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Outer-membrane protein PhoE from *Escherichia coli* forms anion-selective pores in lipid-bilayer membranes

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Porin PhoE of the outer membrane of *Escherichia coli* was isolated and purified. Reconstitution experiments with lipid bilayer membranes showed that this protein formed pores which had a single channel conductance of 210 pS at 0.1 M KCl. The PhoE pores were obviously not voltage-controlled or regulated. In contrast to pores formed by the OmpF porin from *E. coli* the PhoE channel was found to be anion-selective at neutral pH. Chloride is about three to ten times more permeable through the pore than alkali ions. On the basis of the observed pH dependence of the permeability ratio of anions and cations, this anionic selectivity is explained by the assumption that the PhoE pore contains an excess of fixed positive charges.

The outer membrane of *Escherichia coli* acts as molecular filter with a defined exclusive limit for hydrophilic substrates. The sieving properties of this molecular filter are determined by a variety of major polypeptides with the common name 'porins' which form hydrophilic channels crossing the outer membrane. To date, the porins which have been described in *E. coli* include the OmpC, OmpF [1–3,8] LamB [4,11], PhoE [1,3,6,7,9] and protein K [5] porins. Each of these has been purified and studied in reconstituted model membrane systems [2–5,8,9,11] although only limited information is available about the PhoE protein pore [3,9]. Reconstitution of the OmpF and OmpC protein into lipid-bilayer membranes shows that these porins form large channels in the membranes [2] which are more permeable to cations than anions.

The PhoE protein is synthesized under conditions of phosphate deprivation and is coregulated with other phosphate-starvation-inducible proteins, including alkaline phosphatase [10]. The gene for PhoE has been cloned and the nucleotide and primary amino acid sequences determined [12,13]. DNA/DNA hybridization studies and sequence comparison have suggested that the PhoE gene bears strong homology with the OmpC and OmpF genes [13,14]. In addition, chemical crosslinking studies have suggested that the PhoE protein, like other *E. coli* porins is a trimer [15]. *In vivo* characterization of cells containing PhoE as the only outer membrane porin has indicated that PhoE has a preference for anions and may contain a phosphate-binding site [6,7]. Therefore, in this study we purified and reconstituted into lipid bilayer membranes, the PhoE protein. The data confirm that the PhoE protein unlike other bacterial porins studied to date (with the exception of protein P from *Pseudomonas aeruginosa* [9]) contains an anion-selective channel.

MATERIALS AND METHODS

Bacterial strains and porin isolation

Strain CGSC6042 (JF 694) [*proC*-24, *ompF*-254, *his*-53, *ompC*-263, *purE*-41, *nmpA*, *ilv*-277, *met*-65, *lacY*-29, *xyl*-14, *rpsL*-97, *cycA*-1, *cycB*-2, *tsx*-63, λ^-], was received separately from Dr B. Bachmann (Coli Genetic Stock Centre, Yale University, New Haven, CT, USA) and from Dr U. Henning (Max-Planck-Institut, Tübingen, FRG). This strain made PhoE protein as its sole major porin. PhoE protein was purified exactly as described for protein P [9] (Fig. 1). In addition some experiments were performed with the osmotic shock fluid obtained from strain CGSC6042 as published previously [16] with the exception that the 100 ml shock fluid obtained from 500 ml culture were used directly after the centrifugation at $100000 \times g$ for 1 h. Protein P was purified from *Pseudomonas aeruginosa* by K. Poole in our laboratory as described previously [9]. The OmpF porin was purified from *Escherichia coli* strain JF701 as described by Nikaido and Rosenberg [3].

Fig. 1. *Sodium dodecyl sulfate/polyacrylamide gel electrophorogram of purified PhoE porin after solubilization at 100 °C for 10 min (lane 1) or 30 °C for 10 min (lane 2).* The apparent relative molecular masses of the monomer (lane 1) and oligomer (lane 2) form of PhoE protein were 38000 and 80000 respectively

Lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously in detail [16]. The apparatus consisted of a teflon chamber with two compartments connected by a small hole (0.1 – 2 mm^2). A membrane was formed across the hole by painting on a 1% (w/v) solution of different lipids dissolved in *n*-decane. Bilayer formation was indicated by the membrane turning optically black to incident light. The temperature was kept at 25 °C, unless indicated otherwise.

All salts, besides 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, Sigma, analytical grade), tetramethylammonium hydroxide and tetraethylammonium hydroxide (Fluka, purum) were obtained from Merck (Darmstadt, FRG, 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 1 mM citrate or 1 mM Tris. Tris and citrate did not have any influence on the results in these concentrations. Aqueous solutions 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 to prevent protein inactivation.

A variety of different lipids were used for membrane formation: Monoolein (Nu Check Prep., Elysian, MN, USA); diphytanoyl glycerophosphocholine (Avanti Biochemicals, Birmingham, AL, USA); brain phosphatidylserine was isolated and purified according to standard methods [17]; oxidized cholesterol was prepared as described earlier [18]. Conductance through the pores was measured after application of a given voltage using a pair of Ag-AgCl electrodes or of calomel electrodes with salt bridges (if no chloride was present in the aqueous solution) inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a preamplifier (Keithley 427) monitored on a storage oscilloscope (Tektronix 5115) and recorded on a strip chart recorder. Zero current potential measurements were performed as described previously [9]. In brief, the membranes were formed in a 10 mM salt solution. After the membrane was in the optically black state the porins were added in such a concentration that a 100 – 1000-fold conductance increase was observed within 20 – 30 min. Then the instrumentation was switched to the measurements of zero current potentials and the salt concentration on one side of the membrane was raised by adding small amounts of concentrated solution. The membrane potential reached its final value within 5 – 10 min.

RESULTS

Macroscopic conductance

When purified PhoE porin in sodium dodecyl sulfate solution was added in small quantities (10 nM) to the aqueous phase bathing a lipid bilayer membrane, the conductance of the bilayer increased by several orders of magnitude. The kinetics of the conductance increase was similar to that described before for other bacterial porins [2,5,8,9], with an initial rapid increase for 15 – 20 min followed by a slower rate of increase which continued until membrane breakage occurred. When the rate of conductance increase was relatively slow (30 min after the membrane turned black), it could be shown that the membrane current was a linear function of the applied voltage for voltages up to 150 mV, suggesting that the PhoE protein conductance units were not voltage gated or voltage regulated in our experimental approach.

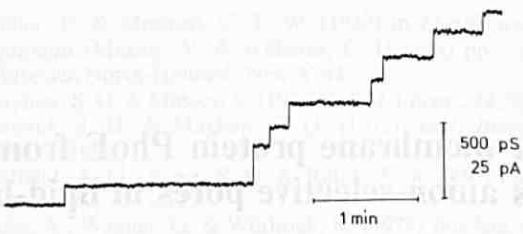

Fig. 2. *Stepwise increase of the membrane current after the addition of PhoE porin.* The aqueous phase contained 0.1 M KH_2PO_4, pH 6, 4 ng/ml PhoE protein and about 40 ng/ml sodium dodecyl sulfate. The membrane was formed from diphytanoyl glycerophosphocholine dissolved in *n*-decane. The applied membrane potential was 50 mV; $t = 25$ °C

As controls we demonstrated that addition of detergent alone in 1000-fold higher concentrations than those used here, caused no substantial rise in membrane conductance. Furthermore we could show that the addition of shock fluid from strain CGSC 6042 to lipid bilayer membranes led to a similar macroscopic conductance to that described above for isolated PhoE porin. 10 – 100 µl shock fluid added to 100 ml 1 M KCl solution were sufficient to increase the specific conductance of the membranes by about three orders of magnitude (from 0.01 – 0.1 µS cm^{-2} to 10 – 100 µS cm^{-2}) without the addition of any detergent. Whereas the membrane conductance was a linear function of the concentration of the isolated PhoE porin in the aqueous phase, we observed a high power of the concentration up to three in the presence of the shock fluid porin. The nature of the porin from the osmotic shock fluid remains still unclear so far. It is interesting to note, however, that bacterial cell cultures and their supernatants also exhibit a strong pore-forming activity.

Single channel experiments

Addition of smaller amounts of PhoE protein (0.1 nM) to lipid bilayer membranes allows resolution of step increases in conductance (Fig. 2). By analogy with other channel experiments, these step conductance increases were assumed to represent the incorporation of single channels into the membrane. Most of the conductance increments were directed upwards and the single channel lifetime was long. Like other porins [2,5,8], these conductance increments were distributed over a 2 – 3-fold range (Fig. 3) with an average conductance in 0.1 M KH_2PO_4 (pH 6.0) of 161 pS. There was no basic difference in the results obtained using purified PhoE protein in sodium dodecyl sulfate solution or using shock fluid from *ompC ompF nmpA* cells as a source of PhoE porin. Incorporation of purified PhoE porin caused the membranes to become noisy, but the average single channel conductances measured for a few different salts were the same for both preparations. The single channel increments observed in the presence of whole cell cultures or their supernatants were generally much larger than those obtained in the presence of the isolated porin or in the presence of the shock fluids. This may be explained in the first case by the insertion of pieces of the outer membrane into the lipid bilayer membrane, which contain several pores.

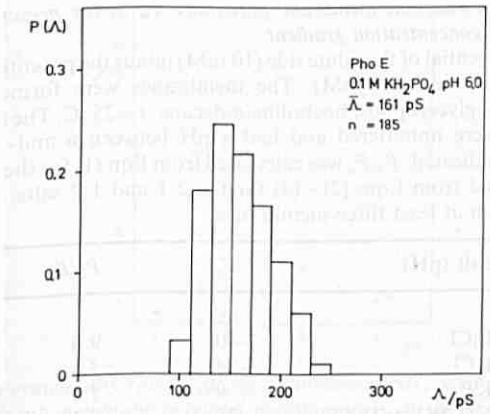

Fig. 3. *Histogram of conductance fluctuations observed with diphytanoyl glycerophosphocholine/n-decane membrane in the presence of PhoE porin.* The aqueous phase contained 0.1 M KH_2PO_4, pH 6. The applied voltage was 50 mV, $t = 25\,^{\circ}C$. The mean value of all conductance fluctuations was 161 pS for 185 single channel events

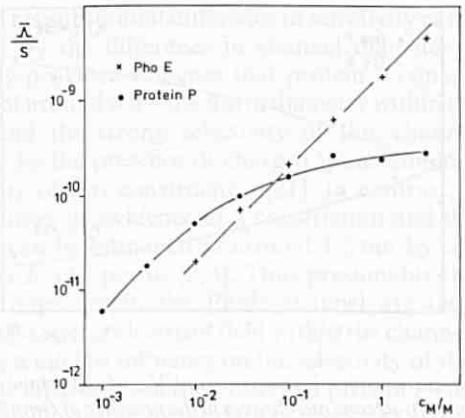

Fig. 4. *Average single channel conductance of PhoE porin from* E. coli *and protein P from* P. aeruginosa *as a function of the aqueous KCl concentration.* The protein concentration was around 10 – 100 ng/ml for both proteins. The membranes were formed from diphytanoyl glycerophosphocholine/*n*-decane. The applied voltage was 50 mV; $t = 25\,^{\circ}C$

Table 1. *Dependence of the average single channel conductance $\bar{\Lambda}$ on the concentration and type of the salt solution*
The aqueous phase contained 5 – 20 ng/ml PhoE protein and less than 0.2 μg/ml sodium dodecyl sulfate. The pH was between 5.5 and 7.0 if not indicated otherwise. Membranes were formed either from diphytanoyl glycerophosphocholine/*n*-decane or from oxidized cholesterol/*n*-decane; $t = 25\,^{\circ}C$. The applied potential was 50 mV. *n* is the number of events from which $\bar{\Lambda}$ was calculated. The standard deviation of the single channel conductances was in all cases around ±15% of the average value

Salt (pH)	*c*	$\bar{\Lambda}$	σ	$10^{-8} \times \bar{\Lambda}/\sigma$	*n*
	M	nS	mS cm^{-1}	cm	
LiCl	1	1.2	71	1.7	435
NaCl	1	1.7	84	2.0	360
KCl	0.01	0.020	1.4	1.4	49
	0.03	0.069	4.6	1.5	178
	0.1	0.21	12	1.8	221
	0.3	0.63	34	1.9	152
	1.0	1.8	112	1.6	245
	3.0	4.6	250	1.8	105
KF	1.0	0.90	76	1.2	85
KBr	1.0	1.9	110	1.7	158
KJ	1.0	2.0	115	1.7	52
NH_4Cl	1.0	1.9	112	1.7	459
RbCl	1.0	1.8	115	1.6	399
CsCl	1.0	1.9	115	1.7	491
KH_2PO_4	0.1	0.16	9.5	1.7	185
(pH 6)	1.0	0.72	43	1.7	45
K_2HPO_4	0.1	0.15	17	0.9	133
(pH 8.2)	1.0	0.69	80	0.9	83
$MgCl_2$	0.5	0.93	64	1.5	37
K_2SO_4	0.5	0.80	76	1.1	139
$MgSO_4$	0.5	0.28	33	0.9	121
Na^+Hepes^- (pH 9.0)	0.5	0.21	18	1.2	63
$Tris^+Cl^-$	0.5	0.41	30	1.4	58
$Tris^+Hepes^-$	0.5	0.079	7.2	1.1	38
$N(C_2H_5)_4^+$ $Hepes^-$	0.5	0.035	4.8	0.7	73

whereas single pores are inserted into the membranes in the presence of isolated PhoE porin or of the shock fluid.

Single channel conductances were measured for a large variety of different salt solutions (Table 1). The standard deviation of the results of the single channel measurements was in all cases around ±15% of the mean value. This indicates that the differences found for the average single channel conductances of the PhoE-pore in the presence of NaCl, KCl, RbCl and CsCl were insignificant. On the other hand, the differences of the single channel conductances found in the presence of LiCl or KF as compared with KCl were significant. Despite the fact that the specific aqueous conductance of these solutions (i.e. the conductance per unit length in the absence of a lipid bilayer membrane) varied by over two orders of magnitude, the ratio of the single channel conductance to the specific aqueous conductance ($\bar{\Lambda}/\sigma$) varied less than threefold. Within these data two trends were observed. Firstly the $\bar{\Lambda}/\sigma$ ratio was generally lower in the presence of divalent anions (0.9 – 1.1 instead of 1.4 – 2.0 for monovalent anions), except in the case of the highly hydrated anion F^-. This could possibly be explained by assuming binding sites in the channel with some affinity for anions. Secondly, very large salts like $N(C_2H_5)^+Hepes^-$ had a lower $\bar{\Lambda}/\sigma$ ratio presumably due to some steric hindrance of the movement of these large ions. As expected for a large water-filled channel, the average single channel conductance was a linear function of the salt concentration (Fig. 4). This indicated that ions exhibited relatively free diffusion within the channel. As a comparison, results for the equivalent phosphate-starvation-inducible outer membrane protein P of *Pseudomonas aeruginosa* are also shown in Fig. 4. This channel, which is anion selective [19] demonstrates saturating single channel conductance with increasing salt concentration.

Zero current membrane potentials

In order to measure directly the selectivity of the PhoE porin, a concentration gradient of salt was established across membranes into which around 100 PhoE pores (corresponding to 10^4 pores/cm^2) had been incorporated. Ions diffused across the channels according to the selectivity properties of these channels until the voltage (V_m) due to the preferential movement of one ion relative to the other balanced the chemical potential gradient of the ions. At this stage the net flow of ions through the channel is zero. As shown in Fig. 5 the zero current membrane potential V_m of membranes doped

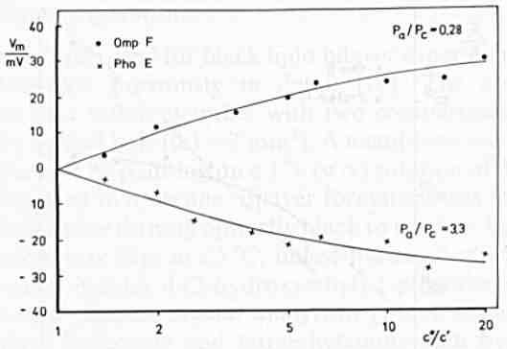

Fig. 5. *Zero-current membrane potential* V_m *of diphytanoyl glycerophosphocholine/*n*-decane membranes in the presence of OmpF and PhoE porins from* E. coli *measured as a function of a KCl gradient across the membrane.* V_m is defined as the difference of the potential at the dilute side (c') minus the potential at the concentrated side (c''). c' was kept at 10 mM. The lines were drawn according to Eqn (1) with the indicated values for P_a/P_c. Results of four different membranes are shown in each case; $t = 25$ °C, pH 6

Table 2. *Zero-current membrane potentials* V_m *in the presence of a 10-fold salt concentration gradient*

V_m is the potential of the dilute side (10 mM) minus the potential of the concentrated side (100 mM). The membranes were formed from diphytanoyl glycerophosphocholine/*n*-decane; $t = 25$ °C. The aqueous solutions were unbuffered and had a pH between 6 and 7 if not otherwise indicated. P_a/P_c was calculated from Eqn (1) for the 1:1 and 2:2 salts and from Eqns (2)–(4) for the 2:1 and 1:2 salts. V_m was derived from at least three membranes

Salt (pH)	V_m	P_a/P_c
	mV	
LiCl	−40	9.0
KCl	−24	3.3
KF	− 9.0	1.6
KH_2PO_4 (pH 4.8)	−35	6.3
KH_2PO_4 (pH 6)	−21	2.9
K_2HPO_4 (pH 8)	− 9.6	2.1
K_2SO_4	−14	3.1
$CaCl_2$	−26	5.6
$MgSO_4$	− 9.4	2.5

with PhoE porin became increasingly negative on the more dilute side (concentration c') of the membrane as increasing salt gradients c''/c' were established across the membrane. This indicated that anions had preferentially moved across the membrane in response to the concentration gradient. The results given in Fig. 5 were analyzed with the Goldman-Hodgkin-Katz equation (full line):

$$V_m = \Psi' - \Psi'' = \frac{RT}{zF} \ln \frac{c''/c' + P_a/P_c}{1 + (P_a c'')/(P_c c')}, \quad (1)$$

where Ψ' and Ψ'' are the potentials at the dilute and concentrated side, respectively. R is the gas constant, T the absolute temperature, F the Faraday constant and z the valency of the z-z salt. The analysis suggested that the PhoE channel exhibited a 3.3-fold preference for Cl^- over K^+, i.e. the ratio of the permeabilities P_a (anion) and P_c (cation) was 3.3. In contrast, purified OmpF porin from *Escherichia coli* was about 3-fold more selective for cations over anions (Fig. 5).

Zero current potentials were also measured for a variety of different salts. The results of these measurements are summarized in Table 2. The ratio of the permeabilities P_a/P_c was calculated from Eqn (1) for the 1:1 and the 2:2 salts. The Goldman-Hodgkin-Katz equation cannot be used for the evaluation of the permeability ratios for salts where the valency of the anion, z_a, is different from that of the cation, z_c. The permeability ratio P_c/P_a may be calculated in this case from the fluxes of the anions and the cations, ϕ_a and ϕ_c, through the channel under zero current conditions:

$$F(z_a\phi_a - z_c\phi_c) = 0, \quad (2)$$

where ϕ_a and ϕ_c are given by

$$\phi_a = P_a z_a u \frac{c_a'' e^{z_a u} - c_a'}{e^{z_a u} - 1}, \quad (3)$$

$$\phi_c = P_c z_c u \frac{c_c'' e^{z_c u} - c_c'}{e^{z_c u} - 1}, \quad (4)$$

c_a'', c_a' and c_c'', c_c' are the concentrations of the anion and the cation on both sides of the membrane, respectively. $u = V_m \cdot F/RT$ is the reduced voltage. The insertion of Eqs (3) and (4) into Eqn (2) leads to a closed solution for V_m in the case of 2:1 salts or 1:2 salts, which was used for the calculation of the permeability ratio P_a/P_c. The results are also summarized in Table 2.

The PhoE channel remained anion-selective for all four different anions tested although clearly the mobility of the anion played an important role since the channel was less permeable to the highly hydrated anion F^- than to the other anion tested. In addition, the higher selectivity for Cl^- measured for the salt LiCl could reflect the low mobility of Li^+ ions in the aqueous phase. It was demonstrated that as the pH was increased from 4.8 to 8 with potassium phosphate solutions, the selectivity for phosphate anions decreased 3-fold. This could be due to a binding site with a preference for $H_2PO_4^-$, over HPO_4^{2-}, which would presumably represent the basis of the anion selectivity. To help distinguish between these possibilities, we studied the effect of pH on selectivity using KCl as the salt. Altering the pH from 3 to 9 caused no change in the average single channel conductance for 0.1 M KCl, suggesting that no gross disturbance of PhoE protein structure occurred in this pH range. In contrast, pH had a substantial effect on selectivity (Fig. 6). Between pH 3 and pH 9, the anion selectivity was reduced over tenfold, with the major alterations in selectivity occurring around pH 3–5 and pH 7–9. At pH 9 the PhoE porin had only extremely low selectivity for anions.

Effect of lipid composition and temperature

In order to test whether the selectivity of the PhoE channel was due to the influence of charged groups outside the channel, we measured the single channel conductance with different lipids. The nature of the lipid did not influence the average single channel conductance (Table 3) although it did influence the rate of reconstitution of channels as described previously for *Salmonella* porins [2, 20]. In addition, changing the temperature between 10 °C and 40 °C did not influence the single channel conductances (Table 3) implying that the mobility of the lipid fatty acyl chains did not influence the permeability properties of the PhoE porin.

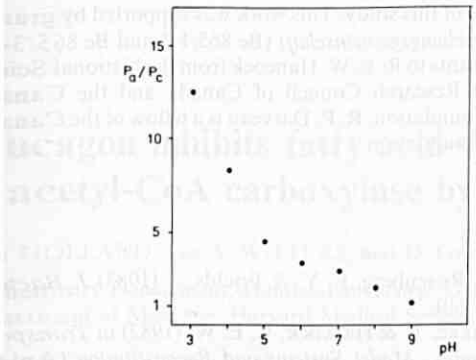

Fig. 6. *Permeability ratio* P_a/P_c *as a function of pH, as obtained from zero-current membrane-potential measurements in the presence of a 10-fold KCl gradient across membranes from diphytanoyl glycerophosphocholine*/n-*decane membranes.* P_a/P_c was calculated from Eqn (1). In addition to the salt, the aqueous phase contained 500 ng/ml PhoE porin and 5 μg/ml sodium dodecyl sulfate. The aqueous solution was buffered with 1 mM citrate at pH 3, 4 and 5 and with 1 mM Tris at pH 9; $t = 25$ °C

Table 3. *Dependence of the average single channel conductance* $\bar{\lambda}$ *on the type of lipid used for membrane formation and on temperature*
The aqueous phase contained 0.1 M KCl, 5 – 50 ng/ml PhoE protein and less than 0.5 μg/ml sodium dodecyl sulfate, pH 6. The transmembrane potential was 50 mV. *n* is the number of events, from which $\bar{\lambda}$ has been calculated

Lipid	*t*	$\bar{\lambda}$	*n*
	°C	nS	
Monoolein	25	0.21	123
Diphytanoyl glycerophosphocholine	10	0.19	105
	25	0.21	221
	40	0.21	227
Oxidized cholesterol	25	0.19	185
Brain phosphatidylserine	25	0.20	89

DISCUSSION

This paper clearly demonstrates that the PhoE protein forms anion-selective channels in lipid bilayer membranes. The results of our conductivity measurements are thus in basic agreement with data obtained from whole cells [1,6,7] and reconstituted vesicles [3]. In the latter model system studies, the authors pointed out considerable theoretical problems in interpretation of their experiments. Thus we decided to study the PhoE protein in lipid bilayers in which interpretation of the experimental results is more straightforward due to the use of simple salt solutions as experimental tools.

Our data clearly demonstrate (Fig. 3) that the PhoE porin contains large water-filled channels with similar dimensions to the OmpC, OmpF, LamB [2] and protein K [5] porins of *Escherichia coli* as judged by their similar conductances in 1 M KCl (1.8 – 2.2 nS). However in strong contrasts to all other bacterial porins [2,3] with the exception of protein P of *Pseudomonas aeruginosa* [9,19] PhoE shows weak selectivity for anions (Fig. 5). Whereas PhoE showed only a 1.2 – 9-fold preference for anions over cations, depending largely on the mobility of the respective anion and cation (Table 2) as well as the pH of the salt solution (Fig. 6), *P. aeruginosa* protein P showed over 100-fold-higher preference for anions over cations. This substantial difference in selectivity can be largely explained by the difference in channel diameter. We have previously provided evidence that protein P contains a constriction of around 0.5 – 0.6 nm in diameter within the channel [19,21] and the strong selectivity of this channel can be explained by the presence of charged lysine amino groups in the vicinity of this construction [21]. In contrast, the PhoE protein shows no evidence of a constriction and the channel diameter can be estimated at around 1.2 nm by comparison with other *E. coli* porins [2,3]. Thus presumably the charged amino groups within the PhoE channel are incapable of generating a strong electrical field within the channel and thus have only a modest influence on the selectivity of the channel. The major differences in the PhoE and protein P channels are graphically illustrated by the data in Fig. 4. As salt concentration is increased in the aqueous phase bathing the bilayer, and presumably within the channel, there is a linear increase in conductance. Such a result would be expected for bulk conductance (i.e. conductance between two electrodes in the absence of a membrane) or for a pore exhibiting relatively 'free' diffusion. In contrast the conductance of the protein P channel is larger than that of the PhoE channel at low salt concentrations due to the high electrical field strength generated by the presumably closely spaced charged amino groups within the protein P channel. With higher salt concentrations, as demonstrated previously [17,19,21], the protein P channel is saturated, a behaviour expected for a channel containing a binding site with a significant influence on conductance. By redrawing the data from Fig. 4 as an Eadie-Hodstee plot, one can calculate a K_m for Cl^- of around 30 – 50 mM.

The molecular state of the porin from the osmotic shock fluid is still not clear. It could be arranged as trimers which are part of small pieces of outer membrane released from the bacteria by the osmotic shock procedure. This possibility is unlikely because of the centrifugation of the shock fluid at high speed for a long time (100000 × *g* for 1 h). On the other hand, the shock porin could also be released from the periplasmic space as has been proposed earlier [16]. The latter possibility is consistent with the finding that the membrane conductance is dependent on high power of the shock fluid concentration (up to third power), which would indicate an association-dissociation equilibrium between monomers and conducting trimers in the membrane. In any case, the close analogy between the pore properties observed in the presence of the shock fluid and of purified protein indicated that the use of a detergent has only little influence if any on the structure of the PhoE pore.

The discussion of whether a porin trimer from *E. coli* outer membrane contains one or three pores has been controversial in the literature [18,22]. Schindler and Rosenbusch [22] proposed that a trimer contains three pores which switch on together at about 100 mV and switch off separately at transmembrane potentials between 150 mV and 200 mV. In our experimental approach the porin pores from *E. coli* were never voltage-regulated [2,16,18,25]. Furthermore, the single channel conductance of a single unit appeared always too small to account for three pores with a diameter which would be large enough for the permeation of solutes with molecular weights up to 600 [25]. Recently, Garavito et al. [23] and Dorset et al. [24] have presented evidence that the OmpF porin of *E. coli* outer membrane is basically one pore with three openings faced to the outside. There exists a close analogy between the sequences of the OmpF and the PhoE porins [13,14] and we would expect a similar structure for both pores. The three openings on the outside of a porin trimer could lead

to an increase of the active diffusion area of the molecular filter properties of the outer membrane.

We also attempted to answer the question as to whether there was a specific binding for phosphate within the PhoE channel, since the PhoE protein is induced under phosphate-deficient conditions [10] and probably has a role in phosphate transport via the inorganic phosphate transport system. The anion binding site seems to have at best a slight preference for phosphate. For example the increase in the selectivity for phosphate over K^+ compared to Cl^- over K^+ was less than 20% at pH 4.8, and no preference for phosphate over Cl^- could be measured at pH 6 or pH 8 (Table 2, Fig. 6). Thus our data do not favour a specific phosphate-binding site within the pore. Presumably, as the diffusing anion becomes larger, the charged amino groups in the channel will have a stronger influence on the relative mobility of the anion through the channel. While we attempted to demonstrate this using pyrophosphate and polyphosphate as anions in selectivity measurements, the data was difficult to interpret theoretically due to uncertainties in the actual charge on the anions. However, we feel that it is unlikely (although, as yet, not excluded) that there is a specific polyphosphate binding site within the channel which does not interact with other multivalent anions. Presumably the channel could be rendered relatively specific to phosphate and polyphosphate by an interaction between the periplasmic phosphate binding protein [26], and the outer membrane PhoE protein. The existence of a high-affinity phosphate binding site on the phosphate-binding protein in close conjunction to the PhoE channel would thermodynamically favour phosphate movement towards the periplasm. Under these circumstances, the binding of phosphate to amino groups on the channel interior would be a potential intermediate step in phosphate uptake.

In order to probe the nature of the groups resulting in anion selectivity we determined the effect of pH on selectivity (Fig. 6). The 2.5-fold change in selectivity between pH 7 and pH 9 could represent deprotonation of lysine amino groups, with the channel becoming virtually non-selective at pH 9, at which pH lysines can be entirely deprotonated. Interestingly, although the PhoE protein has strong homology to the OmpC and OmpF proteins, it has substantially more lysine residues (i.e. 23 as compared to 15 and 18 for OmpC and OmpF, respectively [13, 14]). Possibly one or more of the 'extra' lysine residues are responsible for the anion selectivity of the channel.

An additional fourfold change in selectivity occurred between pH 3 and pH 6. This effect may be due to the effects of carboxyl groups which would become deprotonated in this pH range and would thus inhibit the preference for the movement of anions through the channel. Clearly, in order to fully resolve the molecular nature of the pore interior of PhoE, further experiments are required. With this in mind we are currently attempting to chemically alter the selectivity of the PhoE protein.

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