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The selectivity filter of voltage-dependent channels formed by phosphoporin (PhoE protein) from *E. coli*

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Phosphoporin, an *Escherichia coli* outer membrane-spanning protein re-incorporated in phospholipid planar bilayers generates aqueous channels similar to those of matrix porin. One phosphoporin trimer contains three pores which are induced simultaneously but fluctuate separately between open and closed states. Membrane potential shifts this two-state equilibrium in favour of closed channels. This negative resistance occurs at lower potentials than with matrix porin channels. The phosphoporin channel is poorly anion selective for small solutes. Polyphosphates and other phosphorylated molecules specifically inhibit phosphoporin pore conductance to small ions, a property which is specific to phosphoporin. There is an excellent correlation between the effect of such solutes measured in planar bilayers and their inhibitory effect on β-lactam antibiotic uptake *in vivo* by phosphoporin. It is concluded that the phosphoporin channel contains a selectivity filter which is only efficient for larger molecules, most probably through basic residues.

Key words: phosphoporin/PhoE protein/voltage-dependent channels/selectivity

Introduction

General pore proteins such as the porins (OmpF and OmpC proteins) confer well-defined permeability properties to the outer membrane of *Escherichia coli* (for a review, see Nikaido and Vaara, 1985). These proteins form aqueous channels allowing the passage of solutes to an apparent exclusion size of ~600 daltons. Other porin-like proteins are synthesized under particular growth conditions which induce them. Thus, phosphate starvation causes derepression of the phosphate regulon and hence expression of the products of the *pho* operon, including the *phoE* gene. Its product, the pore-forming PhoE protein (Tommassen and Lugtenberg, 1982), is also called phosphoporin. PhoE protein shares a number of properties with the porins: the homology among the three sequences (Mizuno *et al.*, 1983) is high, the native form of each is a trimer interacting tightly with lipopolysaccharide (LPS), and all act as phage receptors. A comparative permeability study of OmpF, OmpC and PhoE proteins incorporated in liposomes (Nikaido and Rosenberg, 1983) has indicated that negative charge on the solute reduces the diffusion rate through OmpF and OmpC channels while it accelerates it through PhoE channels. In studies using intact cells, Korteland *et al.* (1982) have shown the preference for phosphate and phosphorylated compounds of the PhoE protein and more recently its general anionic selectivity (Korteland *et al.*, 1984). Benz *et al.* (1984) have also reported the anionic selectivity of PhoE protein in black lipid membrane experiments.

In the present study, we report the incorporation of the highly purified PhoE protein and demonstrate its functional reconstitution in planar bilayers. Its pore properties are characterized by negative resistance, and significant effects of phosphorylated compounds on PhoE protein channels are observed. These results allow structure–function relationships to be considered and the channel properties of porin and phosphoporin (OmpF protein and PhoE protein) to be compared.

Results

General pore properties of PhoE protein

Phospholipid vesicles containing purified PhoE protein, upon transformation into planar bilayers, exhibit well-defined conductance properties which display a characteristic stepwise profile. Figure 1 shows successive induction of 1.8–2 nS conductance steps in 1 M NaCl with applied potentials of 80 or 100 mV. In addition, stepwise fluctuations corresponding to one-third of the initiated steps (0.6 ± 0.1 nS at 1 M NaCl) consistently occur as soon as channels have been induced (Figure 1, insert B). The

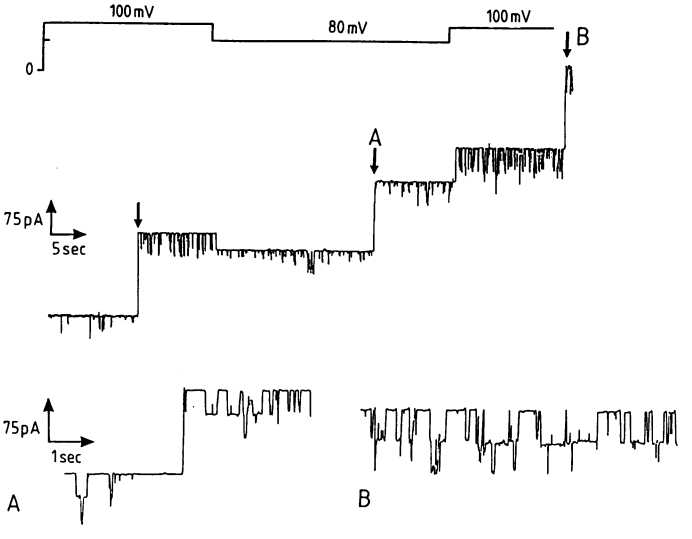

Fig. 1. Successive induction of three PhoE trimers in asolectin planar bilayers at +100 mV or +80 mV membrane potential. Asymmetric planar membranes containing ~30 trimers per bilayer area were formed by apposition of a monolayer containing PhoE trimers (*cis* side) and a pure asolectin phospholipid monolayer (*trans* side). The arrows indicate the three successive inductions of identical conductance steps after application of membrane potential of +80 or 100 mV (relative to the *cis*-compartment). Note the current fluctuation on top of the conductance increments. The bottom two traces show **A** and **B** at an enlarged time scale. **A** shows the induction of the second PhoE trimer (1.8–2 nS conductance); **B** is the current trace after induction of the third PhoE trimer. Stepwise current fluctuation corresponding to about one third of a trimer conductance (0.6 ± 0.1 nS) are clearly resolved. The small pore fluctuations occur immediately after induction of the first trimer. Tris-HCl 10 mM, NaCl 1 M, $MgCl_2$ 10 mM, NaN_3 3 mM, pH 7.4.

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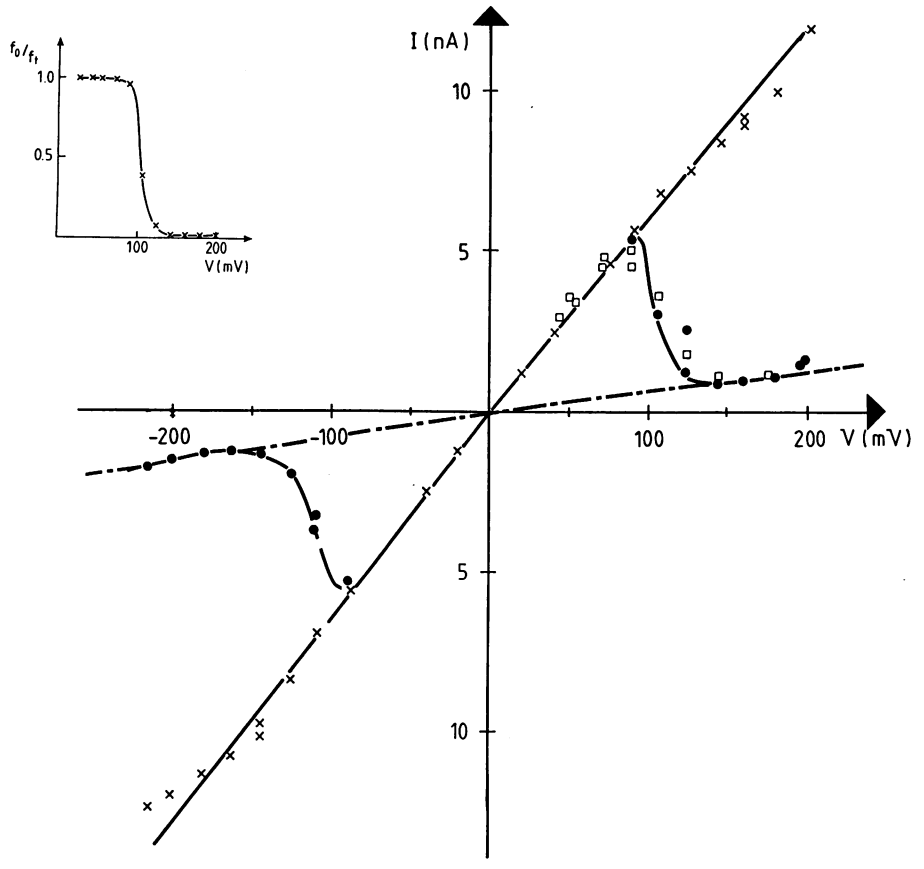

Fig. 2. Current voltage curve of PhoE protein trimers contained in a phospholipid bilayer. The experimental conditions are identical to those in Figure 1. (—x—x—) and (—●—●—): instantaneous current voltage curve and steady-state voltage curve, respectively, after voltage steps from 0 mV up to a given potential; □—□: steady-state current voltage curve after voltage steps from 200 mV down to a given potential. **Insert**: Voltage dependence of the fraction of open pores (fo/ft) at steady-state. The fraction of open pores was calculated from the instantaneous current, and from the steady-state current voltage curve indicated in the main figure, assuming that the residual current (-·-·-) is proportional to membrane potential when all pores are closed.

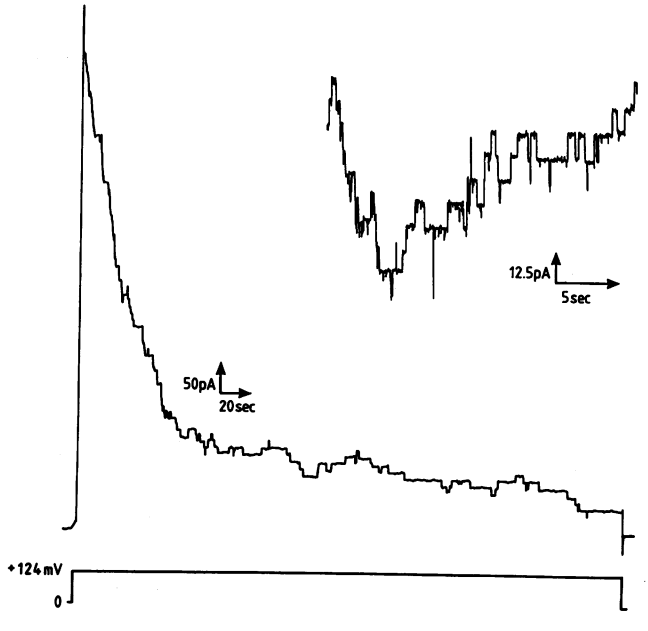

Fig. 3. Closing of PhoE protein pores upon application of a voltage step. After induction of 23 trimers (not shown), a voltage step from 0 to 124 mV is applied. The current decreases in an exponential fashion, reaching a steady-state level after ~3 min. Fluctuations of the 0.06 ± 0.01 nS pores at steady-state are well resolved (see **insert**). Closing of trimers appears to occur exclusively through individual closing of the 0.06 nS pores. Tris-HCl 10 mM, NaCl 0.1 M, $MgCl_2$ 10 mM, NaN_3 3 mM, pH 7.4.

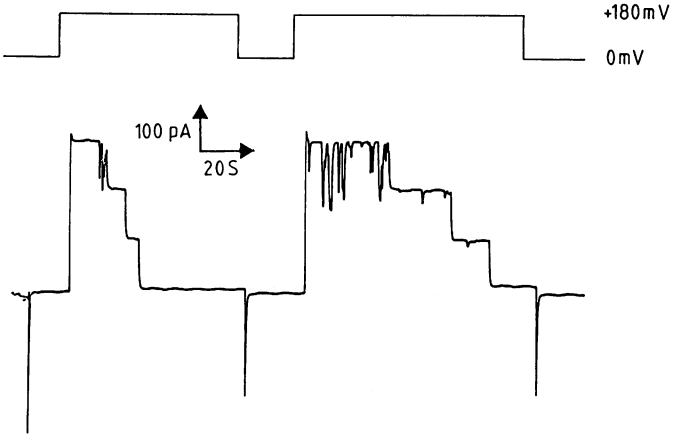

Fig. 4. After induction of one PhoE trimer conductance in a phospholipid planar bilayer, membrane repeated potential steps from 0 to 180 mV were applied. Complete closing of the triplet pore occurred in three identical conducting steps of 0.6 ± 0.1 nS conductance. Note that some residual conductance remains in the closed state. Conditions as in Figure 1.

dominant 2 nS conductance increments may be attributed to the opening of a single PhoE protein trimer for the following reasons: (i) induction of conductance always occurs through 2 nS conducting steps or a multiple thereof; (ii) the optimal number of these 2 nS conductance steps induced at maximal activation corresponds to 80% of the total number of trimers included, on

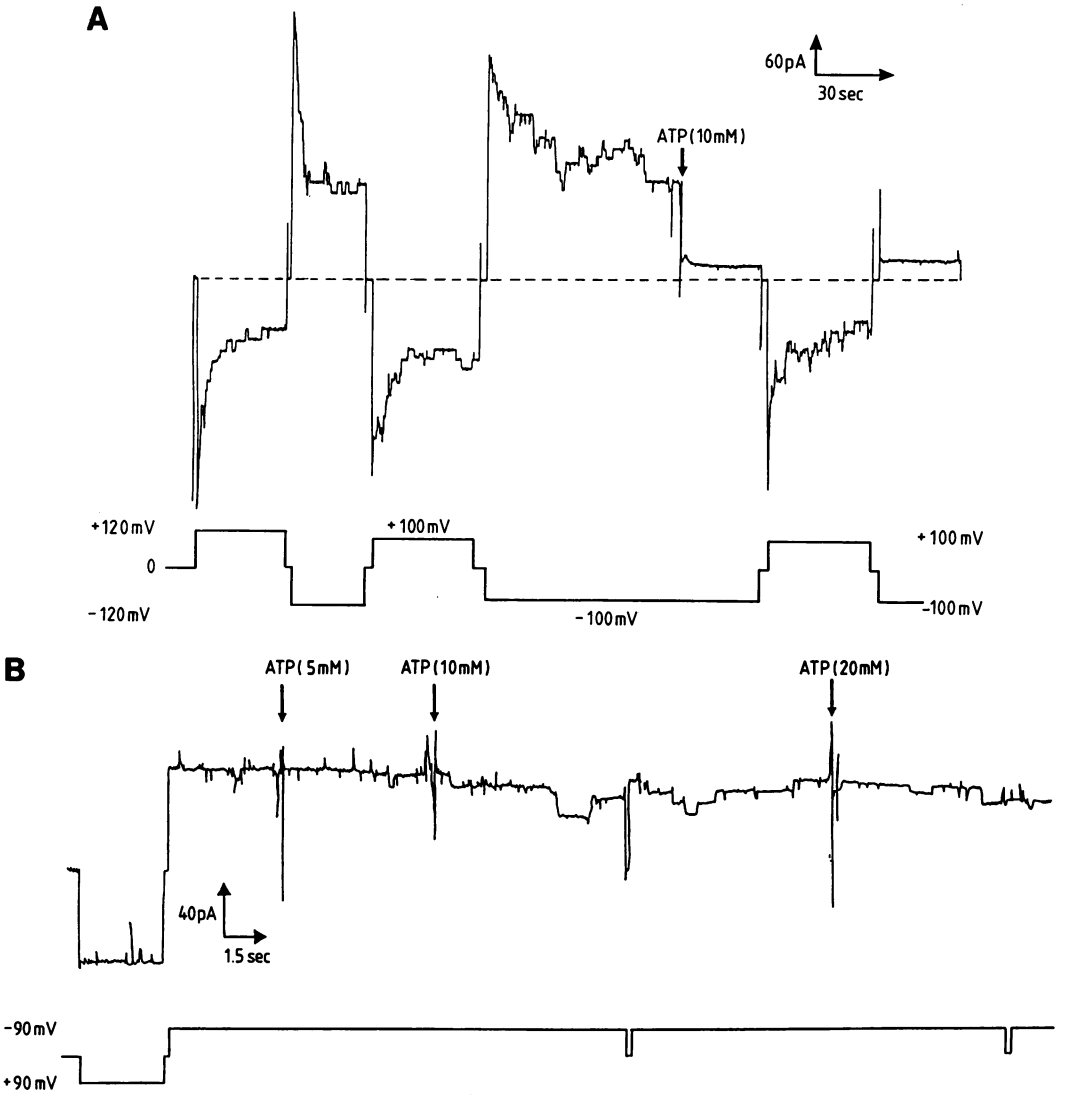

Fig. 5. (**A**) Inhibition of PhoE pore conductance by ATP. The left part of the current trace shows a typically symmetrical response to a voltage step applied across a planar bilayer containing 12 induced trimers. At the time indicated by the arrow, 10 mM ATP (final concentration) was added in the *cis* side at −100 mV applied voltage potential. Note that ATP does not affect the current response at inverse polarity. PhoE trimers were inserted asymmetrically by apposition of a monolayer containing PhoE trimers with a pure asolectin monolayer (Tris-HCl 10 mM, NaCl 0.1 M, $MgCl_2$ 10 mM, NaN_3 3 mM, pH 7.4). (**B**) Control experiment with the unspecific porin. Conditions were as in **A** except that homogeneous OmpF protein trimers substituted for PhoE trimers. The figure also demonstates the significantly lower voltage at which phosphoporin exhibits negative resistance (no inactivation in **panel B**).

average, per bilayer area. Results similar to those obtained with planar phospholipid bilayers containing ~30 trimers (Figure 1) were observed with 300 trimers per bilayer area. Unlike porin (Schindler and Rosenbusch, 1978, 1981), the initiation of channels does not require the application of high voltage across the bilayer nor the presence of lipopolysaccharide (LPS) in the planar bilayer.

The channel conductance of the PhoE protein is voltage-dependent. The steady-state current resulting from the ionic flux across the channel is proportional to the applied voltage up to a value of $V_{in} = \pm$ 100 mV, after which it decreases (Figure 2). This property, the so-called negative resistance, indicates that the pores have a tendency to close at high voltage, a phenomenon which is fully reversible. Indeed, when the voltage is decreased from 200 mV to a lower value, the equilibrium level of conductance is reached again. Figure 3 shows an exponential decay occurring after application of a potential step from 0 to 124 mV and reaching a steady-state consisting of fluctuation between open and closed states of unit step conductance size of 0.06 ± 0.01 nS in 0.1 M NaCl. This negative resistance of the pore demonstrates that one trimer is composed of three pores of identical conductance. The complete closing of one phosphoporin trimer upon application of a high membrane potential always occurs through three steps of 0.6 ± 0.1 nS conductance in 1 M NaCl (Figure 4).

Single conductance measurements were performed either with few trimers within the bilayer area, or during steady-state fluctuations at potentials where negative resistance occurs. As expected for a large water-filled channel, the average single channel conductance of the PhoE protein is strictly proportional to the salt concentration. Thus, the single channel conductance levels in KCl, NaCl and sodium phosphate (0.6 ± 0.05 nS, 0.6 ± 0.01 nS, 0.33 ± 0.02 nS in 1 M KCl, 1 M NaCl and 0.7 M $NaHPO_4$, pH 7.4, respectively) are in fair agreement with the single trimer conductances reported by Benz *et al.* (1984). In order to measure the ionic selectivity of PhoE protein, a 10-fold NaCl gradient (1 M:0.1 M) was established across the bilayer. The zero current potentials of −18.8 ± 0.9 mV obtained indicate a 2.5-fold preference for anion over cation. Similar ex-

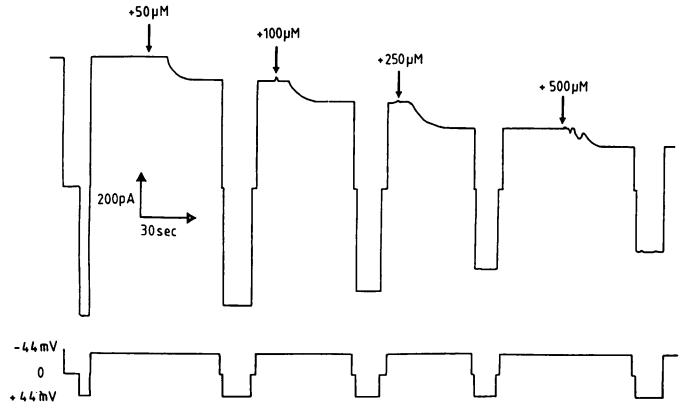

Fig. 6. Inhibition of PhoE pore conductance by increasing amounts of polyphosphate (P_5; cf. Materials and methods). Conditions are those as given in Figure 4. At times indicated (arrows), increasing amounts of polyphosphate type P_5 were injected in the *cis*-compartment. Note that, unlike ATP (Figure 5), polyphosphates P_5 inhibit the current independently of the polarity of membrane potential.

Table I

Phosphorylated compounds	NaCl (M) salt	Inhibition constants (M)	
		Injection in *cis*-compartment	Injection in *trans*-compartment
G6P	0.1	$32 \pm 3 \times 10^{-2}$	ND
AMP	0.1	$21 \pm 2 \times 10^{-2}$	ND
ADP	0.1	$34 \pm 2 \times 10^{-3}$	ND
ATP	0.1	$2 \pm 0.1 \times 10^{-3}$	$2.2 \pm 0.1 \times 10^{-3}$[a]
	1	$21 \pm 2 \times 10^{-3}$	ND
Polyphosphates type P_5	0.1	$3.4 \pm 0.3 \times 10^{-4}$	$3.2 \pm 0.3 \times 10^{-4}$
Polyphosphates type P_{15}	0.1	$1.7 \pm 0.1 \times 10^{-4}$	$1.5 \pm 0.1 \times 10^{-4}$

[a]At inverse polarity.

periments performed with the OmpF protein give the opposite selectivity: 2.5-fold preference for cation over anion. Selectivity measurements determined according to an alternative method described by Raymond *et al.* (1985) gave identical values (data not shown).

Identical channel characteristics of the PhoE protein (single conductance, size, fluctuations, voltage dependence) have been observed when, instead of incorporating the protein through vesicle spreading and monolayer self-assembly, the PhoE protein was injected under agitation in solubilized form in the presence of a detergent (octyl-POE) to the solution compartment of a preformed bilayer.

Inhibition of PhoE protein channels by phosphorylated compounds

Overbeeke and Lugtenberg (1982) reported a preference for P_i and P_i-related compounds of PhoE protein in intact cells. We have investigated this using PhoE protein reconstituted asymmetrically into phospholipid planar bilayers. Under such experimental conditions, the orientation of the protein in the bilayer should be unique (see Schindler and Quast, 1980). In the absence of a phosphate-containing compound, PhoE trimers respond symmetrically to 120 mV (or 100 mV) potential steps. The injection of 10 mM ATP (final concentration) in the *cis*-compartment at −100 mV membrane potential leads to an immediate and drastic drop in the ionic current crossing the bilayer (Figure 5A). Upon reversal of the applied potential, current intensities and current relaxation were identical to those in the absence of ATP.

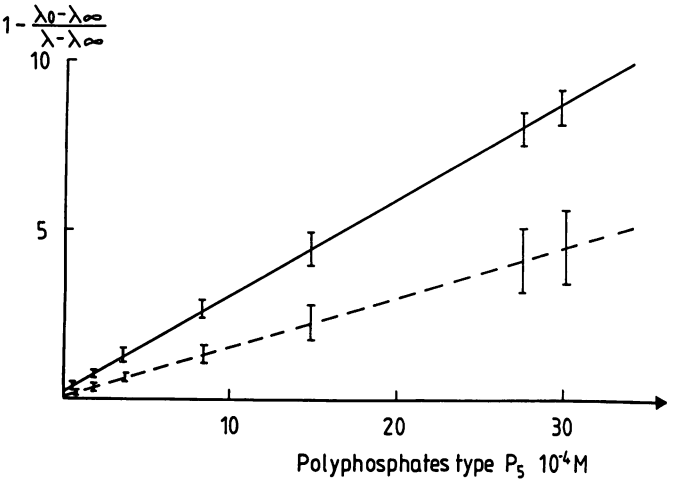

Fig. 7. Determination of the apparent inhibition constants of the polyphosphates type P_{15}. The residual conductance is plotted versus the concentration of polyphosphates. In the expression of residual conductance, λ is the conductance at a given polyphosphate concentration, and λ_0 and λ_∞ are the conductances at zero and infinite polyphosphate concentration (maximal inhibition) respectively. A linear plot is expected for a single class of independent binding sites.

The injection of ATP in the *trans*-compartment inhibits the current in a similar way, but only if the polarity of the applied voltage is reversed. This effect of ATP is unique to the PhoE pore protein: as is shown in Figure 5B, ATP up to a final concentration of 20 mM does not affect the conductance of porin. Other phosphorylated compounds such as AMP, ADP, glucose-6-phosphate and polyphosphates of various size (P_5 and P_{15}; see Materials and methods) yield similar results. As shown in Figure 6, stepwise increase of polyphosphate (P_5) concentration in the *cis*-compartment leads to a gradual decrease in the current. Note that at the applied potential of 44 mV, the membrane conductance is affected by polyphosphate compounds irrespective of the direction of the potential. Apparent inhibition constants, listed in Table I, were calculated (Woodhull, 1973) by plotting the residual conductance as a function of the concentration of phosphorylated molecules (Figure 7). With increasing state of phosphorylation of the adenosine moiety, the inhibiting efficiency increased drastically. The effect of polyphosphates, irrespective of size, is 10 times larger than that of ATP under identical conditions. As found with ATP, the injection of P_i-related compounds either in the *cis*- or in the *trans*-compartment gives identical results. A study of the effect of salt concentration showed that increasing NaCl concentrations from 0.1 M to 1 M results in proportional decreases in the apparent affinity of ATP for the phosphoporin channel (Table I), suggesting competition between salt ions and ATP within the channel. The inhibition of pore conductance by ATP is enhanced by increasing the membrane potential with its sign favouring electrophoresis of ATP molecules through the membrane. In 1 M NaCl at 80 mV, the inhibition is twice as effective as at 50 mV.

The existence of a binding site for P_i and P_i-related compounds within the PhoE channel has been postulated on the basis of *in vivo* experiments. In our study the linearity of residual conductance shown in Figure 7 is consistent with a single class of binding sites in the PhoE protein. For quantitative description, equilibrium dialysis binding experiments were carried out with ^{14}C-labelled ATP, with the PhoE protein either in a soluble state (as a protein−detergent micelle complex), or re-incorporated in phospholipid vesicles. Under the experimental conditions used,

a binding site with an apparent dissociation constant as high as 10 mM would have been detected. However, no ATP binding was observed with the PhoE protein either in its soluble or lipid-bound form; neither were the spectroscopic properties of this protein influenced by the presence or absence of polyphosphates P_5 or ATP.

Discussion

The general pore properties of phosphoporin (PhoE protein) are related in many respects to those of the unspecific porin, as expected from a protein with a high degree of sequence homology. Thus, the smallest activatable conducting step (1.8–2 nS in 1 M NaCl) corresponds to the induction of one trimer of PhoE protein, analogous to the finding with the OmpF porin (Schindler and Rosenbusch, 1978, 1981). This resemblance includes the observation that one trimer contains three pores of comparable conductance (0.6 ± 0.1 nS in 1 M NaCl) which are voltage-dependent and close reversibly at high potential. The stoichiometries, found by conductance measurements are in good agreement with the low resolution structure deduced from two-dimensional crystals of OmpF protein (Engel *et al.*, 1985) and PhoE protein (unpublished). Also similar to the unspecific porin (Schindler and Rosenbusch, 1981), the voltage dependence is linearly related to the ionic strength and exhibits a slight, yet distinct dependence on the state of aggregation of the trimers in the bilayer (data not shown). Although protein-containing vesicles were added asymmetrically in the *cis*-compartment, PhoE protein pores responded perfectly symmetrically to the membrane potential. The following quantitative dissimilarities were revealed upon close examination of the channel properties. Induction of phosphoporin trimers in the bilayer does not require the application of high potential. Negative resistance occurs at a significantly lower voltage than for porin. Conductance fluctuations (0.6 ± 0.1 nS in 1 M NaCl) are observed frequently and are clearly visible at 80 mV. As reported recently by Benz *et al.* (1984), the PhoE protein pore exhibits a 2.5-fold preference for small anions over small cations unlike the OmpF protein which is cation-selective (2.5-fold). Moreover, the bacterial LPS did not exhibit an effect on phosphoporin channel induction and properties. Since this glycolipid binds very tightly to phosphoporin, contamination of the protein may be responsible for the apparent absence of the requirement of LPS for pore activity.

Diffusion of small ions through PhoE protein pores is specifically inhibited by polyphosphate and related molecules, a property not observed with the unspecific porin. There is an excellent correlation between the inhibiting capacities of phosphorylated molecules measured with artificial planar bilayers and the capacities of such molecules to inhibit β-lactam antibiotics uptake by *E. coli* cells, as reported by Overbeeke and Lugtenberg (1982). In the *in vitro* study, increasing the number of phosphate groups in the adenosine moiety resulted in a stronger inhibition of the ionic current flowing through the PhoE protein channels. In view of the high sequence homology between PhoE protein and the unspecific OmpF and OmpC porins (65% strict homology, 85% conserved amino acid residues), this functional difference is striking. In this context, it is noteworthy that the PhoE sequence contains eight positively charged di- and tripeptides (such as Arg–Lys, Lys–Lys and Arg–X–Lys). Of these, four are unique to the PhoE sequence in comparison with the OmpF sequence. In addition, three basic residues are found in the segment between residues 29 and 65 of the PhoE protein. This would be compatible with experimental evidence that the amino-terminal domain is involved in the anion specificity of the PhoE protein pore as it was postulated by Tommassen *et al.* (1984, 1985) on the basis of different OmpF–PhoE and OmpC–PhoE hybrid proteins.

Our observations suggest specific interactions of the negatively charged solute with basic residues within the channel. Our failure to detect a binding site for ATP in the PhoE protein does not contradict this hypothesis. Although analogies with respect to channel functioning and enzymatic catalysis have been drawn (Läuger, 1973; Latorre and Miller, 1983), channel selectivity should be discussed in terms of channel occupancy time and energy profiles rather than in terms of binding sites, as they are found in active sites of enzyme (Eisenman and Horn, 1983). With ions of the size of ATP or polyphosphate P_5, part of the pore lumen may act as a 'selective filter' as defined by Bezanilla and Armstrong (1972) and by Hille (1973). These selective filters may be viewed as the narrowest part of the channel able to dehydrate permeant ions partially or totally. Due to the pore size (1–2 nm) of the phosphoporin channel, the apparent selectivity may exert its effect only on large ions. This could explain the poor anion selectivity of the phosphoporin channel in NaCl or KCl or conducting salts. Small ions move inside the pore almost as fast as in water (Benz *et al.*, 1985).

The inhibition of pore conductance by phosphorylated molecules should not be considered as blocking effect. Upon diffusion within the lumen of the pore, the phosphorylated molecules presumably interact with positively charged residues such as arginine or lysine of the channel wall. As long as these molecules reside within the channel, Na^+ and Cl^- may be hindered in diffusing across the pores, leading to an apparent block of channel conductance. The current generated by the phosphorylated molecules themselves is likely to be negligible as compared with their inhibitory effect on Na^+ and Cl^- currents. The positively charged residues appear accessible from both sides of the membrane, even though incorporation of the PhoE protein was asymmetrical, since injection in either the *cis* or the *trans* side of the asymmetric bilayer yielded identical results. A full understanding of the influence of membrane potential on the inhibiting effect of ATP and relative molecules requires a more quantitative study. Most probably the electrical field built up by the membrane potential has a synergetic or antagonistic effect on the concentration gradient of the negatively charged solutes across the membrane.

Determination of inhibition constants by conductance measurements as a function of size, charge and the chemical nature of the transported species, should provide a very precise tool for determining the degree of specificity of the PhoE protein pore. Moreover, the availability of PhoE protein modified chemically (Page and Rosenbusch, 1986) or by genetic engineering will make investigations of this pore structure on a molecular level a challenging goal.

Materials and methods

Protein preparation and chemicals

PhoE protein was extracted and purified to homogeneity from *E. coli* CE 1197 (kindly supplied by B.Lugtenberg) using the non-ionic detergent octyl-POE (Garavito and Rosenbusch, 1985) in a manner similar to that described for porin and for maltoporin (Neuhaus *et al.*, 1983). ATP, AMP, ADP and polyphosphates were purchased from Sigma.

Vesicle formation

Phospholipid vesicles were made using soy bean lecithin (Sigma Type IV) as described by Schindler and Quast (1980). Proteoliposomes were reconstituted from phospholipids and solubilized homogeneous PhoE protein by detergent dialysis (Dorset *et al.*, 1983). The molar protein–lipid trimer ratio was 10^{-6}. Proteoliposomes were further diluted with phospholipid vesicles to give final molar ratios of 10^{-8} and 10^{-9} corresponding to 300 and 30 trimers per bilayer area.

Formation of the bilayer and electrical measurements

Phospholipid vesicles and vesicles containing PhoE protein were transformed into planar membranes via monolayer self-assembly as described by Schindler (1980). The bilayers were formed over a circular aperture (2×10^{-4} cm^2). Electrical measurements and the criteria for bilayer formation and characteristics were as described by Schindler (1980). The sign of electrical potentials always refers to the *cis*-compartment to which protein-containing vesicles were added. Ag/AgCl electrodes, inserted in the aqueous compartments on both sides of the bilayer, were used in experiments performed with chloride salts. V_m current membrane potentials were measured using calomel electrodes, as described by Raymond *et al.* (1985). All experiments were carried out at room temperature with 10 mM Tris-acetate (pH 7.4) containing 5 mM $CaCl_2$ or 5 mM $MgCl_2$ and 3 mM NaN_3 in addition to various salt concentrations.

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