Biochimica et Biophysica Acta 860 (1986) 699–707
Elsevier

BBA 73289

Demonstration and chemical modification of a specific phosphate binding site in the phosphate-starvation-inducible outer membrane porin protein P of *Pseudomonas aeruginosa*

Robert E.W. Hancock [a] and Roland Benz [b]

[a] *Department of Microbiology, University of British Columbia, Vancouver, British Columbia, V6T 1W5 (Canada) and* [b] *Lehrstuhl für Biotechnogie, Universität Würzburg, D-8700 Würzburg (F.R.G.)*

(Received 1 April 1986)

Key words: Porin; Protein P; Phosphate binding site; Membrane reconstitution; Anion channel; (*P. aeruginosa*)

The interaction of phosphate ions with the *Pseudomonas aeruginosa* anion-specific protein P channel was probed. The single-channel conductance of protein P incorporated into planar lipid bilayer membranes in the presence of 0.3 M $H_2PO_4^-$ was shown to be 6.0 pS, demonstrating that protein P channels allowed the permeation of phosphate. When large numbers of protein P channels were incorporated into lipid bilayer membranes in the presence of 40 mM Cl^-, addition of small concentrations of phosphate resulted in reduction of macroscopic Cl^- conductance in a dose- (and pH-) dependent fashion. This allowed calculation of an I_{50} value of e.g. 0.46 mM at pH 7.0, suggesting that the affinity of protein P for its normal substrate phosphate was at least 60–100-fold greater than the affinity of the channel for other ions such as chloride. Pyrophosphate and the phosphate analogue, arsenate, also inhibited macroscopic Cl^- conductance through protein P with I_{50} values at pH 7.0 of 4.9 mM and 1.3 mM, respectively. To probe the nature of the phosphate binding site, the ε-amino groups of available lysine residues of protein P were chemically modified. Acetylation and carbamylation which produced uncharged, modified lysines destroyed both the anion (e.g. Cl^-) binding site and the phosphate binding site as determined by single-channel experiments and macroscopic conductance inhibition experiments respectively. Nevertheless, the modified proteins still retained their trimeric configuration and their ability to reconstitute single channels in lipid bilayer membranes. Methylation, which allowed retention of the charge on the modified lysine residues, increased the K_d of the channel for Cl^- 33-fold and the I_{50} for phosphate inhibition of macroscopic Cl^- conductance 2.5–4-fold. A molecular model for the phosphate binding site of the protein P channel is presented.

Introduction

The outer membrane of Gram-negative bacteria constitutes a size-dependent permeability barrier. The components which determine the properties of this barrier are a class of proteins termed porins [1] which generally form defined, water-filled channels with weak ion selectivity [2]. Thus the permeation of small hydrophilic molecules below a given size, the exclusion limit of the porin protein, occurs by a simple diffusion mechanism [1,2]. To date, only two well-defined exceptions to this general scheme are known, the maltose/maltodextrin porin LamB of *Eschericha coli* [3] and the anion-specific protein P channel of *Pseudomonas aeruginosa* [4–6].

Upon growth of *Pseudomonas aeruginosa* in low concentrations of phosphate (0.2 mM or less), we previously demonstrated that a new major outer membrane protein P of monomer M_r 48 000,

Correspondence address: Department of Microbiology, University of British Columbia, Vancouver, BC, V6T 1W5, Canada.

is induced [4]. Chemical crosslinking experiments suggested that in its native state protein P, like other porins, is a trimer [7]. The protein is coregulated with the components of a high-affinity phosphate transport system, as demonstrated by physiological and mutant studies [4,8,9], and has a role in high-affinity phosphate transport as confirmed by the isolation of a Tn501 insertion sequence mutant lacking protein P and deficient in phosphate transport [10].

When purified and reconstituted into black lipid bilayer membranes [4,6], protein P forms well-defined channels with a considerably smaller single-channel conductance in 1 M salt solutions (e.g. 250 pS in 1 M KCl) than other porin proteins (usually larger than 1.5 nS). Ion selectivity and other measurements demonstrated that this protein is more than 100-fold selective for anions over cations [5] due to the presence of an anion binding site (K_d for $Cl^- = 40$ mM) within the channel [5,6] involving lysine ϵ-amino groups [6]. This high selectivity for anions allowed an estimation of the effective diameter of the channel, 0.6 nm, based on the permeability of the channel for anions of different sizes [5].

In this paper we have utilized the ability of phosphate to block chloride movement through the channel to demonstrate that protein P has a binding site for phosphate ions with an affinity at least 80-100-fold higher than the affinity of protein P for chloride or other ions. Chemical modifications of lysines suggested that they are involved in this phosphate binding site.

Methods

Purification of Protein P. A modification of our previously-described method [4] allowed purification of protein P in a single column run. Outer membranes of *P. aeruginosa* strain H103, grown under phosphate-deficient conditions, were isolated as previously described [4]. They were then suspended by extensive sonication in 2% Triton X-100, 10 mM Tris-HCl (pH 8.0) at a protein concentration of 10 mg/ml, followed by centrifugation at 180 000 × *g* for 1 h. The supernatant was discarded and the above cycle of suspension in Triton/Tris and centrifugation repeated twice. The sonication steps (30 s at the highest setting of a Fisher sonic dismembranator Model 300 (Fisher Scientific Ltd., Vancouver) were found to be necessary to prevent subsequent contamination of protein P with porin protein F. The pellet after the third centrifugation was resuspended in 2% Triton X-100, 10 mM sodium EDTA, 20 mM Tris-HCl (pH 8) with the aid of a syringe and 21 gauge needle. This suspension was not sonicated. The supernatant after centrifugation for 2 h at 180 000 × *g* was applied to a DEAE-Sephacel column (1.6 × 10 cm) which had been pre-equilibrated with 0.1% Triton X-100, 10 mM EDTA, 20 mM Tris-HCl (pH 8) (column buffer). The column was eluted with 2 column volumes of column buffer followed by 4 volumes of column buffer containing 0.1 M NaCl. Fractions containing almost pure protein P were eluted after approximately 2 column volumes of the salt wash.

Bilayer experiments. The basic apparatus consisted of a teflon chamber separated into two compartments by a thin teflon divider containing a small hole (0.1 mm^2 for single-channel measurements, 2 mm^2 for all other measurements). Calomel electrodes dipped into the solutions on either side of the teflon divider, and the two compartments could be stirred using small round magnetic stir bars that were rotated by a revolving magnet mounted under the Faraday chamber in which the teflon chamber was located. A membrane was painted across the hole in the teflon divider by wiping a solution of either 1% oxidized cholesterol or 1% diphytanoyl phosphatidylcholine in *n*-decane over the area of the hole. The results of lipid bilayer experiments were not influenced by the composition of the membrane, as determined in control experiments, although more rapid reconstitution rates were obtained with oxidized cholesterol. Experiments were initiated when the lipid in the hole thinned out and turned optically black to incident light, indicating bilayer formation.

For a single-channel measurements, one electrode was connected to a millivolt voltage source. The other was connected to a Keithley 427 amplifier to boost the output 10^{10}-fold, a Tektronik 5103N/5A22N storage oscilloscope to monitor the amplified output, and a Rikadenki R-01 strip chart recorded. For macroscopic conductance inhibition experiments and zero-current potential measurements, one electrode was again connected to a

voltage source while the other was connected to a Keithley 610B electrometer. In all cases the circuits were completed through earth. Macroscopic conductance inhibition experiments were initiated by adding detergent-purified protein P to the bathing solutions (usually 40 mM KCl/1 mM Tris-HCl (pH 7.0)) on either side of the lipid bilayer membrane. The increase in conductance (measured at current increase) was followed for 20–30 min or until the rate of increase had slowed down considerably. At this time the membrane conductance had generally increased 2–4 orders of magnitude. The bathing solutions in both compartments of the chamber were stirred gently (approx. 60 rev./min) with a magnetic stir bar and aliquots of inhibitor added carefully with Eppendorf pipettors to both compartments. Sufficient time (usually 30–90 s) was allowed for the new current level to be established before addition of the next aliquots.

For zero-current potential measurements, experiments were initiated exactly as described above for macroscopic conductance inhibition experiments using a bathing salt solution of 50 mM KCl. After the membrane conductance had increased two orders of magnitude, the applied voltage was turned off and the Keithley 610 electrometer switched to measure voltages. Aliquots (100 μl) of 3 M KCl solution were added to the compartment on one side of the membrane (the concentrated side) and equal aliquots of 50 mM KCl added at the same time to the other side. The solutions in the two compartments were stirred (approx. 120 rev./min) to allow relatively rapid equilibration. In these experiments, the concentration gradient of KCl across the membrane provided a chemical potential which was a driving force for ion movement. Ions then diffused across the porin channels according to the ion selectivity characteristics of the channel until the opposing electrical potential created by the preferential movement of one of the ions balanced the chemical potential. At this stage the zero-current potential was measured and fitted to the Goldman-Hodgkin-Katz equation [2] to determine the relative permeabilities of anions (P_a) and cations (P_c).

Chemical modifications of protein P. All methods were slight modifications of previously published procedures. The alterations were designed to allow smaller amounts of protein to be chemically modified due to the limited amount of protein available and the rather small amounts required for bilayer analyses, however, the relative concentrations of all chemicals were exactly as described in the published procedures.

Reductive methylation was performed as described by Jentoft and Dearborn [11]. Stock solutions of 100 mM sodium cyanoborohydride (A), 20 mM formaldehyde (B), 40 mM $NiCl_2 \cdot 6H_2O$ (C) and 1 M sodium Hepes (pH 7.5) (D) were prepared. 25 μl of each of A and B were added to 12.5 μl of each C and D and 175 μl of protein P (1.2 mg/ml in 0.2% Triton X-100, 2 mM Tris-HCl (pH 8)), and the resulting solution incubated for 2 h at 22°C. After this period, the solution was dialysed against 1000 volumes of 2 mM Hepes buffer (pH 7.5) for 4 h followed by a further 20 h dialysis against 1000 volumes of fresh 2 mM Hepes buffer (pH 7.5).

Carbamylation was performed as described by Plapp et al. [12] using 0.2 M KOCN as recommended by Stark [13]. 150 μl of the above protein P solution were added to 150 μl of 2 M triethanolamine-HCl buffer (pH 8) and the reaction started by the addition of 4.9 mg of solid potassium cyanate. The mixture was then incubated for 4 h at 37°C, followed by exhaustive dialysis against 10 mM Tris-HCl (pH 8) to stop the reaction. Acetylation was performed by Keith Poole, University of British Columbia, exactly as described previously [6]. Trinitrophenylation using trinitrobenzenesulphonic acid was performed as described by Fields [14] except that the amounts of all solutions added were adjusted such that the final volume after addition of solution D was 1.51 ml.

Results

Single-channel conductance measurements in the presence of phosphate

Addition of small amounts (10^{-13}M) of protein P to one or both sides of a lipid bilayer membrane in the presence of 0.3 M $H_2PO_4^-$ (potassium salt) caused stepwise increases in conductance (Fig. 1) with an average single-channel conductance (for 299 measured single-channel events) of 6.02 pS. This led to somewhat of a dilemna since the maximal conductance of chloride at a similar pH

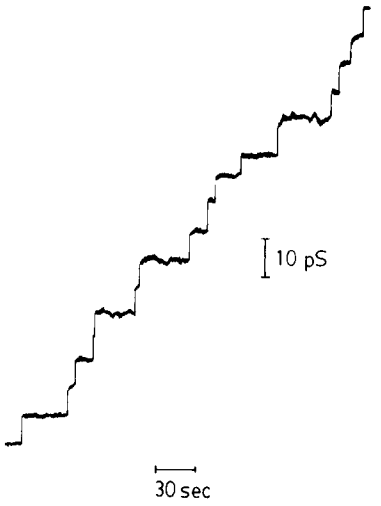

Fig. 1. Stepwise increases in conductance of lipid bilayer membranes after addition to 10^{-13}M protein P to the aqueous phase (1M KH_2PO_4) bathing the membrane. The membrane was made from 1% diphytanoylphosphatidylcholine in *n*-decane; the applied voltage was 50 mV.

was 400 pS, more than 60-fold greater than the maximal phosphate conductance, and yet this channel had a role in phosphate uptake [10]. Attempts to perform single-channel measurements in the presence of equal concentrations of phosphate and chloride (either at 1 M or 0.1 M) gave rise to extremely noisy current records with the only defined single-channel jumps being of the order of 10 pS (data not shown). The only conductance increases approaching the 400 pS expected if only chloride was present, appeared as spikes which rapidly relaxed to baseline or to a new level of around 10 pS. This suggested to us that phosphate was severly inhibiting the movement of chloride through protein P channels. To investigate this further we performed macroscopic conductance inhibition experiments.

Inhibition of chloride flux by phosphate

Large bilayer membranes (2 mm^2) were formed in the presence of 40 mM KCl buffered to the appropriate pH with a low concentration of potassium phosphate (at pH 4 or 6) or Tris-HCl (at pH 7 or 8). Protein P was added to one or both sides of a black lipid bilayer membrane, and the conductance started to rise rapidly for 5–20 minutes and thereafter continued to rise at a decreasing rate (Fig. 2). When the rate of macroscopic conductance increase was quite slow, the membrane conductance represented more than 100 channels incorporated into the membrane. At this time aliquots of phosphate were added to each chamber and the conductance decreased to a new level over a period of about 30–90 s (Fig. 2).

There was an apparent inverse hyperbolic relationship between the amount of phosphate added and the percent decrease in conductance (Fig. 3). From these data, an I_{50} value (concentration of inhibitor giving 50% inhibition) could be obtained using a Dixon plot (Fig. 3, inset). This suggested that phosphate and chloride competed for the same site on protein P (since the lines formed at two different levels of incorporated protein P intercepted above the X axis at a negative value). In

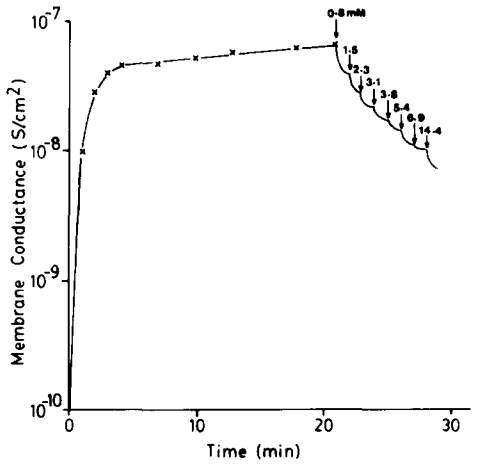

Fig. 2. An example of a macroscopic conductance inhibition experiment. At time 0, after the membrane had turned black, protein P was added to a final concentration of 10^{-10}M to the salt solution (40 mM KCl/0.1 mM potassium phosphate buffer (pH 6.0)). The increase in membrane conductance due to insertion of porin pores was followed until the rate of increase had slowed (in this case 20 min). At this time aliquots of concentrated potassium phosphate buffer (pH 6) (0.1 M or 1 M) were added to the bathing salt solutions (volume 6 ml) in both compartments of the lipid bilayer chamber (i.e., to each side of the membrane) and stirred as described in Methods until the conductance stabilized (in this case 1 min). After a stable conductance level was achieved additional aliquots were added to each side of the membrane. The arrows indicate the time of addition of phosphate solution to the indicated cumulative final concentration in mM.

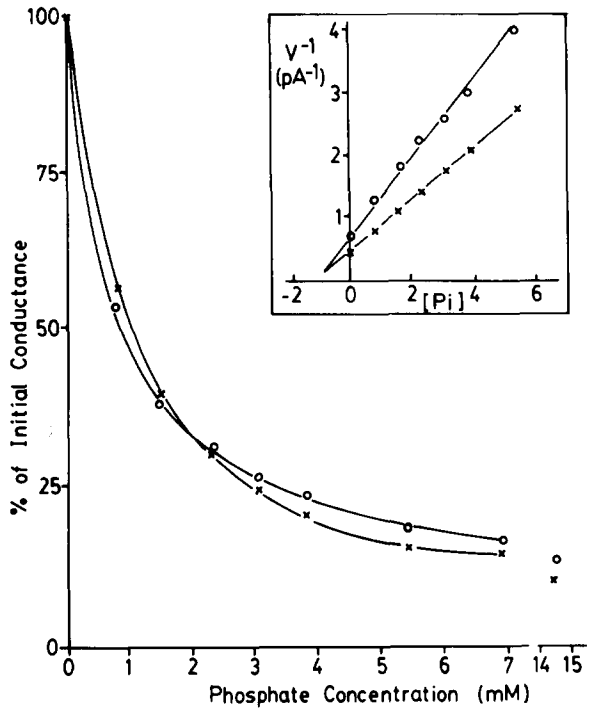

Fig. 3. Kinetics of phosphate inhibition of macroscopic chloride conductance. The crosses are data taken from the experiment described in Fig. 2, the circles represent another experiment. In the insert Dixon plot, the inverse of the measured membrane current (at 20 mV applied voltage) was graphed against the cumulative phosphate concentration in mM.

TABLE I

INHIBITION CONSTANTS (I_{50}) FOR THE INHIBITION OF MACROSCOPIC CONDUCTANCE IN THE PRESENCE OF 40 mM KCl

The experiments were performed as described in Methods and the I_{50} values determined as described in Results and in the legend to Fig. 3. The pH of the 40 mM KCl solution was established by the addition of 0.1 mM potassium phosphate (pH 4.05 or pH 6.0) or 1 mM Tris-HCl (pH 7.0 or 8.0), respectively, for the above-described experiments at pH 4.05, 6.0, 7.0 or 8.0. The pH of the phosphate solutions was established by appropriate mixing of monobasic and dibasic potassium phosphate; the pH of the sodium pyrophosphate solution was established by titrating with concentrated HCl (resulting in contamination with 40 mol% Cl^-; N.B. chloride addition did not decrease chloride macroscopic conductance); the pH of the KH_2AsO_4 solution was brought to pH 7 by titrating in KOH; the pH of the KNO_3 solution was established by adding 50 mM Tris-HCl (pH 7) to a 1 M KNO_3 solution. All macroscopic conductance inhibition experiments were performed at least three times.

Porin	Inhibitor	pH	I_{50}(mM)
P	Phosphate	4.05	3.3
		6.0	0.93
		7.0	0.46
		8.0	0.38
	Pyrophosphate	7.0	4.9
	Arsenate	7.0	1.3
	Nitrate	6.0	>100
PhoE	Phosphate	7.0	>100

addition, the point of intercept provided an estimate of $-I_{50}$. At pH 6 for example, the I_{50} value was estimated as 0.93 mM (Fig. 3) at least 50-fold lower than the K_d for chloride at this pH. The inhibition of chloride transport by phosphate was found to be pH-dependent, in that the apparent I_{50} decreased more than 8-fold as the pH was increased from 4 to 8 (Table I).

To demonstrate that these results were due to a specific interaction of phosphate with protein P, the following control experiments were performed. Firstly, the effects of the lipid forming the membrane, either oxidized cholesterol or diphytanoylphosphatidylcholine, were tested. Despite differences in reconstitution rate and membrane stability with these lipids, the measured I_{50} values were almost identical being 0.46 mM for oxidized cholesterol and 0.49 mM for diphytanoylphosphatidylcholine at pH 7. Secondly, when the phosphate starvation inducible *E. coli* PhoE porin, which has been shown to lack a phosphate binding site [16], was substituted for protein P, no inhibition of conductance was observed when when 112 mM phosphate was added (Table I). Thirdly, when nitrate, which as a K_d of binding to protein P of 40 mM (Benz and Hancock, unpublished data), was substituted for phosphate, no inhibition of Cl^- conductance was observed even when 118 mM nitrate was added (Table I). If after addition of 118 mM nitrate, 1.6 mM phosphate was added, the membrane conductance decreased more than 5-fold over the subsequent 1 min.

Two other substrates which might be considered to be phosphate analogues, arsenate and pyrophosphate, were tested for their ability to inhibit chloride movement in macroscopic conductance inhibition experiments. Both were able to impede

chloride movement, although the measured I_{50} values were substantially greater than the I_{50} for phosphate (Table I).

Chemical modification of protein P

We had previously demonstrated that chemical acetylation of available protein P lysines destroyed the anion binding site of this protein [6]. To obtain evidence for the role of lysines in phosphate binding, the lysine ϵ-amino groups were chemically modified in three ways by acetylation, as previously described [6], by carbamylation or by methylation. Under the reaction conditions utilized, the modifications were quite specific for lysine ϵ-amino groups [15] with only the α-amino group at the N-terminus of the protein P molecule expected to be also modified. Due to the fairly substantial number of available lysine ϵ-amino groups (14.8 ± 4.0 estimated using trinitrobenzenesulphonate) most of which would not be expected to be within the channel at the critical anion binding site, the number of lysine residues modified was determined only in the case of methylated protein P (approx. 12.6 residues per polypeptide chain). Nevertheless, SDS-polyacrylamide gel electrophoresis (Fig. 4) and functional studies (see below) of the chemically modified proteins strongly indicated that protein P had been modified. All modified proteins ran as oligomer (trimer [9]) bands when solubilized at low temperatures and monomer bands when solubilized at high temperatures, as previously described for native protein P [4] (see also Fig. 4, lanes 1, 2 and 5), acetylated [6] and trinitrophenylated [10] (Fig. 4, lane 8) protein P. However, the modified porins demonstrated small but uniform shifts in electrophoretic mobility.

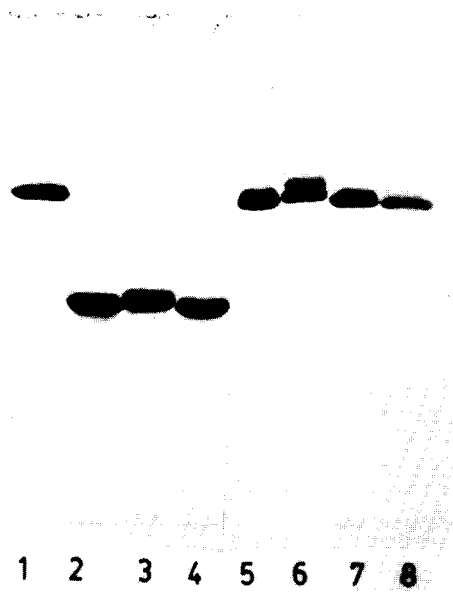

Fig. 4. SDS-polyacrylamide gel electrophoretograms of the native and chemically modified forms of protein P. Lanes 1 and 5–8: samples dissolved in SDS containing solubilization buffer at room temperature to reveal the trimer form. Lanes 2–4: samples solubilized at 95°C for 10 min to reveal the monomer form. Lanes 1, 2, 5, native protein P; lanes 3, 6, methylated protein P; lanes 4, 7, carbamylated proten P; lane 8, protein P derivatized with trinitrobenzenesulphonate.

The anion binding site in chemically-modified protein P molecules

To define the presence or absence of an anion binding site in the chemically-modified forms of protein P, similar experiments were performed to those described previously for acetylated protein P [6]. Addition of small amounts of methylated, acetylated and carbamylated protein P to the aqueous phases bathing lipid bilayer membranes caused stepwise increases in conductance (similar to those shown in Fig. 1), however, the average conductance increases were smaller than those observed for native protein P (Table II; Fig. 5). For all experiments measuring single-channel conductances, approx. 85–98% of the 183–378 individual channels measured formed a uniform probability histogram [4,6] suggesting that the analyzed channels were quite uniformly chemically modified. Substantial alterations in the single channel conductances (Fig. 5) suggested the modification of lysines critical to conductance through protein P channels (cf. trinitrophenylation which apparently modified only peripheral lysines [18]).

Both native and methylated protein P demonstrated an anion binding site as revealed by the saturation curves of single-channel conductance as a function of salt concentration (Fig. 5) [6] and the high anion selectivities of the channels (Table II). However, the binding affinity for Cl^- (revealed by replotting the fig. 5 data as an Eadie-Hofstee plot) was considerably decreased in the case of methyl-

TABLE II

SINGLE-CHANNEL CONDUCTANCE IN 1 M KCl, K_d FOR CHLORIDE BINDING AND SELECTIVITY FOR ANIONS OVER CATIONS OF NATIVE AND CHEMICALLY MODIFIED PROTEIN P MOLECULES

The results for native and acetylated protein P were taken from Ref. 6 except for the selectivity data which was measured in this study. The small difference in the measured ion selectivity of acetylated protein P compared to our previous study [6] may be due to differences in the average level of chemical modification in the two studies. Single-channel conductances in 1 M KCl were measured as described in Methods from current traces similar (but with steps of different magnitude) to those seen in Fig. 1. The mean single-channel conductance was averaged over more than 200 single-channel events. The K_d for chloride binding was estimated from the data in Fig. 5 by replotting the data as an Eadie-Hofstee plot. The notation > 3 M means that there was a linear relationship between mean single-channel conductance and salt concentration up to 3 M KCl. Selectivity was measured as described in Methods by fitting the zero-current potentials at various different salt gradients to the Goldman-Hodgkin-Katz equation [2] and thus calculating the relative permeabilities of anions (P_a) and cations (P_c). The salt used for these measurements was KCl. The results for native and methylated protein P are described here as > 70 although in this measurement range slight differences in the zero-current potential had dramatic effects on the calculated ion selectivity and the zero current potentials as a function of the chloride gradient could be fitted reasonably well to the Nernst equation as described previously [5].

Form of protein P	Mean single-channel conductance in 1 M KCl (pS)	K_d for chloride binding (M)	Selectivity (P_a/P_c)
Native	260	0.03	> 70
Acetylated	25	> 3	7.85 ± 0.96
Carbamylated	43	> 3	9.02 ± 1.91
Methylated	140	1.0	> 70

ated protein P ($K_d = 1$ M Cl^-) as opposed to native protein P ($K_d = 40$ mM Cl^-). In contrast, acetylated [6] and carbamylated protein P apparently lacked the anion binding site as revealed by a linear relationship between salt concentration and conductance (Fig. 5) [6] and the lower anion selectivities of the channels (Table II), phenomena typical of a general diffusion porin channel like OmpF [2].

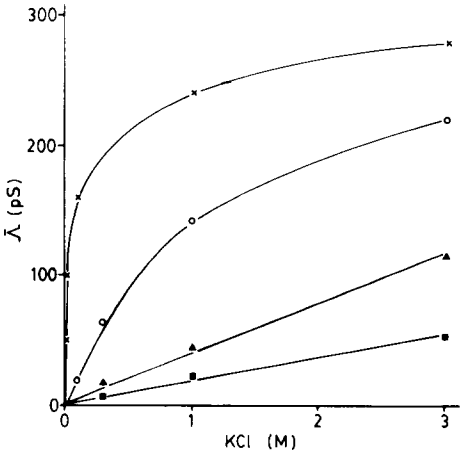

Fig. 5. Average single-channel conductance as a function of the KCl concentration for the native and chemically modified forms of protein P. x, native protein P from Ref. 6; 0, methylated protein P; ▲, carbamylated protein P; ■, acetylated protein P (from Ref. 6). For each point, the single-channel conductances were averaged for 100–300 individual step increases in conductance, observed in experiments similar to that shown in Fig. 1.

Macroscopic conductance inhibition studies with chemically-modified protein P molecules

As described above, macroscopic conductance due to native protein P, in the presence of 40 mM

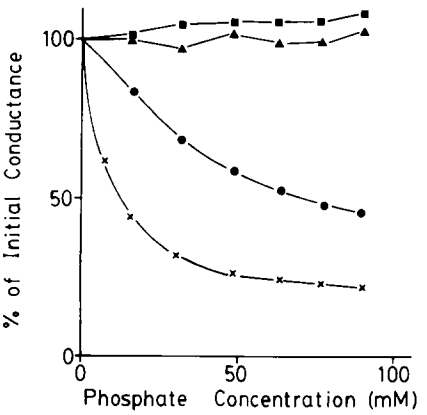

Fig. 6. Macroscopic conductance inhibition experiments using native and chemically modified forms of protein P. The experiments were performed as described in Methods and in the legends to Fig. 2 and 3, except that the bathing solution was 1 M KCl buffered to pH 7 with 1 mM Tris-HCl (pH 7.0). ×, native protein P; ●, methylated protein P; ▲, carbamylated protein P; ■, acetylated protein P.

Cl^-, pH 7, could be inhibited by phosphate ions with a I_{50} value of 0.46 mM. Under the same conditions the I_{50} for phosphate inhibition of chloride flux through methylated protein P was 1.25 mM. Due to poor incorporation of the other modified porins at low concentrations we compared all channels at 1 M KCl.

As shown in Fig. 6, at this higher salt concentration phosphate still caused inhibition of macroscopic chloride conductance for native protein P ($I_{50} = 15$ mM) and methylated protein P ($I_{50} = 65$ mM). The increases in the I_{50} values were presumably due to the higher Cl^- concentration. Macroscopic conductance through carbamylated and acetylated protein P was not inhibited by concentrations of phosphate up to 90 mM (Fig. 6).

Discussion

Our previous model membrane studies on porin protein P of *Pseudomonas aeruginosa* provided definitive evidence for an anion binding site within this channel [5,6]. However, protein P has a role in phosphate uptake (see Introduction). The existence of an anion binding site in protein P is consistent with, but does not fully explain, this role. The results in this paper suggest that protein P has a specific phosphate binding site which has an affinity for phosphate at pH 7 at least 100-fold greater than the affinity of the anion binding site for Cl^- which binds to this anion binding site with a higher affinity than all other tested anions (Benz and Hancock, unpublished data). For phosphate to bind with such a high affinity, it probably binds in a sterospecific manner; binding to the anion binding site in the same manner as Cl^- would not seem to be sufficient to explain the observed higher affinity. It should be noted here that although the affinity of protein P for phosphate is discussed here, we were actually measuring apparent I_{50} values of inhibition of macroscopic Cl^- conductance. Solving the I_{50} values at two different Cl^- concentrations, according to Michaelis-Menten kinetics, gave a K_d value for phosphate of 0.31 mM at pH 7.

The effects of phosphate on macroscopic Cl^- conductance can be explained if the anion binding site comprises a part of the phosphate binding site. Evidence for this includes the observation that Dixon plots for phosphate inhibition of chloride flux indicated competitive inhibition (e.g., Fig. 3 inset). Furthermore, destruction of the anion binding site by acetylation or carbamylation of lysine ϵ-amino groups (Table II) caused loss of the ability of phosphate to inhibit macroscopic conductance (Fig. 6) without influencing the trimeric association of the protein (Fig. 4) or the ability of the protein to form channels in lipid bilayer membranes (Table II). Similarly methylation of lysine ϵ-amino groups, which does not considerably effect the pK_a and hence the charge of these groups [15], caused an increase in the K_d of this channel for Cl^- (Table II) as well as an increase in the I_{50} for phosphate inhibition of macroscopic conductance (Fig. 6).

The results discussed above allow the construction of a more sophisticated model for the interior of the protein P channel, than those previously presented [6]. The model suggests the presence of a ring of three lysine side chains within a narrow part of the channel of protein P. The lysine ϵ-amino groups, being positively charged at neutral pH, would form a positively charged cloud shell that would attract anions and repel cations from the channel. The effect of pH on the I_{50} of macroscopic conductance inhibition by phosphate is consistent with the concept that HPO_4^{2-} ions bind better than $H_2PO_4^-$ ions (Table I). Examination of three-dimensional models of the HPO_4^{2-} ion (constructed using CPK Precision Molecular Models from Ealing Co., Watford, U.K.) revealed that this ion has 3-fold symmetry around the phosphate atom if one considers the two ionized oxygen atoms and the double-bonded oxygen atom. Since this double-bonded oxygen atom has a negative dipole moment, we propose that the symmetrically arranged lysine ϵ-amino groups interact with these three negative (or partial negative) charges. The reason that phosphate binds considerably more strongly than e.g. chloride is that phosphate is coordinated by this binding site whereas Cl^- having only a single negative charge would bind diffusely within the cloud shell.

The model is consistent with all data presented here and in previous papers. Examination of the single-channel conductance as a function of unhydrated anion size [5], as well as trinitrophenylation

experiments [17], suggested that the effective sieving diameter of the channel was around 0.5–0.6 nm. Since the HPO_4^{2-} ion has a reported diameter of 0.5 nm [16], the diameter of the circle formed by the three lysine ε-amino groups in this model would be in the order of 0.6 nm (allowing for van der Waals radii). Furthermore, the presence of three symmetrically arranged lysines can be easily explained if the trimer [7] of porin protein P contains a single-channel per trimer unit. We are currently attempting to define the structure of protein P in more detail, to allow testing of this model.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council to R.H. and the Deutsche Forschungsgemeinschaft (Be865/1-2 and Be865/3-1) to R.B. In addition, we wish to acknowledge the support of an International Collaborative Research Grant from the Natural Sciences and Engineering Research Council and a Study Visit Award from the Deutsche Akademischer Austauschdienst which supported R.H. during a visit to the laboratory of R.B., to perform the lipid bilayer experiments described herein.

References

1 Nikaido, H. and Nakae, T. (1979) Adv. Microbiol. Physiol. 21, 163–250
2 Benz, R., Schmid, A. and Hancock, R.E.W. (1985) J. Bacteriol. 162, 722–727
3 Luckey, M. and Nikaido, H. (1980) Proc. Natl. Acad. Sci. USA 77, 167–171
4 Hancock, R.E.W., Poole, K. and Benz, R. (1982) J. Bacteriol. 150, 730–738
5 Benz, R., Gimple, M., Poole, K. and Hancock, R.E.W. (1983) Biochim. Biophys. Acta 730, 387–390
6 Hancock, R.E.W., Poole, K., Gimple, M. and Benz, R. (1983) Biochim. Biophys. Acta 735, 137–144
7 Angus, B.L. and Hancock, R.E.W. (1983) J. Bacteriol. 155, 1042–1051
8 Poole, K. and Hancock, R.E.W. (1983) FEMS Microbiol. Lett. 16, 25–29
9 Poole, K. and Hancock, R.E.W. (1984) Eur. J. Biochem. 144, 607–612
10 Poole, K. and Hancock, R.E.W. (1986) Mol. Gen. Genet. 202, 403–409
11 Jentoft, N. and Dearborn, D.G. (1980) Anal. Biochem. 10, 186–190
12 Plapp, B.V., Moore, S. and Stein, W.H. (1971) J. Biol. Chem. 246, 939–945
13 Stark, G. (1972) Methods Enzymol. 25, 579–584
14 Fields, R. (1972) Methods Enzymol. 25, 464–469
15 Lundblad, R.L. and Noyes, C.M. (1984) Chemical Reagents for Protein Modification, Vol. I, pp. 127–170, CRC Press, Inc., Boca Raton, FL
16 Stern, K.H. and Amis, E.A. (1959) Physiol. Rev. 59, 1–64
17 Hancock, R.E.W., Schmid, A., Bauer, K. and Benz, R. (1986) Biochim. Biophys. Acta 860, 263–267