{-10} M trimers from SH 5551 (40K), 2.5×10^{-7} g/ml SDS and 1 M KCl; $T = 25$ °C; $V_m = 10$ mV. For experiments of the same type the reproducibility of λ (t) was about ± 30%

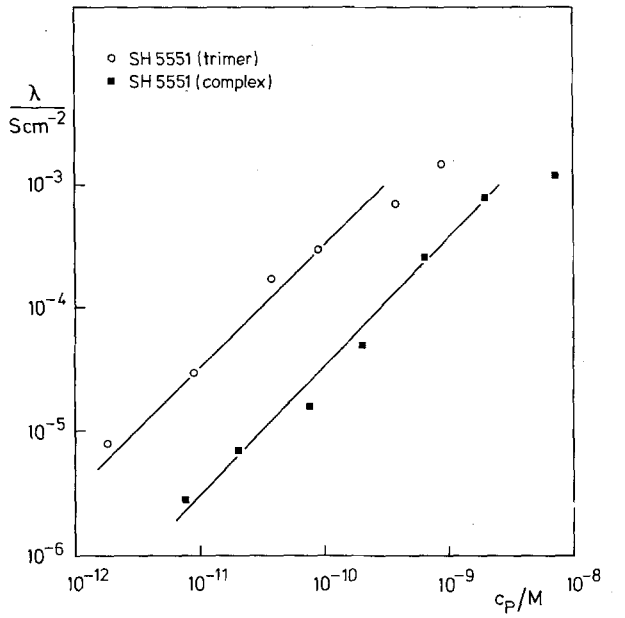

Fig. 2. Specific membrane conductance λ as a function of the protein concentration c_P of SH 5551 trimers (40K) and oligomers in the aqueous phase. Membranes were formed from oxidized cholesterol dissolved in *n*-decane. The aqueous phase contained 1 M KCl and a SDS-concentration, which was always less than 5×10^{-7} g/ml; $T = 25$ °C; $U = 10$ mV. The single points represent the data from at least 4 membranes measured 20 min after blackening

breakage. This behavior did not change whether the porins were added into one or both aqueous compartments. In addition, the use of the different porin trimers and oligomers (complex I) did not result in an alteration of the time course of the conductance increase. However, the absolute level of the conductance at a given time was somewhat dependent on the type of the trimers (38K, 39K or 40K) and it was considerably lower when oligomers were used. In experiments with porin oligomers from *E. coli* the concentration of the detergent used showed a large influence on the incorporation of porin and therefore on the conductance increase [3]. A similar effect was not found for *Salmonella* porins.

The most striking difference from the experiments using porin oligomers from *E. coli* is the observation that porin trimers and oligomers from *Salmonella* showed a linear relationship between the conductance at a certain time (usually 20 min) after blackening and the protein concentration in the aqueous phase. With *E. coli* porin, the corresponding conductance increase was largely independent of protein concentration [3]. Figure 2 shows a concentration dependence of the macroscopic conductance in the presence of 1 M KCl and a different concentration of porin trimers and oligomers from strain SH 5551 (40K). As can be seen, there is a linear dependence of the conductance on the protein concentration in the aqueous phase. The conductance in the presence of the oligomers is about a factor of ten lower than in the presence of trimers. This difference can in principle be explained by the assumption that a certain amount of free trimers are present in the oligomers or that the oligomers may dissociate slowly into trimers in the aqueous phase with a rate that is a linear function of the protein concentration. On the other hand, some porin-trimers in the oligomeric aggregate may only produce ion conductance, while the others may simply be aggregated on the surface of the conducting trimers and do not contribute to the conductance. A similar dependence of the conductance on the protein concentration was also found for the other porin trimers and oligomers (strains HN 407 (39K) and HN 6017 (38K) (Fig. 3)). However, in the case of 39K porin (HN 407) and the 38K porin (HN 6017) the conductance in the presence of the oligomers was only a factor of 3 and 5 lower, respectively. The reason for this difference is not clear, but the above-mentioned explanations may well be dependent on the type of porin.

The magnitude of the conductance increase in the presence of the porins from *Salmonella* was strongly dependent on lipid composition of the membrane (Fig. 4). With membranes made from oxidizied cholesterol (ox. chol) or of monoolein the conductance λ in the presence of the porins is by several orders of magnitude larger than that of the membrane in the absence of porins. A much smaller increase of the conductance in the presence of porin trimers from the strain TA 1014 is observed (in the given time

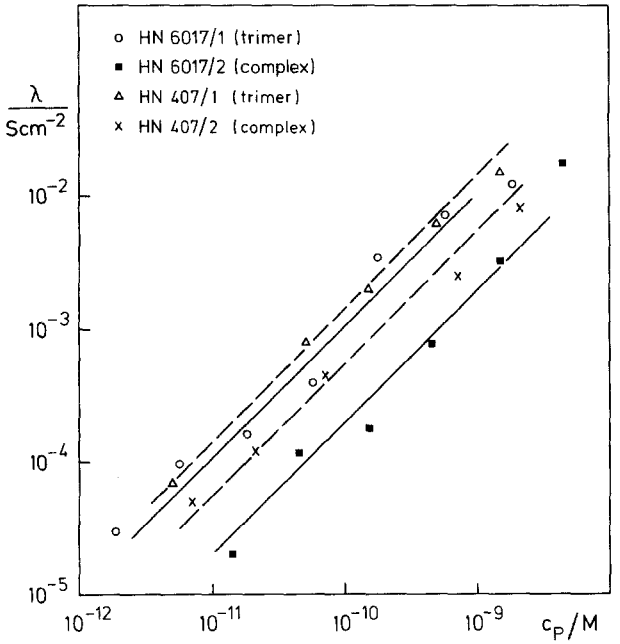

Fig. 3. Specific membrane conductance λ as a function of the protein concentration c_P in the aqueous phase. Trimers and oligomers of the strains HN 6017 (38K) and HN 407 (39K) were added to an aqueous solution of 1 M KCl bathing the membranes from oxidized cholesterol in *n*-decane; $T=25$ °C; $U=10$ mV. The concentration of SDS in the aqueous phase was always less than 5×10^{-7}g/ml. The results were derived from at least 4 membranes 20 min after blackening

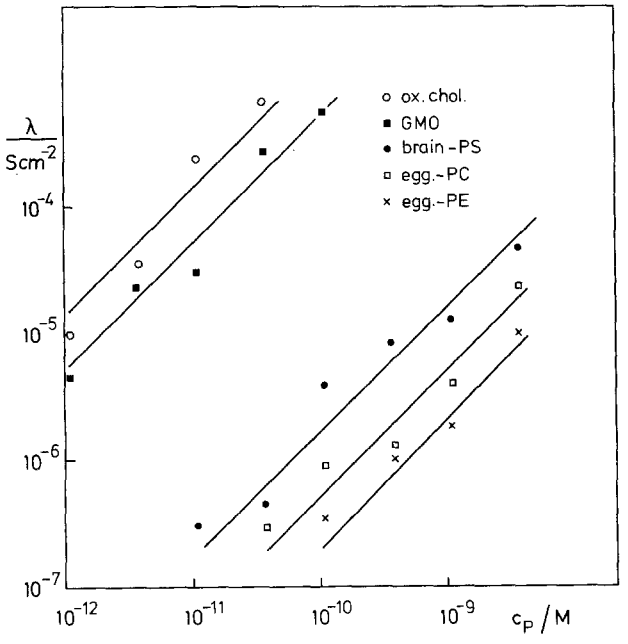

Fig. 4. Specific membrane conductance λ as a function of the protein concentration c_P of trimers from the strain TA 1014 (38K, 39K and 40K) in the aqueous phase. The membranes were formed from different lipids dissolved in *n*-decane. The aqueous phase contained 1 M KCl and less than 5×10^{-6}g/ml SDS. The results were derived from at least 4 membranes 20 min after blackening; $T=25$ °C; $V_m=10$ mV

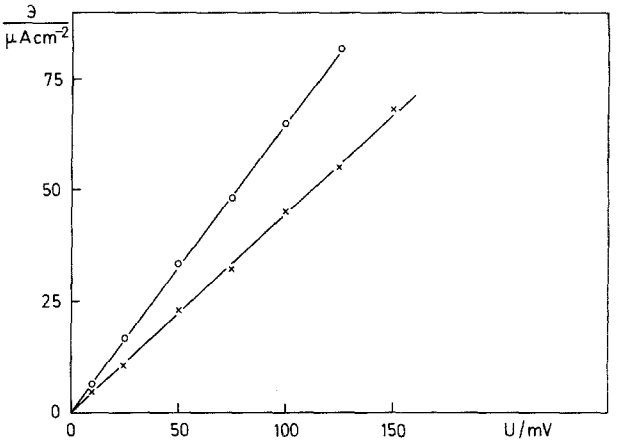

Fig. 5. Current vs. voltage characteristic of an oxidized cholesterol membrane doped with 4.7×10^{-11}M porin trimers from strain TA 1014 (38K, 39K and 40K). The aqueous phase contained 1 M KCl and 5×10^{-8}g/ml SDS. The results from two different membranes are shown; $T=25$ °C

of the experiment, 20 min) with brain-PS, egg-PC, and egg-PE (Fig 4). But also in these cases a linear dependence of the conductance on the protein concentration was observed.

The effect of salt concentration on λ was studied by measuring the conductance 20 min after the formation of the membranes at a given protein concentration. It was found that the conductance is a linear function of salt concentration within the limit of the experimental error. This showed that the rate of incorporation of the protein was not dependent on the aqueous salt concentration, a result which may indicate that the interaction of the protein with a membrane is of hydrophobic nature. Figure 5 shows a current voltage behavior of two membranes from oxidized cholesterol/*n*-decane in the presence of porin trimers from the strain TA 1014 (38K, 39K and 40K). As can be seen from Fig. 5 the membrane current was a linear function of the applied voltage up to at least 150 mV. For short voltage pulses in the few millisecond range, this could be proved up to 300 mV. The conductance after a voltage jump of 100 mV showed only a small increase in the time range between 1 msec and several seconds. These experiments showed that the properties of the single conductance unit are virtually independent of voltage.

Single-Channel Experiments

When porins from *Salmonella* were added in low concentrations (10^{-11} to 10^{-12}M) to the aqueous solution, the membrane conductance started to increase in a stepwise fashion. An example for the porin trimers from strain SH 5551 (36K) is given in Fig. 6.

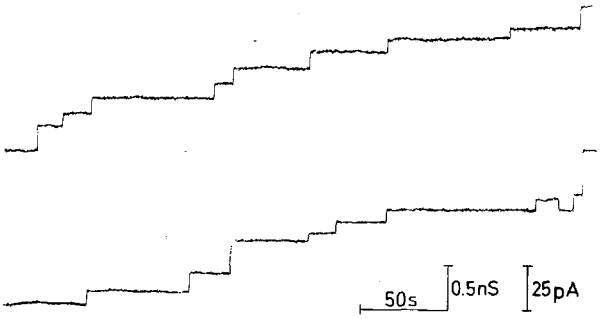

Fig. 6. Stepwise increase of the membrane current after the addition of 9×10^{-11} M porin trimers from strain SH 5551 to the aqueous phase containing 0.1 M NaCl; $T=25$ °C. The membrane was formed from a 1% (wt/vol) solution of egg-phosphatidylcholine in *n*-decane. The applied voltage was 50 mV; the current prior to the addition of porin was about 0.1 pA. The bandwidth of the measurements was 1 kHz. The record starts at the left end of the lower trace and continues in the upper trace

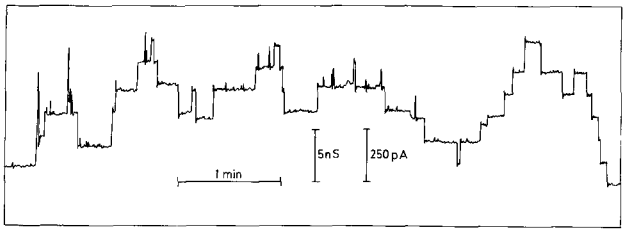

Fig. 7. Fluctuations of the membrane current in the presence of about 10^{-12} M porin trimers from strain HN 6017 (38K). The record begins about 10 min after the addition of the protein, and 19 pores are open at the start of the record (left-hand side). The membrane was formed from oxidized cholesterol in *n*-decane; the aqueous phase contained 1 M KCl; T=25 °C. The applied voltage was 50 mV, and the bandwidth of the experiment was 3 kHz

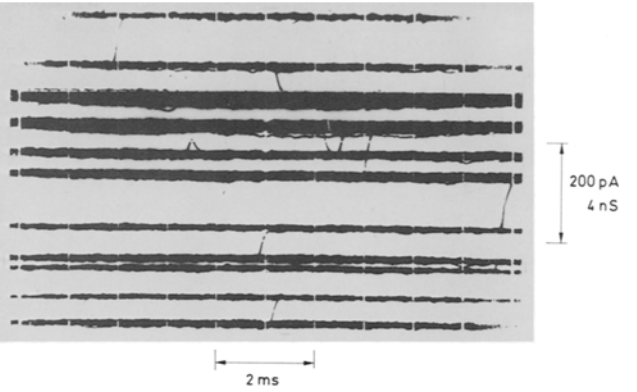

Fig. 8. Oscillographic record of current fluctuations in the presence of about 10^{-12} M porin trimers from strain HN 407 (39K). The record begins about 5 min after the addition of the porin to the aqueous phase at the lower left-hand side of the record. The membrane was formed from oxidized cholesterol in *n*-decane; the aqueous phase contained 1 M NaCl; $T=25$ °C. The applied voltage was 50 mV, and the bandwidth of the experiment was 3 kHz

The occurrence of these steps is specific for one of the porin trimers and is not seen when only the detergent SDS is present in a concentration equivalent to that in the porin experiments. In addition, at these SDS concentrations alone no conductance increase was observed and the noise level of the background conductance was not altered. The magnitude of the conductance steps was not changed when one of the different porins was added only to one side of the membrane or to both sides. In both cases no change in the scatter of the experimental data could be detected. As can be seen from Fig. 6, most steps were directed upwards, whereas terminating events are rarely observed, an observation which has also been found for *E. coli* porins [3]. However, when about 20 to 30 pores are open and a quasi-stationary level of the number of open pores was reached (Fig. 7), the pores formed from *Salmonella* porins seem to close more often than those of *E. coli* porin [3]. From records extending over a long time, the average lifetime of the single conductive unit may be estimated to be in the order of 1 to more than 5 min, varying somewhat for the particular porin. The shortest lifetime (~1 min) was found for the porin trimers of strain HN 6017 (38K), whereas the longest (~5 min) for trimers from strain SH 5551 (40K). The short lifetime of pores from trimers of HN 6017 (38K) and HN 407 (39K) is hard to understand.

Changes in the salt concentration and in the lipid composition had no influence on the lifetime of the single conductance unit, although in the case of membranes from brain-PS, egg-PC, and especially egg-PE much more protein (in the order of 10^{-10} M) had to be added in order to obtain a reasonable number of pores. The risetime of the single conductance step was always faster than the time resolution of the preamplifier. Figure 8 shows that within a time resolution of about 0.5 msec the conductance rise and fall was always smooth without any indication of smaller intermediate steps.

Similar to the finding with *E. coli* porin [3], the single conductance increments are not uniform in size but distribute over a certain range (Figs. 6–8). Histograms of the conductance steps observed with the different *Salmonella* porins are presented in Fig. 9. As can be seen from Fig. 9, the distribution of the conductance increments is quite similar for the different species of *Salmonella* porins. In each case there was at least a factor of three from the smallest conductance step to the largest one. In addition, the histogram of the porin trimers from the strain TA 1014 (38K, 39K and 40K) shows a broader distribution of pores than the histograms of the trimers containing single species of polypeptides (Fig. 9).

Table 1

Porin	Lipid/solvent	Salt	$\frac{\bar{A}_{on}}{n\,S}$	n_{on}	$\frac{\bar{A}_{off}}{n\,S}$	n_{off}
HN 6017/1 (38K)	Ox. chol./C_{10}	LiCl	0.97	452	0.99	99
		NaCl	1.5	658	1.3	165
		KCl	2.4	369	2.1	38
		NH_4Cl	2.5	361	2.3	189
		RbCl	2.3	350	2.1	232
		CsCl	2.1	582	2.0	371
HN 6017/2		KCl	2.5	317	2.2	72
HN 407 (39K)		LiCl	1.1	148	1.1	111
		NaCl	1.6	187	–	–
		KCl	2.2	399	2.1	270
		NH_4Cl	2.6	255	–	–
		RbCl	2.2	428	2.1	395
		CsCl	2.4	534	2.5	433
HN 407/2		KCl	2.2	340	1.8	142
SH 5551/1 (40K)		LiCl	0.90	503	0.78	134
		NaCl	1.8	484	–	–
		KCl	2.4	948	–	–
		NH_4Cl	2.6	329	–	–
		RbCl	2.2	196	–	–
		CsCl	2.3	249	–	–
SH 5551/2		KCl	2.3	459	–	–
SH 5551/1	Egg-PC/C_{10}	NaCl	1.4	96	–	–
	Brain-PS/C_{10}	NaCl	2.0	138	–	–
	Monoolein/C_{10}	KCl	2.5	119	–	–
	Monoolein/C_{16}	KCl	2.4	142	–	–
	Egg-PC/C_{10}	KCl	2.3	131	–	–
	Egg-PE/C_{10}	KCl	2.2	48	–	–
	Brain-PS/C_{10}	KCl	2.7	149	–	–
Ta 1014/1	Ox. chol./C_{10}	LiCl	1.0	149	–	–
		NaCl	1.8	223	–	–
		KCl	2.1	185	–	–
		NH_4Cl	2.3	169	–	–
		RbCl	2.1	150	–	–
		CsCl	2.2	155	–	–

Average conductance increments $\bar{A}_{on}$ and $\bar{A}_{off}$ for different porins from *Salmonella typhimurium* and different 1 M alkali chlorides. The membranes were formed from the lipids as indicated. The lipids were dissolved either in *n*-decane (C_{10}) or in *n*-hexadecane (C_{16}); $T=25\,°C$; $U=50\,mV$. n_{on} or n_{off} are the numbers of events from which $\bar{A}_{on}$ and $\bar{A}_{off}$ have been calculated.

Table 1 presents the results of single-channel experiments with a variety of different alkali chlorides and lipids in the presence of the trimers (denoted as /1) and the oligomers (denoted as /2). As can be seen, there was in all cases little difference between the single-channel conductances for the different porin species. Only in the case of LiCl and NaCl $\bar{A}_{on}$ was somewhat lower than for the other alkali chlorides. Besides for LiCl, the same ratio between pore conductance $\bar{A}_{on}$ and specific conductance of the bulk aqueous phase σ was found (*see also* Table 2). This result indicates that the porin pores behave like a channel with a bulk aqueous phase.

As mentioned before, pores formed from the porins of *Salmonella* (especially HN 6017 (38K) and HN 407 (39K)) switch off occasionally. Therefore it was also possible to determine magnitude and distribution of the switching off of the pores (Table 1). As can be seen from the average conductance increment $\bar{A}_{off}$, there is no difference in the conductance between an opening pore and a closing pore. The number of closing pores shows that the lifetime of pores from trimers of the strains HN 6017 and HN 407 lies within the duration of a single-channel experiment (about 15 min), whereas the lifetime of a SH 5551 pore is so long that a sufficient number

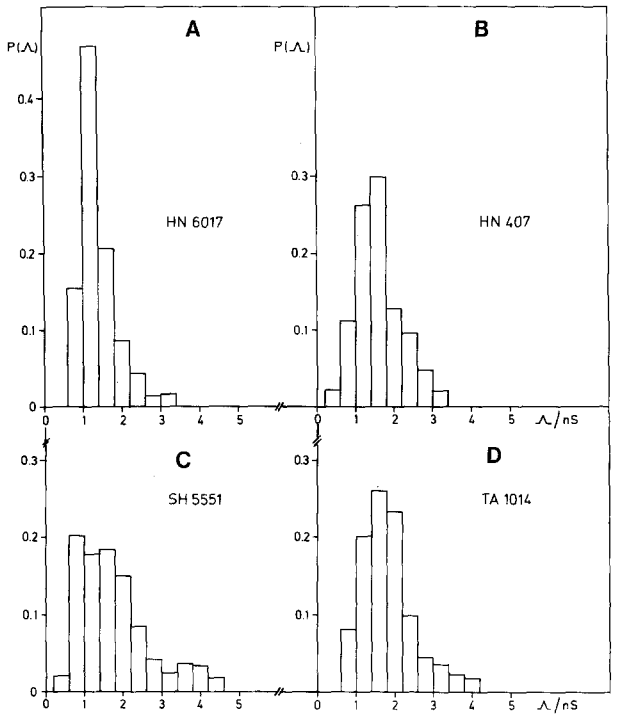

Fig. 9. Histogram of the conductance fluctuations observed with membranes from oxidized cholesterol in the presence of (A): trimers from strain HN 6017 (38K), $n_{on}=658$; $\bar{\Lambda}_{on}=1.5\times10^{-9}$S (1 M NaCl); (*B*): trimers from strain HN 407 (39K), $n_{on}=187$; $\bar{\Lambda}_{on}=1.6\times10^{-9}$S (1 M NaCl); (*C*): trimers from strain SH 5551 (40K), $n_{on}=484$; $\bar{\Lambda}_{on}=1.8\times10^{-9}$S (1 M NaCl); (*D*): trimers from strain TA 1014 (38K, 39K and 40K), $n_{on}=223$; $\bar{\Lambda}_{on}=1.8\times10^{-9}$S (1 M NaCl). The applied voltage was 50 mV

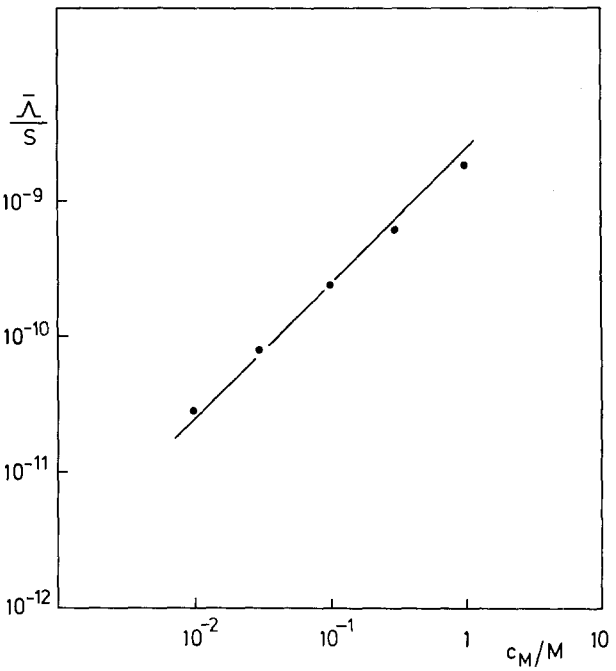

Fig. 10. Average conductance increment $\bar{\Lambda}_{on}$ as a function of NaCl concentration in the aqueous phase; $T=25$ °C; $U=50$ mV. Oxidized cholesterol/*n*-decane membranes. The aqueous phase contained about 10^{-12}M porin trimers from strain SH 5551 (40K) and 10^{-9}g/ml SDS

of closing pores could not be recorded. The reason for this different behavior is not clear, but we do not think that it has any physiological relevance. The lipid composition of the membranes had no influence on the magnitude of pore conductance in the case of trimers of SH 5551. However, the pore forming activity of the porin trimers was drastically reduced in membranes from some lipids and more than two orders of magnitude more protein had to be added to the aqueous solutions in these cases in order to receive a reasonable number of pores within a given time range of a single channel experiment (15 min). It is interesting to note that the lowest activity of porin was observed for membranes from phosphatidylethanolamine, the major lipid of gram-negative bacteria.

The pore conductance was pH independent and between pH 3 and pH 9 the same activity of porin trimers from strain SH 5551 was observed. The same results were also found for single-channel experiments. In membrane from oxidized cholesterol/*n*-decane $\bar{\Lambda}_{on}$ had the following values for the different pH and 1 M KCl:

pH 9: $\bar{\Lambda}_{on}=2.3$ nS ($n_{on}=343$)

pH 6: $\bar{\Lambda}_{on}=2.4$ nS ($n_{on}=948$)

pH 3: $\bar{\Lambda}_{on}=2.1$ nS ($n_{on}=310$).

This result was quite different from those results obtained with *E. coli* porin [4]. There a strong influence of the pH on single-channel conductance was observed.

Figure 10 contains the results obtained for different NaCl-concentrations in the aqueous phase bathing a membrane in the presence of trimers from strain SH 5551. The average conductance increment $\bar{\Lambda}_{on}$ was found to be a linear function of the concentration of the salt in the aqueous phase, a result expected for a large water-filled pore. The same conclusion may also be drawn from single-channel experiments in the presence of various electrolytes differing in charge and size of the anions and cations listed in

Table 2

Salt	c /(M)	$\bar{\Lambda}_{on}$ /(nS)	σ /(mS cm^{-1})	$\bar{\Lambda}/\sigma$ 10^{-8}cm	n
LiCl	1	0.9	71	1.3	503
NaCl	1	1.8	84	2.1	462
KCl	1	2.4	112	2.1	948
NH_4Cl	1	2.6	112	2.3	329
RbCl	1	2.2	115	1.9	196
CsCl	1	2.3	115	2.0	249
$MgCl_2$	0.5	0.62	64	1.0	145
$CaCl_2$	0.5	0.73	78	0.94	124
K_2SO_4	0.5	1.4	76	1.8	251
$MgSO_4$	0.5	0.32	33	1.0	136
Na^+Hepes$^-$ (pH 9)	0.5	0.39	18	2.2	431
Tris$^+$Cl$^-$	0.5	0.38	30	1.3	196
Tris$^+$Hepes$^-$ (pH 8)	0.5	0.088	7.2	1.2	217
Glucosamine$^+$ Cl (pH 3)	1	0.60	45	1.3	119
$N(CH_3)_4^+Cl^-$	1	0.82	71	1.2	153
$N(CH_3)_4^+$ Hepes$^-$ (pH 8.5)	0.5	0.12	15	0.80	103
$N(C_2H_5)^+$Hepes$^-$	0.5	0.045	4.8	0.94	231

Average conductance $\bar{\Lambda}_{on}$ in different salt solutions of concentration c. The aqueous solution contained besides the salt about 10^{-12} M trimers from SH 5551 and 10^{-9}g/ml SDS; the pH was between 6 and 7, if not otherwise indicated; $T=25$ °C; $U=50$ mV. The salts with large cations and anions did not contain Na^+ or Cl^-. The membranes were made from oxidized cholesterol/*n*-decane $\bar{\Lambda}_{on}$ was determined by recording a large number n_{on} of conductance steps and averaging. σ is the specific conductance of the aqueous phase [4].

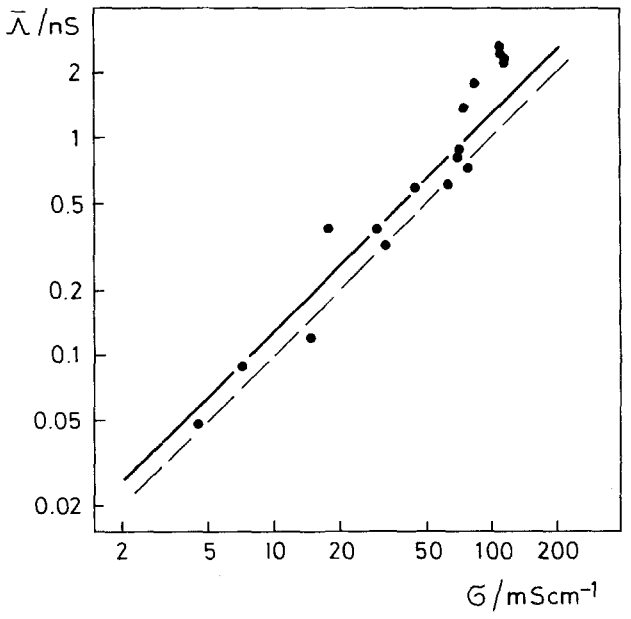

Fig. 11. Average pore conductivity $\bar{\Lambda}_{on}$ of trimers from strain SH 5551 given as a function of the specific conductance of the corresponding aqueous salt concentration; $T=25$ °C. The data were taken from Table 2. The broken line corresponds to the result obtained with *E. coli* porin oligomers [4]

Table 2. In addition to $\bar{\Lambda}_{on}$, the specific conductance of σ of the aqueous phase, as well as the ratio Λ/σ are given in Table 2. It is seen from the data that, despite a variation of $\bar{\Lambda}_{on}$ by a factor of about 60, the ratio $\bar{\Lambda}_{on}/\sigma$ varies only by a factor of less than 3. Therefore, the single-channel conductance in the presence of trimers of strain SH 5551 (40K) follows reasonably well the conductivity of the aqueous phases. This is in principle also reflected in Fig. 11. The results for the single-channel conductance can be fitted by a straight line with the slope one. Even large ions such as $N(C_2H_5)_4^+$ or Hepes$^-$ are permeable through the channel. This can be derived from the high channel conductance in the presence of the large ions and by measurements of diffusion potentials in the presence of salt gradients (R. Benz, *unpublished results*). In these experiments the pore of SH 5551 (40K) was at neutral pH about three times more permeable for cations (glucosamine, Tris$^+$, $N(C_2H_5)_4^+$, Na^+) than for anions (Cl^-, Hepes$^-$). $N(C_2H_5)_4^+$ has a nearly spherical shape with a diameter of 0.75 nm, Hepes$^-$ may be represented as an ellipsoid with axes of 1.4, 0.6 and 0.5 nm. If it is assumed that the pore is a cylinder with spherical cross-sections and is filled with an aqueous solution of the same conductance as the external bulk phase, according to

$$\bar{\Lambda}=\sigma\pi r^2/l \qquad (1)$$

the average pore diameter d ($=2r$) may be calculated for a certain length of the pore l. Assuming a pore length of 7.5 nm (corresponding to the thickness of the outer membrane), which seems to be likely [10, 21], the pore diameter d is calculated to be about 1.4 nm. This value for the diameter of the pore is a little larger, as has been found for the porin pore of *E. coli* [3, 4]. The finding that ions like Hepes$^-$ are permeable through the channel is consistent with the estimate of the channel diameter.

Discussion

The experiments described here show that the porins from *Salmonella* are able to form pores in lipid bilayer membranes similar to those of the porins and the λ-receptor of *E. coli* [3, 4, 6]. The conductance increased several orders of magnitude, and moreover at very low protein concentrations in the aqueous phase the conductance of the membrane increased in discrete steps. This finding indicates that the single conductance unit consists in the formation of channels. The single-channel conductance for a porin pore from *Salmonella* is about 2.3 nS in 1 M KCl. Assuming a pore length of 7.5 nm (which seems to be very likely

[35]) and that the pore is filled with a solution of the same specific conductance as the external solution, the pore diameter is calculated to be 1.4 nm. This diameter is large enough to account for the permeability, which has been found for the nonelectrolytes in outer membranes [22] and reconstituted vesicles [15, 27]. In addition, for the diameter of the trisaccharides, which have been found to be partially permeable through the pores in our experiment [17], a value of at least 1.0 nm may be obtained from molecular models.

The results presented here support the assumption of a large water-filled pore formed by porins from *Salmonella typhimurium*. For instance, the current *vs.* voltage characteristic of a wide, unselective channel should be ohmic, a behavior observed for the alamethicin channel [7]. The selectivity of the pores was found to be poor for the different porin pores. The single-channel conductance varied in the series LiCl to CsCl by less than a factor of three, and the salt concentration dependence showed no saturation. The main result of the selectivity studies is the finding that the channel induced by the porin trimers from strain SH 5551 (40K) is permeable to ions as large as $Hepes^-$ or $glucosamine^+$. From the dimension of these molecules a minimum diameter for the pore of 0.8 nm is estimated. In addition, the single channel conductance $\bar{A}$ is proportional to the conductance in the aqueous phase for all salts studied here.

In the experiments with porin oligomers from *E. coli,* a large variety of conductance fluctuations has been observed [2, 3, 4]. It has been assumed that this originates either from different porin molecules (*E. coli* K 12 contains the porins Ia and Ib [29]) or that the channel is an oligomeric structure composed of several of the 36,500-dalton subunits of the single porin molecule and the number of subunits may vary from channel to channel similar to that found for the alamethicin channel [19]. According to the results presented here for the channels formed from *Salmonella,* both explanations seem to be rather unlikely to account for the broad distribution found for *Salmonella* channels. First, the porin trimers are obtained from strains which contain only one porin species (38K, 39K or 40K) and second, porin trimers from *E. coli* are rather stable and considerable energy is needed to dissociate them into monomers [36]. The same may also be valid for porins from *Salmonella.* In addition, isolated monomers have been found to be completely ineffective in the vesicle permeability assay [36]. The observation that the conductance of the single channel does not depend on the composition and thickness of the membrane suggests that the pore is longer than the thickness of all membranes used here. This result is in agreement with the assumption that the porin molecules should span about 7.5 nm [10, 21, 35; T. Tanake, M. Ueki, and T. Nakae *to be published*].

In the case of porin oligomers from *E. coli,* the macroscopic conductance has been found to be independent of protein concentration in the aqueous phase down to 2 ng/ml [2, 3]. The results described here (i.e., a linear dependence of conductance on protein concentration) may be explained by the assumption that the trimers from *S. typhimurium* are fairly stable in the aqueous phase and show no aggregation tendency. They interact with the lipid bilayer membrane presumably by hydrophobic interaction and the rate of the adsorption to the membrane is a linear function of the concentration in the aqueous phase. In the case of the oligomers it may well be that they either contain a certain amount of trimers and the exchange between oligomers and trimers is slow or that the release of trimers from oligomers is directly related to the concentration of oligomers. Both assumptions are able to explain the finding that the conductance in the presence of the oligomers is a fixed fraction of the conductance in the presence of trimers.

The reconstitution rate was found to be strongly dependent on the type of the lipid used for membrane formation, whereas the conductance of the single conductance unit, the trimer, was independent from the lipid. Considering the different lipids, it is evident from the results with membranes from phosphatidylethanolamine (the major lipid of gram-negative bacteria) that the reconstitution of porin trimers with lipid bilayer membranes is not a question of a specific lipid-protein interaction. Highest reconstitution rates were observed with lipids with a small polar headgroup; therefore it seems likely that the insertion of porin trimers in the membrane is governed by a kinetic process. Porin trimers have approximately a cross section of about 50 nm^2 [9, 21, 35]; that means that 100 lipid molecules (area 0.5 nm^2) on each side of the membrane were replaced by one single trimer. It is not known how large the surface pressure is in a lipid bilayer membrane. However, assuming a value of 20 dyn/cm for the surface pressure, an energy of 1.2×10^6 J/mol is needed for the insertion of molecules with 8 nm diameter into the membrane. It is easy to imagine that cells have developed a special mechanism for the insertion of protein into membranes [10, 25]. Pores obtained from *E. coli* porin have also been studied in experiments with lipid bilayer membranes [28] formed by a different technique. In these experiments planar bilayers have been formed from reconstituted vesicles. However, no pores were found to be open at zero voltage [28] and a considerable potential difference was needed to switch on

($V_m > 100$ mV) and to switch off ($V_m \geqq 200$ mV) the pores [28]. In our experimental approach no voltage dependence of pore conductance and pore lifetime was observed either for *E. coli* porins [3] nor for the results with *Salmonella* porins reported here. A voltage dependence for opening and closing pores *in vivo* is difficult to understand. The channel density in the outer membrane of bacteria is very high (10^{12} pores/cm^2 [10, 21]) and the specific resistance very low (10^{-2} Ω cm^2 in 0.1 M salt). Assuming a specific capacity of 1 μF/cm^2 for the outer membrane, the *RC*-time constant is 10 nsec and a potential difference across this membrane will drop to zero within 1 μsec. Similar considerations apply to the action of Donnan potentials, which were found to be in the order of 20–30 mV across the outer membrane (negative inside) [33]. Therefore, we believe that a voltage-gated pore in the outer membrane of gram-negative bacteria is rather unlikely.

It is still on open question whether one porin trimer contains three pores [21] or only one pore [10]. According to our results, it seems more likely that one porin trimer contains only one pore. In the case of three pores, the diameter of one single pore would be only about 0.8 nm (pore length 7.5 nm, 1 M KCl), and this value seems to be too small to account for the permeability of large sugars through the porin channel. The finding that porin monomers are not able to increase the permeability for sugars, although their structure seems not to be changed [19], seems to support this hypothesis. However, at present it cannot be excluded that the conductance of the porin pore is considerably lower than the size of the channel would predict. In this case, the data presented here would also agree with three channels in one trimer of porin molecules.

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