The Role of Specific Lysine Residues in the Passage of Anions through the Pseudomonas aeruginosa Porin OprP*

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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 Lys126 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 Pseudomonas aeruginosa Pho regulon is the 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 traverse 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 Pi 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 third 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). Additionally, 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 Pi 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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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 were found to be free of errors.

| Plasmid | Codon | Amino acid |
|---------|-------|------------|
| pOPE13  | AAG   | GAG        |
| pOPE15  | AAG   | GAG        |
| pOPE25  | AAG   | GAG        |
| pOPE30  | AAG   | GAG        |
| pOPE44  | AAG   | GAG        |
| pOPE109 | AAG   | GAG        |
| pOPE121 | AAG   | GAG        |
| pOPE126 | AAA   | AAA        |
| pOPE181 | AAG   | GAG        |
| pOPE74  | AAG   | GAG        |
| pOPE121 | AAA   | AAA        |
| pOPE126 | AAA   | AAA        |
| pOPE121 | AAA   | AAA        |
| pOPE126 | AAA   | AAA        |

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, Lys74, Lys121, and Lys126 were replaced with glycines, Lys121 and Lys126 were replaced with glutamines, and a triple mutant in which Lys74, 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 (Lys74-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 (Lys74, Lys121, and Lys126) 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 mM 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.

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 P0-binding 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 mM 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 I50 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 Lys74 mutant had a slightly lowered affinity for Pi, with a maximum inhibition of 58% and an I50 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 → Glu, 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 Glu 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 conductance of this mutant in 0.1 M KCl was lower than 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 Glu 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 Glu 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-
induced inhibition of conductance of this mutant channel was in the range of the Lys$^{211}$ single mutants.

**DISCUSSION**

It has been demonstrated previously that the channel characteristics of several general diffusion porins 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 thre...
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