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MODIFICATION OF THE CONDUCTANCE, SELECTIVITY AND CONCENTRATION-DEPENDENT SATURATION OF *PSEUDOMONAS AERUGINOSA* PROTEIN P CHANNELS BY CHEMICAL ACETYLATION

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Protein P, an anion-specific channel-forming protein from the outer membrane of *Pseudomonas aeruginosa* was chemically modified by acetylation and succinylation of its accessible amino groups. The chemically modified protein retained its ability to form oligomers on sodium dodecyl sulfate polyacrylamide gels, whereas only the acetylated protein formed channels in reconstitution experiments with lipid bilayers. Acetylated protein P demonstrated a substantially reduced mean single channel conductance (25 pS at 1 M KCl) compared to the native protein P channels (250 pS at 1 M KCl) when reconstituted into black lipid bilayer membranes. The homogeneous size distribution of single-channel conductances suggested that all of the protein P molecules had been acetylated. Zero-current potential measurements demonstrated that the acetylated protein P channel was only weakly selective for anions and allowed the permeation of cations, in contrast to the native protein P channels, which were more than 100-fold selective for anions over cations. The dependence of conductance on salt concentration was changed upon acetylation, in that acetylated protein P demonstrated a linear concentration-conductance relationship, whereas native protein P channels became saturated at high salt concentrations. These data strongly suggested that the basis of anion selectivity for native protein P channels is fixed amino groups. In agreement with this, we could demonstrate a 2.5-fold decrease in single-channel conductance between pH 7 and pH 9, between which pH values the ϵ -amino groups of amino acids would start to become deprotonated. Two alternative schemes for the topography of the protein P channel and localization of the fixed amino groups are presented and discussed.

Introduction

The mechanism of anion permeation across membranes is poorly understood, since only a few anion-selective channels such as band 3 from red blood cells [3] and the chloride channel in the *Torpedo* electric organ [2] have been extensively studied in the past. We have recently purified to apparent homogeneity protein P from the outer

membrane of *Pseudomonas aeruginosa* [3]. This protein is specifically induced to a level of approx. 10^5 copies per cell by phosphate deprivation [3]. Its mode of induction and coregulation with a high-affinity phosphate uptake system suggest that it has a rôle in phosphate accumulation at low environmental phosphate concentrations. Upon reconstitution into black lipid bilayer membranes, protein P resulted in the formation of channels with a conductance of 160 pS for 0.1 M chloride solutions [3,4]. Since this conductance was relatively independent of the cationic counterion [3], it

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was assumed that the channel was strongly anion selective. This was confirmed by zero-current membrane potential measurements which, when fitted to the Goldman-Hodgkin-Katz equation, suggested that the protein P channel is greater than 100-fold more permeable for chloride than potassium ions [4]. The single-channel conductance was a logarithmic function of the Einstein-Stokes radius of the halide anions $\text{Cl}^- > \text{Br}^- > \text{I}^-$, suggesting that the ions were at least partially dehydrated as they entered the channel [4]. Furthermore, the channel demonstrated a small but measurable single-channel conductance (9 pS) for 1 M H_2PO_4 ions (Benz, R., unpublished data) in agreement with its proposed biological functions. Using either oxidized cholesterol or diphyanoylphosphatidylcholine as the lipid for reconstitution experiments did not influence the measured conductance in a given salt solution [3,4]. The channel became saturated at salt concentrations higher than 10 mM [4], suggesting that only a single anion can occupy the channel at any one time [5], i.e., there is one binding site inside the channel.

Protein P forms oligomers on SDS-polyacrylamide gels when solubilized at temperatures less than 60°C [3], and chemical crosslinking using the cleavable crosslinker disuccinimidyl bispropionate has suggested that the protein is a trimer of subunit molecular weight 48 000 (Angus, B.L. and Hancock, R.E.W., unpublished data). In this regard, it is similar to other bacterial outer membrane channel-forming proteins named porins, although it differs in a number of features. Firstly, protein P, unlike other porins, is not strongly noncovalently associated with the underlying peptidoglycan using the normal criteria for peptidoglycan association [3,6]. Secondly, protein P channels are significantly smaller than those of other bacterial porins which have single-channel conductances in the range 5–20-fold greater [7]. This was confirmed *in vivo* by demonstrating that protein P was unable to act as a channel for a 514 Da β -lactam nitrocefin [8], which was able to pass through the major porin, protein F, of *P. aeruginosa* and through the *Escherichia coli* outer membrane [9]. Thirdly, because of their large size, other porins demonstrate weak selectivity for ions [7,10,11] with most porins being about 2–4-fold selective for cations over anions, although the *E.*

coli *phoE* protein, which like protein P is phosphate starvation inducible, is about 3-fold selective for anions (Benz, R., Gimble, M., Darveau, R. and Hancock, R.E.W., unpublished data). Thus, while protein P has the major advantages of other porins (i.e., high copy number, stability in strongly denaturing detergents and ease of purification), it offers a significant advantage in the study of ion permeation (i.e., high selectivity).

Recently, Tokunaga et al. [12] demonstrated that the *E. coli* B porin protein could be chemically acetylated or succinylated, resulting in apparent 2-fold changes in the permeability of the porin to charged solutes. We have adapted their methods to the study of protein P channels. The results strongly suggest that fixed positively charged amino acids form the basis for the strong anion selectivity through protein P channels.

Materials and Methods

Isolation of outer membranes and purification and acetylation of protein P

Outer membranes were isolated by the one-step procedure of Hancock and Carey [13] using cells grown to late logarithmic phase on phosphate-deficient medium [3]. Protein P was purified as previously described [3] and stored at 4°C. At this temperature, it retained its pore-forming activity for about 3 months. Acetylation and succinylation of protein P was performed as described by Tokunaga et al. [12]. SDS-polyacrylamide gel electrophoresis was performed as previously detailed [13] using a 14% acrylamide running gel and varying the temperatures of solubilization.

Black lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously in detail [7,10]. The apparatus consisted of a Teflon chamber with two compartments connected by a small hole (0.1 mm²). A membrane was formed across the hole by painting on a solution of 1% (w/v) diphyanoylphosphatidylcholine in *n*-decane. Bilayer formation was indicated by the membrane's turning optically black to incident light. Conductance through the pores was measured after application of a given voltage, using a pair of Ag/AgCl electrodes inserted into the aqueous solutions on

both sides of the membrane. The current through the pores was boosted by a preamplifier, monitored by a storage oscilloscope and recorded on a strip chart recorder.

Zero-current potential measurements

Zero-current membrane potentials were measured by a slight modification of a method described earlier [10]. Bilayer membranes were formed in a $1 \cdot 10^{-2}$ M salt solution in the presence of 100 ng of native or acetylated protein P. After the membranes had turned completely black, a voltage of 10 mV was applied and the membrane conductance was observed to increase, within 10–20 min, to a value of about $1 \mu\text{S} \cdot \text{cm}^{-2}$. The applied voltage was then removed and the salt concentration on one side of the membrane was raised by the addition of small amounts of concentrated salt solution. After about 10 min, the zero-current membrane potential (mV) reached a steady-state value and was measured with a Keithley 610C electrometer, using calomel electrodes with salt bridges (Metrohm, Herisau, Switzerland).

Results

pH-dependence of single-channel conductance

In order to test the hypothesis that the ion selectivity of protein P channels is influenced by the presence of fixed charges either in the mouth of the pore or at the selectivity filter, the pH-dependence of permeability was studied (Fig. 1). As the pH was increased from 3 to 9, the average single-channel conductance for 30 mM KCl was decreased 6-fold. In contrast, previous data for the slightly cation-selective large pore formed by the porin protein of *E. coli* B showed a 1.25-fold increase over this range [10]. Titration of the pH of the aqueous phase for protein P suggested that two distinct effects of pH on conductance were observable. The 2.5-fold decrease in conductance between pH 7 and pH 9 may have been due to deprotonation of amino groups, resulting in reduced binding of anions to the channel. In addition, there was a 2.3-fold decrease in conductance between pH 3 and pH 5. Because of the complexity of these data, we chose to acetylate amino groups chemically as a possible method of identifying the basis for selectivity.

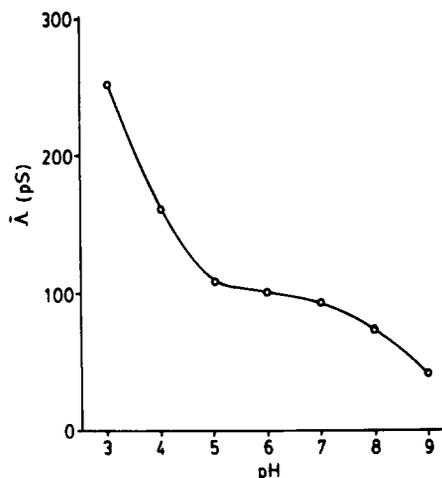


Fig. 1. Average single-channel conductance $\bar{\lambda}$ in 30 mM KCl of native protein P of *P. aeruginosa* as a function of the pH in the aqueous phase. The pH of the aqueous phase was adjusted by the addition of 5 M Hepes buffer (for pH between 7 and 9) and by the addition of dilute HCl (for pH between 3 and 6). The pH of the aqueous phase did not change during the course of the experiment. The applied voltage was 50 mV, a temperature of 25°C was maintained during the experiment, and membranes were made from 1% diphytanoylphosphatidylcholine in *n*-decane.

Acetylation of protein P

Protein P was acetylated using acetic anhydride at pH 7.0. After acetylation was complete and acetylated protein P had separated from unreacted free reagents by gel filtration, protein P retained its ability to form oligomers as judged by SDS-polyacrylamide gel electrophoresis of the protein after solubilization in SDS at 37°C (Fig. 2). The acetylated oligomers of protein P migrated slightly faster and were stained more poorly with Coomassie blue than the unacetylated oligomers, whereas the acetylated monomers migrated more slowly than native protein P monomers. No oligomers or monomers equivalent to native protein P were observed for the acetylated protein P sample. When protein P was succinylated with succinic anhydride, a similar pattern of oligomer formation in SDS after solubilization at 37°C and monomer formation at higher solubilization temperatures was observed (data not shown). Again, small changes in the relative mobilities of monomer and oligomer indicated that protein P had been entirely modified.



Fig. 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of native and acetylated protein P. Lane 1, native protein P solubilized at 37°C (note that although this oligomeric form is probably a trimer, it runs aberrantly on polyacrylamide gel electrophoresis [3]); lane 2, acetylated protein P solubilized at 37°C prior to electrophoresis; lane 3, native protein P solubilized at 100°C; lane 4, acetylated protein P solubilized at 100°C.

When purified protein P or acetylated protein P from a stock solution in SDS were added in small quantities (10 ng/ml) to aqueous salt solutions bathing a lipid bilayer membrane, conductance started to increase in a stepwise fashion (Fig. 3) due to progressive incorporation of single protein P channels. The results for native protein P closely mimicked data previously observed with other preparations of protein P [3,4] and were not caused by SDS alone, even at concentrations 1000-fold greater than those used here. In contrast, acetylated protein P showed much smaller step increases in conductance. Histograms of the conductance increases (Fig. 4) demonstrate that the measured conductance of individual channels was fairly homogeneous with native protein P, giving an average conductance of 250 pS in 1 M KCl, and acetylated protein P demonstrating an average conductance 10-fold lower (25 pS). The similarity of the single-channel conductance steps for acetylated protein P (measured for 185 individual channels) indicated that acetylation was uniform,

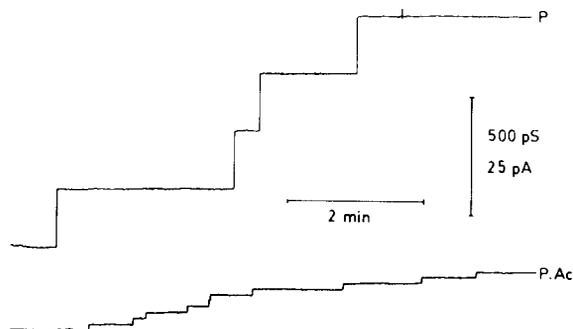


Fig. 3. Stepwise conductance increments caused by the progressive incorporation of single channel-forming units of protein P (upper trace - labelled P) or acetylated P (lower trace - labelled P.Ac) in membranes made from 1% diphytanoylphosphatidylcholine in *n*-decane. The solution bathing the membrane was 1 M KCl (pH 6), the applied voltage was 50 mV, and the temperature 25°C. 10 ng/ml of porin was present in these experiments.

whilst the lack of conductance increases similar to those observed for native protein P suggested that all protein P molecules had been acetylated, in

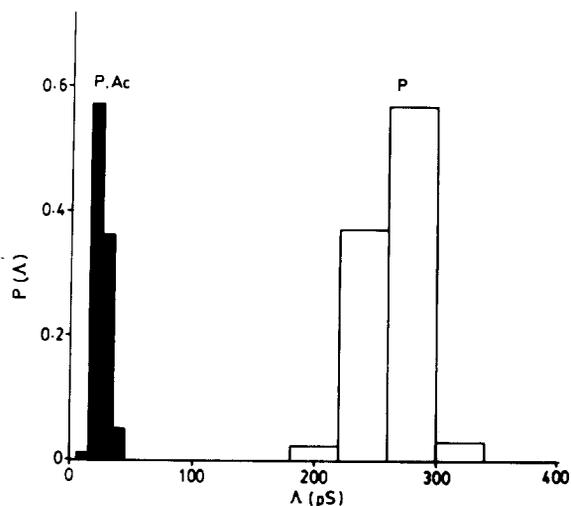


Fig. 4. Histogram of conductance increments observed with membranes made from 1% diphytanoylphosphatidylcholine dissolved in *n*-decane in the presence of native (labelled P - open bars) or acetylated (labelled P.Ac - filled bars) protein P. The aqueous phase contained 1 M KCl (pH 6), the temperature was 25°C and the applied voltage was 50 mV. $P(\Delta)$ is the probability of a given conductance increment, Δ , taken from recorder traces, such as those shown in Fig. 3. The histograms were derived from 185 individual steps for P.Ac and 222 steps for P and the average conductance increment was 25 and 250 pS, respectively.

agreement with the above polyacrylamide gel data.

Addition of succinylated protein P to lipid bilayer membranes did not result in conductance fluctuations or in any increase of the macroscopic conductance of the membranes. As the oligomeric nature of the protein P was unchanged by succinylation (see above), this finding could have only two explanations. The succinylation could have changed the surface of the protein in such a way that the insertion into lipid bilayer membranes was blocked. Alternatively, the bulky succinyl group might block the channel. The former explanation would be consistent with the experimental observation that the lipid bilayer membranes became unstable at protein concentrations around 1 $\mu\text{g}/\text{ml}$ succinylated protein P, if it is taken into account that lipid bilayer membranes usually become unstable if more than 10^6 porin channels per cm^2 are incorporated into the membranes [16].

Concentration dependence of single-channel conductance

Channels through which free diffusion of ions can occur should demonstrate a linear dependence of conductance on the concentration of ions in the aqueous phase [5]. Such behaviour has been demonstrated for the weakly selective bacterial porin pores [7,11]. In contrast, the strongly selective

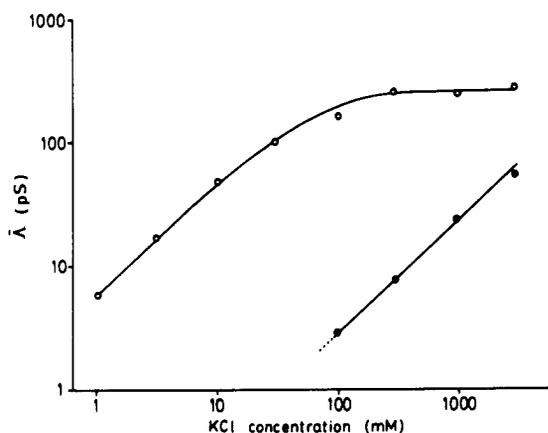


Fig. 5. Dependence of average single-channel conductance (\bar{A}) on the concentration of KCl in the aqueous phase using 10 ng/ml of protein P (open circles) or acetylated protein P (filled circles). Other than the salt concentration, conditions were the same as noted in the legend to Fig. 4.

protein P channel only demonstrates a linear conductance-concentration relationship with KCl concentrations as low as 1 mM to 10 mM (Ref. 4; see also Fig. 5). The single-channel conductance approached saturation for higher salt concentrations and no further conductance increases or decreases occurred above 0.3 M KCl. Such behaviour can be predicted if only a single ion can occupy the channel at a given time. In contrast to the data for native protein P, acetylated protein P demonstrated almost linear dependence of single-channel conductance on KCl concentrations between 100 mM and 3 M (Fig. 5).

Zero-current potential measurements

The above data were consistent with the possibility that acetylation of amino groups altered the channel properties by decreasing the selectivity for anions. This was further confirmed by zero-current potential measurements in which a concentration gradient of salt was established across membranes into which 10 or more protein P channels had been incorporated. Ions diffused across the channels according to the selectivity properties of these channels until the voltage (V_m) due to preferential movement of one ion relative to the other balanced the chemical osmotic potential gradient of the ions. At this stage ions would cease to flow through the channels, resulting in zero-current potentials which could be simply measured with an electrometer. By such measurements. It was

TABLE I
ZERO-CURRENT POTENTIALS, V_m , IN THE PRESENCE OF A 10-FOLD SALT GRADIENT

V_m was the electrical potential of the dilute side (10 mM) of the protein-P-containing membrane minus the potential of concentrated side (100 mM) calculated from at least three separate experiments. Membranes were formed from diphytanoylphosphatidylcholine dissolved in *n*-decane. The data were analysed using the Goldman-Hodgkin-Katz equation (see Ref. 10) to calculate P_a/P_c (the ratio of anion permeability to cation permeability).

Channel	Salt	V_m (mV)	P_a/P_c
Protein P	KCl	-58	> 100
Acetylated protein P	KCl	-10	3.4 ± 1.1
	LiCl	-12	4.3 ± 1.1
	RbCl	-5	1.4 ± 0.3

confirmed that acetylation of protein P reduced the anion selectivity at least 30-fold, although the acetylated channels remained slightly anion-selective (Table I).

Discussion

The results described in this paper indicate that fixed positive charges form the basis of the strong selectivity of the protein P channel for anions. There exist two possibilities for the location of these charges. The channel could consist of a long cylinder with a diameter around 0.6 nm, similar to the gramicidin channel [17]. In this case, one must assume that the charges would be located at the openings of the channel, especially since the charges would then be freely accessible from the aqueous phase and could easily be acetylated or succinylated. It is more difficult to picture how a positively charged group inside a long cylinder could be modified without blockage of the channel and it also seems unlikely that acetic anhydride would have access to the channel under these circumstances. Thus, we consider the location of one or several positively charged groups at the opening of the pore more likely if the pore has the form of a long cylinder (Fig. 6A).

The alternative possibility is that the pore has small constriction which acts as a selectivity and sieving filter. In this case, the positively charged group would be located close to this opening and would provide a binding place for anions (Fig. 6B). Acetylation of this group could reduce the single-channel conductance of the pore in two ways. One way might be partial blockage of the channel by the acetyl group (depending on the geometry of the amino group), whereas the other would be a simple reduction of the electric field by removal of the positive charge. Partial blockage of the channel by the acetyl group could also explain the complete blockage of the channel by succinylation (assuming that the protein was inserted into the membranes in both instances). However, both of the proposed structures are in principle consistent with the experimental results.

Both models would predict the observation that the conductance of individual native protein P channels decreased substantially as the pH was increased from pH 7 to pH 9 (Fig. 1). Over this

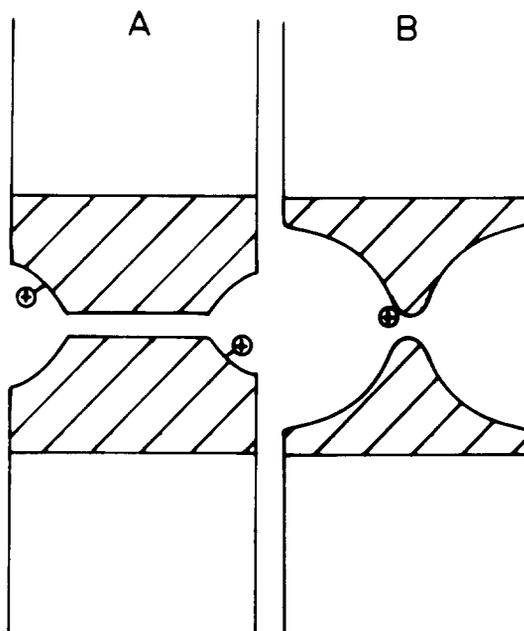


Fig. 6. Alternative models for the protein P channel. Model A follows the general concept proposed for gramicidin [17] with a long narrow channel. In this model, the fixed positive charges are at both mouths of the channel and acetylation of these amino groups would alter the channel selectivity and conductance but might not alter the apparent channel size (see text). Model B demonstrates a different concept in which the small size of protein P channels is due to a constriction in the channel, while the fixed positive charge which results in the channel's anion selectivity is at the rim of the constriction. In this model, a single positive charge can serve as the selectivity filter determining anion specificity of the channel for ions moving in both directions.

pH range, the tertiary amino groups of lysines would become deprotonated. However, due to the strong influence of environment on the pK_a of amino acids, we are unable to conclude with certainty that lysine residues are the basis of selectivity. A second effect of pH on the conductance of the protein P channel was observed at lower pH values. In fact, the overall influence of pH on conductance was reminiscent of pH titrations of such proteins as ovalbumin and β -lactoglobulin [14]. Thus, the increase in conductance between pH 5 and pH 3 may represent protonation of carboxyl groups which might exert, at the surface of the protein, a slight repulsive force on anions at higher pH values. Even if this be true, it should be pointed out that these carboxyl groups must have

little effect on the selectivity filter in either model (Fig. 6), since when the majority of the carboxyls were deprotonated at pH 7, the channel was at least 100-fold selective for anions. An alternative explanation for the effect of low pH on the conductance may be conformational changes in protein P.

Acetylation of protein P resulted in three major alterations in the properties of the channel, although it did not appear to alter the oligomeric structure of protein P or its ability to incorporate into membranes. Firstly, the selectivity was markedly reduced (at least 30-fold). The weak residual selectivity for anions could be explained by secondary, weakly positively charged amino acids in or around the channel or might be due to incomplete modification of protein P amino groups. This latter explanation seems less likely due to the narrow distribution range of single-channel conductances for the acetylated protein P channels (Fig. 3).

A second change in the properties of the acetylated protein P channel was observed in the salt dependence of conductance. Thus, while the native protein P channel became saturated at high salt concentrations, behaviour that can be interpreted in terms of only a single binding site inside the channel [5], the conductance of the acetylated protein P channel showed a linear dependence on salt concentration. Linear conductance-concentration relationships are typical of channels which demonstrate free diffusion of ions due to non-selectivity [5] or weak selectivity (like other bacterial porins [11]). These data, therefore, suggest that the basis of single ion occupancy in the protein p channel is the positively charged selectivity filter.

The third alteration in channel properties was the single-channel conductance. While the difference in conductance was 10-fold at a salt concentration giving easily measurable currents (Fig. 3), this underestimates the magnitude of the alteration caused by acetylation, since native protein P channels are saturated at this salt concentration. Extrapolation of the concentration-conductance data for acetylated protein P to 3 mM KCl suggests the conductance would be approx. 135 fS, less than one-hundredth of the conductance of native protein P at this salt concentration (Fig. 4).

This large difference in single-channel conductance could be explained by blockage of the channel by acetyl groups and/or by the influence of the positively charged groups of native protein P channel on conductance. As an example of the latter effect, one can crudely estimate the conductivity (Λ) of a free diffusion channel with a radius r and length l in the presence of a salt solution of bulk conductivity σ by the formula $\Lambda = \sigma \pi r^2 / l$. Thus, the estimated conductance at 3 mM salt ($\sigma = 0.42 \text{ mS} \cdot \text{cm}^{-1}$) for a channel of radius 0.25 to 0.30 nm and a length of 7.5 nm (equal to the width of the bilayer [11]) should be around 0.86 to 1.24 pS. This is probably an overestimate and does not take into account frictional interactions of chloride ions with the channel walls, relative degrees of hydration of ions in the channel and in the bulk phase, or steric hindrance in approach of the ion towards the channel. If the frictional component is taken into account using the Faxen [15] correction factor ($1 - 2.104a + 2.09a^3 - 0.95a^5$; where a is the ratio of ion radius to channel radius) of 0.114 for an 0.3 nm channel radius, the predicted conductance of native protein P channels at 3 mM KCl would be 141 fS, in close agreement with the extrapolated value for acetylated protein P channels of 135 fS (see above). If these calculations were accurate, assuming the model in Fig. 6A was correct, then the entire 100-fold difference in conductance between the native and acetylated protein P could be accounted for by the influence of the fixed positive charge. On the other hand, if the structure of the protein P channel is as given in Fig. 6B, it is obvious that the above calculations are not valid (since the channel is not a cylinder) and we must assume that the channel is partly blocked by the acetyl group. The absence of conductance fluctuations in the presence of succinylated porin would support these arguments if the channel were blocked by the succinyl group and not simply inactivated. In total, our results demonstrate that the anion selectivity of the protein P channel arises from the presence of positively charged amino groups in or near the mouth of the channel. Although we cannot exclude the possibility that the channel contains a long narrow part (possibility A in Fig. 6) our results support, in principle, a model in which protein P forms a short channel where the sieving and the selectivity filter are combined.

Acknowledgements

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