CHEMICAL MODIFICATION OF THE ANION SELECTIVITY OF THE PhoE PORIN FROM THE ESCHERICHIA COLI OUTER MEMBRANE

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The PhoE porin of Escherichia coli is induced by phosphate deprivation and when purified, forms moderately anion-selective channels in lipid bilayer membranes. To further investigate the basis of anion selectivity, PhoE was chemically acetylated with acetic anhydride. Acetylation modified the mobility and staining characteristics of the PhoE porin on SDS-polyacrylamide gel electrophoresis but the acetylated protein was still found in its normal trimeric state after solubilization in SDS at low temperatures. Furthermore, the acetylated PhoE porin retained its ability to reconstitute into lipid bilayer membranes and the single channel conductance in 1 M KCl was unaltered. Zero-current potential measurements demonstrated that whereas the native PhoE porin was anion-selective, a 30–40-fold increase in preference for cations upon acetylation resulted in the acetylated PhoE porin being cation-selective. Increasing the pH of KCl solutions bathing lipid bilayer membranes from pH 3 to pH 6 caused symmetrical 4-fold increases in the selectivity of both the native and acetylated PhoE proteins for cations. In contrast, increasing the pH from 7 to 9 caused a 2.5-fold increase in selectivity only for the native PhoE porin. These results suggest that the basis of anion selectivity in the native PhoE porin is fixed protonated amino groups (possibly on lysines) in or near the channel, and furthermore indicate that deprotonated carboxyl groups have a strong influence on ion selectivity.

Introduction

The PhoE porin protein of Escherichia coli is produced as a major outer membrane protein under conditions of phosphate deprivation [1] or in revertants of porin-deficient strains [1,2]. This suggests that it may have both a specific permeability function in enhancing the uptake of phosphate into the periplasm, and a general permeability function in that it can functionally replace the other major porins OmpF and OmpC. In agreement with this latter function, the permeability of the purified PhoE protein to a variety of compounds and ions both in vitro and in vivo [2,4–6] has been demonstrated. The data suggest that the PhoE channel has an exclusion limit for hydrophilic compounds similar to other porins with an approximate effective channel diameter around 1.2 nm [3,7]. The proposed specific permeability function has been more difficult to clearly demonstrate. Zero current potential measurements demonstrated that phosphate, sulphate and chloride ions have similar permeability through PhoE channels at physiological pH values [4], although evidence has been obtained in vivo that polyphosphate can inhibit the passage of an anionic β-lactam through the PhoE channel [5,6]. In any case, it has been clearly demonstrated that the PhoE protein channel is anion-selective [2–4], a
property which distinguishes it from all other *E. coli* porin proteins studied to date [2,3,7] which are cation-selective. Only protein P of *Pseudomonas aeruginosa* amongst the well-studied bacterial porins has been shown to favour the passage of anions [8]. This protein has a greater than 100-fold selectivity for anions over cations. In contrast, the PhoE protein is approximately 3-fold selective for phosphate over potassium [4]. This selectivity is markedly affected by pH, suggesting that the basis of anion selectivity of the PhoE channel is charged amino acids within the channel. In order to confirm this hypothesis we have acetylated the PhoE protein with acetic anhydride. The data suggest that charged amino groups within the channel specify anion selectivity and further suggest that carboxyl groups also have a strong influence on the channels' properties.

**Materials and Methods**

*Bacterial strains, porin isolation and acetylation.* Strain CGSC 6042 (JF 694) [proC-24, ompF-254, his-53, ompC-263, purE-41, nmpA-1, ilv-277, met-65, lacY-29, xyl-14, rpsL-97, cycA-1, cycB-2, tsx-63, λ-] obtained from Dr. B. Bachmann (Coli Genetic Stock Centre, Yale University, New Haven, CT), was used for the isolation of PhoE porin since this protein was the only major porin in strain CGSC 6042. *P. aeruginosa* PA01 (strain H103) was used as a source of protein P. Both the PhoE protein and protein P were purified as described previously for protein P [9]. Acetylation of PhoE protein followed the technique of Tokunaga et al. [10]. SDS-polyacrylamide gel electrophoresis utilizing a 14% acrylamide running gel was described previously [11].

*Black lipid bilayer experiments.* The methods used for black lipid bilayer experiments were described in detail previously [12]. 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 a solution of 1% (w/v) lipid (usually di-phytanoylphosphatidylcholine, Avanti Biochemicals, Birmingham, AL) in n-decane. Bilayer formation was indicated by the membrane turning optically black to incident light. Conductance through the pores was measured after application of a given voltage, using either Ag/AgCl or 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 membranes 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] and analysed using the Goldman-Hodgkin-Katz equation [7].

**Results**

*Acetylation and macroscopic conductance behaviour of the PhoE protein*

The phoE protein was acetylated using acetic anhydride as described in Materials and Methods. When analysed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Fig. 1), the apparent molecular weight varied according to the temperature of solubilization in SDS prior to electrophoresis. At low temperatures of solubilization the acetylated protein maintained its trimeric [13] configuration since it comigrated with the unacetylated PhoE trimer (Fig. 1, lanes 3, 4, and 7). However, the acetylated trimers (lane 3) were far better stained by Coomassie Brilliant Blue than native trimers (lane 4). At higher temperatures of solubilization, the acetylated form ran with a lower relative mobility and stained more poorly (Fig. 1, lane 1) than the native PhoE protein (Fig. 1, lane 2). There was little or no native PhoE protein observable in the acetylated PhoE preparation suggesting that acetylation occurred with almost all PhoE molecules. Furthermore, the fact that the acetylated product formed a tight band on SDS-polyacrylamide gels suggested that the acetylation was uniform. Presumably, the alteration in apparent molecular weight might be due to the reduction in positive charge of the protein and consequently in the ability of the protein to bind negatively charged dodecyl sulfate. The consequent lower charge to mass ratio of the protein would cause it to run slower on polyacrylamide gels.

The observation that the acetylated PhoE protein ran as a trimer at low temperatures of solubilization suggested that the structure of the protein
Fig. 1. SDS-polyacrylamide gel electrophoretogram of native and acetylated PhoE porin proteins run on 14% polyacrylamide gels after solubilization at 30°C or 100°C for 10 min. Lane 1, 5 μg acetylated PhoE solubilized at 100°C; lanes 2 and 5, 5 μg native PhoE solubilized at 100°C; lane 3, 5 μg acetylated PhoE solubilized at 30°C; lane 4, 5 μg native PhoE solubilized at 30°C (this band was barely visible on the original gel and its position is indicated by an arrow); lane 6, 20 μg native PhoE solubilized at 100°C; lane 7, 20 μg native PhoE solubilized at 30°C.

was not substantially perturbed. The conductivity measurements below were consistent with this concept. Addition of relatively large amounts of acetylated PhoE protein to the aqueous phases bathing lipid bilayer membranes resulted in a typical (see Ref. 4 for native PhoE protein data), time-dependent rise in membrane conductance (data not shown). Similar amounts of acetylated and native PhoE protein caused similar conductance increases indicating that the ability of the PhoE protein to reconstitute into a lipid bilayer was not greatly perturbed by acetylation. This result supports the assumption that hydrophobic interaction is responsible for the insertion of the PhoE porin into lipid bilayer membranes.

**Single-channel measurements**

Addition of very small amounts ($10^{-10}$ M) of acetylated PhoE protein to the solutions bathing a planar lipid bilayer membrane resulted in stepwise increases in conductance (Fig. 2), as also seen for native PhoE porin [4]. These increases were considered by analogy to other bilayer experiments [7] to represent the time-dependent incorporation of single acetylated PhoE channels into the membrane. A histogram of the conductance increase caused by native and acetylated PhoE protein in the presence of 0.1 M K$_2$HPO$_4$ (pH 8) showed that in each case the step increases were not homogeneous, but distributed over a certain range (Fig. 3). In addition the average conductance increase in this salt for the acetylated PhoE protein was nearly 40% greater than the conductance increase for unacetylated PhoE protein.

Single-channel conductance $\Lambda$ measurements were made for a variety of salts and it could be shown that despite bulk conductances $\sigma$ (i.e., the conductance in the aqueous phase in the absence of a membrane) which varied by two orders of magnitude, only a 2-fold variation in the ratio of single-channel conductance to bulk conductance ($\Lambda/\sigma$) could be observed (Table I). When the $\Lambda/\sigma$ ratio for the acetylated PhoE channel was compared to our earlier data for the native PhoE channel, for many salts no variation was seen (Table I). For some salts including K$_2$HPO$_4$, LiCl,
NaCl, KF, MgCl₂, K₂SO₄ and N(C₂H₅)₄⁺ Heps⁻ up to 40% increase or decrease in the single channel conductance \( \Lambda \) (reflected in the \( \Lambda/\sigma \) ratios in Table I) could be measured for the acetylated PhoE channel. Each of the above salts differed from the other salts in Table I by the fact that one of the two ions comprising the salt had a substantially different mobility to the other in a given electrical field (e.g., \( \text{Li}^+ < \text{Cl}^- \), \( \text{K}^+ > \text{F}^- \) etc.). This suggested that a substantial alteration in ion selectivity had occurred. In order to examine this directly zero-current potential measurements were made.

**Selectivity of the acetylated PhoE channel**

To directly measure the selectivity of the acetylated PhoE porin channel a salt concentration gradient \( (c'/c'') \) was established across a lipid bilayer into which about 100 porin molecules 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 time ions would cease to flow through the channels, and the resultant zero-current potential \( (V_m) \) could be measured simply with an electrometer. As shown previously [4] with the native PhoE porin incorporated into the membrane, the zero-current potential became increasingly negative on the dilute side of the membrane as increasing salt gradients were established across the membrane (Fig. 4). The results analysed according to the Goldman-Hodgkin-Katz equation suggested that the native PhoE channel exhibited a 3.3-fold preference for \( \text{Cl}^- \) over \( \text{K}^+ \). In contrast, with acetylated PhoE protein in the membrane, the zero-current potentials became increasingly positive (Fig. 4), suggesting a 14-fold preference for \( \text{K}^+ \) over \( \text{Cl}^- \). Consistent with this 46-fold change in selectivity for \( \text{K}^+ \) over \( \text{Cl}^- \), we measured a
TABLE I
SINGLE-CHANNEL CONDUCTANCE OF ACETYLATED PhoE IN A VARIETY OF DIFFERENT SALTS

Membranes were made from diphytanoylphosphatidylcholine or oxidized cholesterol in n-decane. Temperature, 25°C; applied voltage, 50 mV.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Conc. (M)</th>
<th>Single-channel conductance, ( \Lambda ) (nS)</th>
<th>Bulk conductance, ( \sigma ) (mS cm(^{-1}))</th>
<th>( \sigma/\Lambda ) (10(^{-8}) cm)</th>
<th>Number of increments measured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acetylated</td>
<td>Non-acetylated</td>
</tr>
<tr>
<td>LiCl</td>
<td>1.0</td>
<td>0.75</td>
<td>71</td>
<td>1.1</td>
<td>(1.7)</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0</td>
<td>1.2</td>
<td>84</td>
<td>1.4</td>
<td>(2.0)</td>
</tr>
<tr>
<td>KCl</td>
<td>0.01</td>
<td>0.020</td>
<td>1.4</td>
<td>1.4</td>
<td>(1.4)</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1</td>
<td>0.19</td>
<td>12</td>
<td>1.6</td>
<td>(1.8)</td>
</tr>
<tr>
<td>KCl</td>
<td>1.0</td>
<td>1.8</td>
<td>112</td>
<td>1.6</td>
<td>(1.6)</td>
</tr>
<tr>
<td>KF</td>
<td>1.0</td>
<td>1.5</td>
<td>76</td>
<td>2.0</td>
<td>(1.2)</td>
</tr>
<tr>
<td>KBr</td>
<td>1.0</td>
<td>1.8</td>
<td>118</td>
<td>1.6</td>
<td>(1.7)</td>
</tr>
<tr>
<td>RbCl</td>
<td>1.0</td>
<td>1.9</td>
<td>115</td>
<td>1.7</td>
<td>(1.7)</td>
</tr>
<tr>
<td>K$_2$HPO$_4$ (pH 6)</td>
<td>0.1</td>
<td>0.18</td>
<td>95</td>
<td>1.9</td>
<td>(1.7)</td>
</tr>
<tr>
<td>K$_2$HPO$_4$ (pH 8)</td>
<td>0.1</td>
<td>0.28</td>
<td>17</td>
<td>1.6</td>
<td>(0.9)</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>0.5</td>
<td>0.55</td>
<td>64</td>
<td>0.86</td>
<td>(1.5)</td>
</tr>
<tr>
<td>K$_2$SO$_4$</td>
<td>0.5</td>
<td>1.4</td>
<td>76</td>
<td>1.8</td>
<td>(1.1)</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.5</td>
<td>0.30</td>
<td>33</td>
<td>0.91</td>
<td>(0.9)</td>
</tr>
<tr>
<td>Tris$^+$ Hepes$^-$</td>
<td>0.5</td>
<td>0.081</td>
<td>7.2</td>
<td>1.1</td>
<td>(1.1)</td>
</tr>
<tr>
<td>N(C$_2$H$_5$)$_4$+ Hepes$^-$</td>
<td>0.5</td>
<td>0.039</td>
<td>4.8</td>
<td>1.2</td>
<td>(0.7)</td>
</tr>
</tbody>
</table>

30-fold increase in the selectivity of Li$^+$ over Cl$^-$ and a 36-fold increase in the selectivity of K$^+$ over F$^-$ (Table II). The actual numerical differences in the relative permeabilities of cations and anions of the acetylated PhoE channel, reflected the relative mobilities of the anions and cations, with the highly hydrated small ions Li$^+$ and F$^-$ having lower mobilities than K$^+$ or Cl$^-$.

In our previous studies [4] we observed, for the native PhoE channel, a complex relationship between pH and ion selectivity in KCl solutions. In particular, a 4-fold decrease in anion selectivity between pH 3 and pH 6 (seen as a 4-fold increase in cation selectivity in Fig. 5), was postulated to represent increasing attraction of cations by carboxyl groups in the channel which would become deprotonated (i.e., charged) in this pH range. In order to confirm this, and to demonstrate the basis of cation selectivity of the acetylated PhoE channel, we determined selectivity between pH 3 and pH 9. Over this pH range, the single-channel conductance was unaltered. In contrast the cation selectivity of the acetylated PhoE channel increased 4.6-fold when the pH of the KCl solution was adjusted from pH 3 to pH 6. Between pH 7 and pH 9, only an about 20% increase in cation selectivity was observed for the acetylated PhoE porin (Fig. 5). In contrast, over this pH range, we measured a 2.5-fold increase in the cation selectivity of the native PhoE channel.

TABLE II
ZERO-CURRENT MEMBRANE POTENTIALS \( V_m \) IN THE PRESENCE OF A 10-FOLD SALT GRADIENT

Membranes were made from diphytanoylphosphatidylcholine or oxidized cholesterol in n-decane, containing 10–100 molecules of the acetylated porin PhoE. The relative permeabilities of cations and anions \( P_c/P_a \) were calculated from the Goldman-Hodgkin-Katz equation.

<table>
<thead>
<tr>
<th>Salt</th>
<th>( V_m ) (mV)</th>
<th>( P_c/P_a )</th>
<th>Factor increase in cation selectivity upon acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl</td>
<td>24</td>
<td>0.11</td>
<td>30</td>
</tr>
<tr>
<td>KCl</td>
<td>45</td>
<td>0.30</td>
<td>46</td>
</tr>
<tr>
<td>KF</td>
<td>50</td>
<td>0.65</td>
<td>30</td>
</tr>
</tbody>
</table>

Discussion
The data presented in this paper provide strong evidence that the basis of anion selectivity of the native PhoE channel is fixed charged amino groups. The specific basic amino acids involved are probably lysine residues. Consistent with this, acetic
anhydride acetylates lysine but not arginine. Acetylation of PhoE porin largely removed the effect on ion selectivity of increasing the pH from 7 to 9 (Fig. 5). In this pH range unacetylated lysine residues would be expected to become deprotonated thus resulting in the loss of the positive charge on the lysines of the native PhoE protein and reducing anion selectivity (see Fig. 5). Interestingly, one of the major differences in amino acid composition between the cation-selective OmpC and OmpF porins and the anion-selective PhoE porin, despite extensive homology, is an increase in the number of lysine residues [16,19,20].

In addition to the charged amino groups which influence ion selectivity in the PhoE channel, two lines of evidence strongly suggested that carboxyl groups are important. Firstly, acetylation of the PhoE porin altered the selectivity of PhoE channel from weakly-anion to moderately-cation selective. Since acetylation would merely neutralize positive charges, this observation can only be explained by assuming the presence of negatively charged amino acids in the channel. Secondly, titration of the pH from pH 3 to pH 6, over which pH range carboxyls would become charged (deprotonated), resulted in approximately 4-fold increases in cation selectivity in both the native and the acetylated PhoE channels (Fig. 5). Thus in the native PhoE channel, charged amino acid residues can exert strong influences on ion selectivity. At physiological pH most carboxyl and amino groups in amino acid side chains would be charged. Presumably the anion selectivity of the PhoE channel results from an excess of charged amino groups over charged carboxyls although the spatial arrangement of these groups would be important. In any case it should be noted that while the relative anion selectivity of the PhoE channel can be decreased 46-fold at pH 7 by acetylation (Fig. 4) or increased 4-fold by decreasing the pH of the aqueous phase to 3 (Fig. 5), neither of these changes in selectivity alters the single-channel conductance in KCl solutions (see for example, Table I). Thus the total ion flux (i.e., the numbers of ions per channel per second) through the channel will remain the same through compensatory increases in the flux of one of the ions as the flux of the ion with opposite charge decreases. This may be explained by the fact that the limiting molar conductivities $\lambda^\infty$ of the two ions in the aqueous phase (i.e., their mobility) are approximately the same ($73.5 \text{ mS} \cdot \text{cm}^{-1} \cdot \text{M}^{-1}$ for K$^+$ and $76.4 \text{ mS} \cdot \text{cm}^{-1} \cdot \text{M}^{-1}$ for Cl$^-$ [17]).

On the other hand we expect a strong influence on the single-channel conductance when a salt with different limiting molar conductivities (mobilities) for the cation and the anion is used for the single-channel experiments (Table I). In the presence of cations of low mobility (such as the small and highly hydrated monovalent cation Li$^+$ and the divalent cation Mg$^{2+}$) acetylation resulted in a 40% decrease in single-channel conductance. In the presence of anions of low mobility (such as the small and highly hydrated monovalent anion F$^-$, the large monovalent anion Hepes$^-$, and the divalent anions HPO$_4^{2-}$ and SO$_4^{2-}$) acetylation of PhoE

![Fig. 5. Permeability ratio $P_c/P_a$ for the native and the acetylated PhoE pore as a function of pH. $P_c/P_a$ was obtained from zero-current membrane potential measurements in the presence of a 10-fold KCl gradient across membranes from diphytanoylphosphatidylcholine/n-decane where about 100 pores were incorporated. The aqueous solutions were buffered with 1 mM citrate at pH 3, 4 and 5 and with 1 mM Tris at pH 9; $t = 25^\circ\text{C}$.

$$P_c/P_a$$

- acet. Pho E
- native Pho E

$pH$
resulted in a similar increase in single-channel conductance. The reason for this is in both cases the change of the pore selectivity by chemical acetylation, which favours in the first case the movement of the less mobile cations and in the second case that of the more mobile cations through the channel.

These data underline an important principle. The relative ion selectivity of a channel can vary in response to the mobility of the ions in question (Table II). Thus one might expect, in the absence of specific binding sites, that PhoE would have lower selectivity for polyphosphates than for monophosphates. Unfortunately, as discussed previously [4], it was not possible to measure the selectivity for polyphosphate, given uncertainties in the actual charge on polyphosphate. However, evidence has been presented from in vivo experiments [5,6] that a polyphosphate binding site exists within the PhoE channel and our data does not rule out this possibility, although a specific inorganic phosphate binding site appears to be precluded by our experiments [4].

The results obtained with the chemical modification of the OmpF pore from E. coli [18] are in some contrast to the findings reported here with the acetylated PhoE pore. The OmpF pore had only lost its cation selectivity after amidation and did not become anion selective [18]. This result has been discussed under the assumption that the pores formed by OmpF-porin trimers contain at neutral pH only negatively charged groups and no positively charged residues. The PhoE pore from E. coli outer membrane contains obviously both negatively and positively charged groups in or near the pore. Otherwise we could not explain the selectivity change after chemical acetylation which blocks the positively charged amino groups. As the major change of the PhoE sequence with respect to the OmpF sequence is the insertion of several lysines [16,19,20] it seems very likely that the OmpF and PhoE genes evolved from a common ancestral gene and that most of the additional lysines are located in or near the pore. On the other hand, our results in this and a previous study [18] indicate also that most of the negatively and positively charged groups in the three porins in E. coli (OmpF, OmpC and PhoE) form most probably ion bridges stabilizing the structure of the trimers. These ion bridges are not accessible for chemical modifications. In this respect, it is interesting to note that chemical amidation of the PhoE trimers led to the dissociation and inactivation of the PhoE pore (Darveau and Hancock, unpublished results). This results agree with observations with intensively amidated OmpF trimers, which show also a strong tendency to dissociate [10].

The results of this study demonstrate that acetylation of PhoE channels has a similar but far less dramatic effect than acetylation of the channels of protein P [14], a P. aeruginosa outer membrane protein which like, PhoE, is induced by phosphate deprivation. In the case of protein P, which forms smaller channels than PhoE and has at least 30-fold higher selectivity for anions in its native state [8,9] acetylation converts the channel to a slightly anion selective channel and reduces the single channel conductance 10-100-fold (depending on the salt concentration [14]). These differences can be largely explained on the basis of proteins P and PhoE having a similar distribution of charged amino acids, if protein P has a constriction of about 0.6 nm in diameter (i.e., half the diameter of the PhoE porin channel [4,7]) within the channel and close to the amino groups (see Ref. 14 for discussion). Interestingly, we previously suggested the possibility, on the basis of the effect of pH on the single-channel conductance of protein P, that this protein had charged carboxyl groups which influenced anion movement through the channel [14]. Since the gene for the PhoE porin is now cloned [15], and the amino acid sequence known [16], we feel that directed mutagenesis of the PhoE gene nucleotide sequence (with the aim of altering specific basic and acidic amino acids), followed by ion-selectivity measurements is an exciting possibility for the PhoE porin.

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