Protein Chemistry and Structure:  
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The Role of Specific Lysine Residues in the Passage of Anions through the Pseudomonas aeruginosa Porin OprP

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Anand Sukhan and Robert E. W. Hancock
From the Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

When grown under phosphate-limiting conditions Pseudomonas aeruginosa expresses the phosphate-specific porin OprP. In order to determine whether any of the lysine residues located in the amino-terminal half of the protein play a role in the transport of anions through the channels, the first nine amino-terminal lysine residues of OprP were substituted with glutamates. The mutant proteins were purified and the channels they formed were characterized by reconstituting the purified porins in planar lipid membranes. In comparison to the wild-type protein, the Lys74, Lys121, and Lys126 mutants all displayed reduced levels of conductance at KCl concentrations below 1 M, and the Lys74 and Lys121 mutants no longer exhibited a saturation of conductance at high anion concentrations. In addition, the ability of phosphate ions to inhibit the conductance of Cl− ions through the channels formed by the Lys121 mutant was greatly reduced, while their ability to inhibit the Cl− conductance of the Lys74 mutant was reduced by approximately 2-fold. To clarify the roles that Lys74, Lys121, and Lys126 play in regulating the channel characteristics of OprP, these amino acids were replaced with either glycine or glutamine residues. Analysis of these mutants suggested that both Lys74 and Lys126 may serve to funnel anions toward the binding site, but only the presence of Lys121 is required for the formation of the inorganic phosphate-specific binding site of OprP.

The acquisition of inorganic phosphate (Pi) and phosphorylated compounds is an essential function of growing microorganisms. Many bacteria have been shown to possess a group of phosphate starvation-inducible genes whose expression result in enhanced Pi uptake. This group of genes is often referred to as the Pho regulon (1). One member of the Pho regulon is the P. aeruginosa Pho regulon (2). The known gene that encodes the Pi-specific porin OprP (2).

Like the analogous phosphate starvation-inducible Escherichia coli porin PhoE, OprP has been proposed to exist in the outer membrane as a trimer of three identical subunits, each of which traverses the membrane as a 16-stranded β-barrel (3). However, unlike PhoE, the P. aeruginosa porin forms channels that contain a saturable Pi-binding site (Kd ≈ 30 mM/Cl−; 0.3 mM/Pi) (2, 4). Chemical modification studies suggested that the P1 specificity of OprP is due in part to the presence of one or more lysine residues that may take part in the formation of the binding site (4, 5).

It has been demonstrated previously through both combinatorial and site-directed mutagenesis that the amino acids responsible for determining the ion selectivities of the highly homologous E. coli porins OmpF, OmpC, and PhoE are located exclusively in the amino-terminal halves of these proteins (6–8). One specific PhoE residue (Lys125) located in the second surface-exposed loop appeared to be critical for ion transport. The substitution of glutamate for this residue resulted in a cation- rather than an anion-selective channel (8). Alternatively, mutagenesis of the maltose-specific porin LamB has shown that residues important for the substrate specificity of this protein are concentrated in the amino-terminal end (9, 10).

Of the 23 lysine residues found in OprP, only nine are located in the amino-terminal half of the protein; one is located in the vicinity of the proposed second surface-exposed loop and two are found in the proposed third surface-exposed loop (3). In order to determine whether any of the lysines contained in the amino-terminal half of OprP are necessary for the transport of anions a PCR1-based site-directed mutagenesis protocol was used to individually mutate specific lysine residues. In this study we report the results of the mutagenesis of the nine amino-terminal lysine residues of OprP and the effect these mutations had on the single-channel conductance and P1 binding of this porin.

**Experimental Procedures**

Chemicals—KCl, K2HPO4, and KH2PO4 were purchased from Fisher Canada. KCl was used unbuffered (pH 6.0) while equal molar concentrations of K2HPO4 and KH2PO4 were mixed to achieve a pH of 8.0. Bacterial Strains and Growth Conditions—E. coli DH5α was used for all procedures involved in creating the oprP mutant plasmids. Strain CE1248 was utilized in all expression experiments (11). Cells were grown overnight at 37 °C in LB broth supplemented with ampicillin (50 μg/ml), and in the case of cells grown for the purpose of porin purification, 0.4% glucose.

General Molecular Techniques—Restriction endonucleases, Vent DNA polymerase, and T4 DNA ligase purchased from Life Technologies, Inc. and New England Biolabs Inc. were used in accordance with the accompanying literature. Cells were transformed using the CaCl2 method (12).

Site-directed Mutagenesis—The oprP substitution mutants were created using a recombinant PCR method (13, 14) with the oprP containing plasmid pAS27 (3) used as the template. Mutagenic oligonucleotides contained mismatches that corresponded to a substitution mutation in the encoded amino acid sequence (Table I). The mutagenized fragments of oprP were subcloned back into plasmid pAS27 and sequenced. Oligonucleotides were synthesized on a model 392 Applied Biosystems DNA synthesizer (Applied Biosystems Canada, Mississauga, Ontario, Canada).

DNA Sequencing—Plasmid DNA was sequenced using an Applied Biosystems model 373 fluorescent sequencer and PCR protocols provided by the manufacturer. Template DNA was prepared by the polyethylene glycol precipitation method (12). Primers were synthesized on

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To whom correspondence should be addressed: Dept. of Microbiology and Immunology, University of British Columbia, 300-6174 University Blvd., Vancouver, BC, Canada, V6T 1Z3. Tel.: 604-822-2682; Fax: 604-822-6041; E-mail: bob@cbdn.ca.

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1 The abbreviation used is: PCR, polymerase chain reaction.
Lysine Residues in P. aeruginosa Porin OprP

Mutagenic oligonucleotides containing one or two nucleotide substitutions were used with a recombinant PCR method to individually mutate specific lysine residues. The resulting nucleotide and amino acid substitutions are listed. The entire coding sequence of the mutagenized DNA fragments were sequenced and found to be free of errors.

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<tr>
<th>Codon</th>
<th>Amino acid</th>
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<tbody>
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<tr>
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<td>pOPEQ126</td>
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Expression and Purification of Mutant Proteins—The OprP substitution mutant plasmids were transformed into the porin-deficient strain CE1248, and overnight cultures were used to prepare whole cell lysates. After heating at 100°C for 10 min the samples were electrophoresed, transferred to nitrocellulose membranes, and blotted with anti-OprP antisera. All the mutant proteins were expressed at levels comparable to that of the wild-type protein (data not shown).

Site-directed Mutagenesis of oprP—To assess the role that the first nine amino-terminal lysine residues of OprP play in determining the channel characteristics of this protein, these residues were replaced with glutamates using a PCR-based site-directed mutagenesis method as described under "Experimental Procedures." In addition, Lys121 and Lys126 were replaced with glutamines, Lys121 and Lys126 were replaced with glutamates, and a triple mutant in which Lys121, Lys121, and Lys126 were all replaced with glutamates was also created. The secondary PCR products were digested with either HindIII (Lys13-Lys74) or EcoRV/SphI (Lys109-Lys181) and were ligated to similarly digested and gel-purified plasmid pAS27. Recombinant plasmids having the appropriate restriction enzyme digestion patterns were sequenced to verify the presence of the desired mutation (Table I). No errors in the coding sequence of the mutagenized fragments were detected.

Expression and Purification of Mutant Proteins—The OprP substitution mutant plasmids were transformed into the porin-deficient strain CE1248, and overnight cultures were used to prepare whole cell lysates. After heating at 100°C for 10 min the samples were electrophoresed, transferred to nitrocellulose membranes, and blotted with anti-OprP antisera. All the mutant proteins were expressed at levels comparable to that of the wild-type protein (data not shown).

Single-channel Conductance of Lys → Glu Mutant Proteins—In order to determine whether the substitution of individual lysine residues had an effect on the conductance saturation of OprP, the single-channel conductance of each of the Lys → Glu mutant proteins was assessed at various salt concentrations (Table II). The average conductance of six of the mutant proteins (Lys13, Lys15, Lys25, Lys74, Lys109 and Lys181) was similar to that of wild-type OprP at all tested salt concentrations. In contrast, three of the mutant proteins displayed distinctly altered channel characteristics. In 1 M KCl, the Lys74 and Lys126 mutants exhibited levels of conductance that were approximately one-half of that of the wild-type protein, while the Lys121 mutant possessed a conductance of approximately one-third of that of wild-type OprP (Fig. 1).

The conductance of the channels formed by these three mutant proteins as well as wild-type OprP was plotted as a function of increasing salt concentration (Fig. 2). As shown previously, the single-channel conductance of wild-type OprP plateaus as the salt concentration approaches 1 M. However, both the Lys74 and the Lys121 mutant proteins formed channels that displayed linear concentration-conductance relationships at up to 3 M KCl. The Lys126 mutant channel conductance, although significantly lower than wild-type OprP, tended to follow the same pattern as the wild-type protein, plateauing as the salt concentration approached 1 M.

While it might be expected that mutations which so profoundly affected the channel conductance might also have an effect on anion selectivity, measurements of the ion selectivity of these mutants revealed no significant differences from the wild-type protein (data not shown).

Phosphate-induced Inhibition of Single-channel Conductance of Lys → Glu Mutant Proteins—In order to determine whether any of the Lys → Glu substitutions had an effect on the P_1-bonding site of OprP, the ability of phosphate ions to inhibit the single-channel conductance of the mutant proteins was measured. The single-channel conductance of each mutant protein in 0.1 M KCl was determined prior to the addition of Pi (Table II). Increasing amounts of potassium phosphate were added to the bathing solutions, and the resultant channel conductances were measured. These data were then used to calculate the percent inhibition and the I_{so} concentration of the added phosphate ions (Table III).

The majority of the mutant proteins exhibited degrees of conductance inhibition similar to or greater than the wild-type protein, which displayed a 74% decrease in conductance after the addition of 3.3 mM potassium phosphate. The Lys13 mutant had a slightly lowered affinity for P_i with a maximum inhibition of 58% and an I_{so} concentration of 1.95 mM compared to 0.96 mM for wild-type OprP. The Lys121 substitution had a
profound effect on the ability of the protein to bind phosphate ions. This mutant showed a maximum inhibition of 30%, and while the I50 for this mutant could not be measured under the conditions used to examine the other mutant proteins, additional experiments revealed that it was above 10 mM. The Lys126 mutant channel conductances, although greatly reduced compared to those of the wild-type protein, appeared to be inhibited by the presence of phosphate ions to a similar degree. Fig. 3 shows the channel conductances of the Lys74, Lys121, and Lys126 mutant proteins along with wild-type OprP plotted as a function of increasing phosphate ion concentrations.

Channel Characteristics of Lys → Gly, Lys → Gln, and Lys74, 121, 126 → Glu Mutant Proteins—To further examine the roles Lys74, Lys121, and Lys126 play in determining the electrochemical nature of the channels formed by OprP, these amino acids were substituted with either a Gly or a Gln residue, and the single-channel conductance and phosphate-induced inhibition of chloride conductance was determined for each of these mutant proteins (Table IV). In addition, a triple mutant with Lys74, Lys121, and Lys126 all substituted with glutamates was also created and analyzed. Substituting Lys74 with Gly resulted in a channel with a conductance comparable to the wild-type protein in 1 M KCl. However, the channel conductance of this mutant in 0.1 M KCl was similar to that of the Lys74 → Glu mutant protein. The phosphate-induced conductance inhibition of the Lys74 → Gly mutant was comparable to that of the wild-type protein.

Substituting the Lys126 residue with either Gly or Gln resulted in channels that had reduced levels of conductance in comparison to the wild-type protein. In the case of the Gly substitution, the channel conductance at both 0.1 M and 1 M KCl was lower than that of the Lys126 → Glu mutant. Substituting Lys121 with either Gly or Gln resulted in channels with reduced conductance at both 0.1 M and 1 M KCl. These mutant proteins also formed channels that were as severely impaired in their ability to bind phosphate ions as the initial Lys121 → Glu mutant protein.

The single-channel conductance of the Lys74, 121, 126 → Glu triple mutant was somewhat lower than any of the single mutants in both 0.1 M and 1 M KCl; however, the phosphate-
The induced inhibition of conductance of this mutant channel was in the range of the Lys$_{21}$ single mutants.

## DISCUSSION

It has been demonstrated previously that the channel characteristics of several general diffusion pores are dependent on the presence of one or more amino acids located in their amino-terminal domains (6–8). In this study we have identified three amino-terminal lysine residues in OprP that play a role in defining the channel characteristics of this porin.

In 1 M KCl, the Lys$_{21}$ → Glu mutant channels displayed a 3-fold decrease in conductance, while the Lys$_{74}$ → Glu and Lys$_{126}$ → Glu mutants displayed 2-fold and 2.5-fold decreases, respectively. This is in contrast with the 10-fold reduction in conductance exhibited by chemically modified forms of OprP (18). Even a triple mutant in which all three of these lysine residues had been substituted with glutamates displayed only a 5-fold decrease in conductance. These findings suggest that there may be certain lysine residues contained in the carboxyl-terminal end of OprP that also play a role in determining the channel conductance. Alternatively, the severe reduction in conductance displayed by the chemically modified forms of OprP may have been due to factors unrelated to the actual loss of the positive charges of the modified lysine residues (e.g. the presence of the modifying groups within and/or around the channel).

Lysine-specific acetylation of OprP was shown to produce channels with conductances that were no longer saturated at high anion concentrations (18). This result was explained to be due to a modification of specific residues which are involved in forming the anion-binding site (4). Of the eight Lys → Glu mutant forms of OprP created during the course of this study, only the Lys$_{74}$ and Lys$_{121}$ mutants exhibited losses in the ability to saturate at KC concentrations above 1 M. The Lys$_{126}$ → Glu mutant displayed saturation kinetics similar to that of wild-type OprP despite the fact that the conductance of these channels was as severely affected at low salt concentrations as that of the Lys$_{74}$ and Lys$_{121}$ mutant pores. The conductance patterns of the other six Lys → Glu mutants did not differ significantly from that of the wild-type protein. Apparently only the Lys$_{74}$ and Lys$_{121}$ substitutions had a detrimental effect on the anion-binding site.

The phosphate-induced inhibition of channel conductance of the Lys$_{74}$ → Glu mutant was approximately 2-fold lower than that of the wild-type protein. Substituting this lysine residue with a glycine resulted in a protein with a phosphate-induced inhibition of conductance that was similar to that of the wild-type protein. This result can be explained if Lys$_{74}$ is assumed to occupy a space proximate to the P$_i$-binding site. The positive charge of this residue would not be required for the formation of the binding site; however, the placement of a negatively charged residue at this location may have indirectly affected the interaction of phosphate (and chloride) ions with the binding site. According to our recent OprP topological model, Lys$_{74}$ is located at the top of the fourth β-strand and would presumably face the interior of the channel (3).

Substitution of Lys$_{21}$ with glutamate, glycine, or glutamine residues resulted in proteins with channel conductances that were severely impaired in their abilities to be inhibited by the presence of phosphate ions. This particular residue is located in the third surface-exposed loop according to a recently published topological model of OprP (3). The placement of this residue in the third loop is in agreement with the role of this loop in constraining the interior of the channels formed by several bacterial porins (19–21). The equivalent lysine residue in PhoE (Lys$_{404}$) that has been established as being responsible for determining the anion selectivity of this porin (8) was also shown to be located in the third surface-exposed loop (19). Substituting Lys$_{126}$ with glutamate in OprP had no apparent effect on the P$_i$-binding site, despite the fact that this residue is also predicted to be located in the third surface-exposed loop.

The roles of Lys$_{74}$ and Lys$_{126}$ in OprP appear to be to form an electrostatic funnel that serves to focus the flow of anions toward the binding site. The Lys$_{121}$ residue seems to render a more critical function in this porin. Not only does the presence of this residue serve to increase the flow of anions through the channel, but it appears that this particular amino acid is required for the formation of the anion/P$_i$-binding site. Whether this is the only residue involved in maintaining the P$_i$ specificity of OprP remains to be seen.

### REFERENCES


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<th>Table IV</th>
<th>Channel characteristics of OprP Lys→Gly and Lys→Gln mutants</th>
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<tbody>
<tr>
<td>OprP mutation</td>
<td>Average single-channel conductance$^a$</td>
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<tr>
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<td>0.1 M KCl</td>
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<tr>
<td>Wild-type</td>
<td>103 ± 45</td>
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<tr>
<td>Lys$_{74}^{21}$ → Glu</td>
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<td>Lys$_{74}^{21}$ → Gly</td>
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<tr>
<td>Lys$_{121}^{21}$ → Glu</td>
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<tr>
<td>Lys$_{126}^{21}$ → Glu</td>
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</tbody>
</table>

$^a$ Average of 100 single-channel events in picosiemens ± S.D.

$^b$ Maximal conductance inhibition after the addition of 3.3 mM potassium phosphate.

$^c$ ND, not determined.