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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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 Lys^{74}, Lys^{121}, and Lys^{126} mutants all displayed reduced levels of conductance at KCl concentrations below 1 M, and the Lys^{74} and Lys^{121} 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 Lys^{121} mutant was greatly reduced, while their ability to inhibit the Cl^- conductance of the Lys^{74} mutant was reduced by approximately 2-fold. To clarify the roles that Lys^{74}, Lys^{121}, and Lys^{126} 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 Lys^{74} and Lys^{126} may serve to funnel anions toward the binding site, but only the presence of Lys^{121} is required for the formation of the inorganic phosphate-specific binding site of OprP.

The acquisition of inorganic phosphate (P_i) 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 P_i 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 P_i-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 P_i-binding site (K_d = 30 mM/Cl^-; 0.3 mM/P_i) (2, 4). Chemical modification studies suggested that the P_i 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 (Lys^{125}) 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 PCR[1]-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 P_i binding of this porin.

EXPERIMENTAL PROCEDURES

Chemicals—KCl, K_2HPO_4, and KH_2PO_4 were purchased from Fisher Canada. KCl was used unbuffered (pH 6.0) while equal molar concentrations of K_2HPO_4 and KH_2PO_4 were mixed to achieve a pH of 8.0.

Bacterial Strains and Growth Conditions—*E. coli* DH5-α 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 $CaCl_2$ 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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[1] The abbreviation used is: PCR, polymerase chain reaction.

TABLE I
Codon changes of oprP substitution mutants

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	
	Wild-type	Mutant	Wild-type	Mutant
pOPE13	AAG	GAG	Lys^{13}	Glu^{13}
pOPE15	AAG	GAG	Lys^{15}	Glu^{15}
pOPE25	AAG	GAG	Lys^{25}	Glu^{25}
pOPE30	AAG	GAG	Lys^{30}	Glu^{30}
pOPE74	AAG	GAG	Lys^{74}	Glu^{74}
pOPE109	AAG	GAG	Lys^{109}	Glu^{109}
pOPE121	AAG	GAG	Lys^{121}	Glu^{121}
pOPE126	AAA	GAA	Lys^{126}	Glu^{126}
pOPE181	AAG	GAG	Lys^{181}	Glu^{181}
pOPG74	AAG	GGG	Lys^{74}	Gly^{74}
pOPG121	AAG	GGG	Lys^{121}	Gly^{121}
pOPG126	AAA	GGA	Lys^{126}	Gly^{126}
pOPQ121	AAG	CAG	Lys^{121}	Gln^{121}
pOPQ126	AAA	CAA	Lys^{126}	Gln^{126}

an Applied Biosystems DNA synthesizer.

Western Immunoblotting—Whole cell lysates of *E. coli* CE1248 expressing the mutant forms of OprP were loaded on to 12% polyacrylamide gels and subjected to SDS-polyacrylamide gel electrophoresis. The gels were transferred to nitrocellulose membranes and blotted with monomer-specific anti-OprP rabbit serum as described previously (3).

Purification of Mutant Proteins—Overnight cultures of *E. coli* CE1248 expressing the mutant forms of OprP were pelleted, resuspended in 20% sucrose containing 50 μg/ml DNase, and broken by passage through a French press. Outer membranes were isolated using a two-step sucrose density gradient as described previously (15). The isolated outer membranes were subjected to a stepwise solubilization with octyl-polyoxyethylene (16), and the detergent-purified mutant proteins were loaded on to preparative SDS-polyacrylamide gels and electrophoresed. The nondenatured proteins were excised and eluted overnight at 4 °C into 10 mM Tris-HCl (pH 8.0) containing 0.1% SDS.

Planar Lipid Bilayer Experiments—Analysis of the channel characteristics of the OprP mutant proteins was accomplished using planar lipid bilayer techniques as described previously (4, 17). Membranes were composed of 2% oxidized cholesterol.

RESULTS

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, Lys^{74}, Lys^{121}, and Lys^{126} were replaced with glycines, Lys^{121} and Lys^{126} were replaced with glutamines, and a triple mutant in which Lys^{74}, Lys^{121}, and Lys^{126} were all replaced with glutamates was also created. The secondary PCR products were digested with either *Hin*dIII (Lys^{13}-Lys^{74}) or *Eco*RV/*Sph*I (Lys^{109}-Lys^{181}) 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 antiserum. 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 (Lys^{13}, Lys^{15}, Lys^{25}, Lys^{30}, Lys^{109}, and Lys^{181}) 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 Lys^{74} and Lys^{126} mutants exhibited levels of conductance that were approximately one-half of that of the wild-type protein, while the Lys^{121} mutant possessed a conductance of approximately one-third of that of wild-type OprP (Fig. 1).

TABLE II
Single-channel conductance of OprP Lys→Glu mutants

The average single-channel conductance of the OprP mutant proteins in various concentrations of potassium chloride was determined using the planar lipid bilayer method. The salt solutions were used unbuffered (pH approximately 6).

OprP mutation	Average single-channel conductance (pS)[a]		
	0.1 M KCl	1 M KCl	3 M KCl
Wild-type	103 ± 45	230 ± 42	261 ± 51
Lys^{13}→Glu	78 ± 25	194 ± 33	220 ± 42
Lys^{15}→Glu	74 ± 24	207 ± 46	ND[b]
Lys^{25}→Glu	88 ± 30	210 ± 51	236 ± 64
Lys^{30}→Glu	93 ± 40	206 ± 60	308 ± 60
Lys^{74}→Glu	41 ± 14	116 ± 34	363 ± 83
Lys^{109}→Glu	85 ± 14	221 ± 34	ND
Lys^{121}→Glu	10 ± 2	74 ± 11	248 ± 36
Lys^{126}→Glu	25 ± 6	99 ± 17	103 ± 23
Lys^{181}→Glu	79 ± 14	220 ± 22	ND

[a] Average of 100 single-channel events expressed in picosiemens ± S.D.
[b] ND, not determined.

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 Lys^{74} and the Lys^{121} mutant proteins formed channels that displayed linear concentration-conductance relationships at up to 3 M KCl. The Lys^{126} 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_i-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 M KCl was determined prior to the addition of P_i (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_{50} 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 Lys^{74} mutant had a slightly lowered affinity for P_i with a maximum inhibition of 58% and an I_{50} concentration of 1.95 mM compared to 0.96 mM for wild-type OprP. The Lys^{121} substitution had a

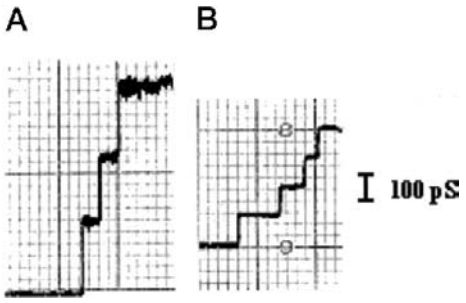

FIG. 1. **Single-channel events of wild-type OprP and Lys^{121} → Glu mutant porin.** Chart recorder tracings of the increasing single-channel steps resulting from the incorporation of wild-type OprP (*A*) and the Lys^{121} → Glu substitution mutant channels (*B*) into lipid membranes. The bathing solution was 1 M KCl (pH 6.0).

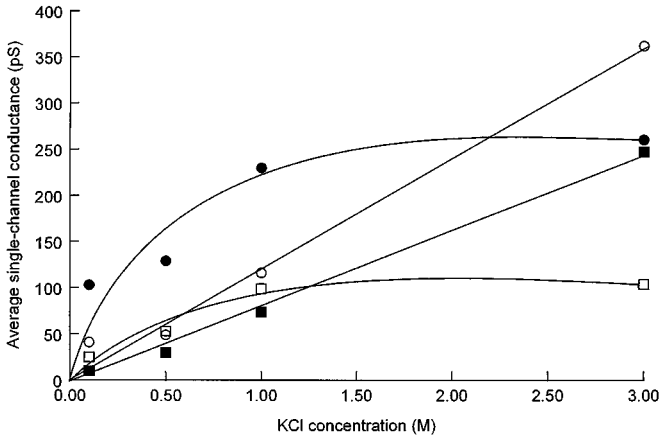

FIG. 2. **KCl concentration-conductance relationship of selected OprP Lys → Glu mutants.** The single-channel conductance of three of the Lys → Glu OprP mutants along with wild-type OprP was determined in a number of KCl concentrations, and the values were plotted as a function of the salt concentration. ●, OprP; ○, Lys^{74} → Glu; ■, Lys^{121} → Glu; □, Lys^{126} → Glu.

profound effect on the ability of the protein to bind phosphate ions. This mutant showed a maximum inhibition of 30%, and while the I_{50} 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 Lys^{126} 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 Lys^{74}, Lys^{121}, and Lys^{126} mutant proteins along with wild-type OprP plotted as a function of increasing phosphate ion concentrations.

Channel Characteristics of Lys → Gly, Lys → Gln, and Lys$^{74, 121, 126}$ → Glu Mutant Proteins—To further examine the roles Lys^{74}, Lys^{121}, and Lys^{126} 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 Lys^{74}, Lys^{121}, and Lys^{126} all substituted with glutamates was also created and analyzed. Substituting Lys^{74} 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 Lys^{74} → Glu mutant protein. The phosphate-induced conductance inhibition of the Lys^{74} → Gly mutant was comparable to that of the wild-type protein.

Substituting the Lys^{126} 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 Lys^{126} → Glu mutant. Substituting Lys^{121} 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 Lys^{121} → Glu mutant protein.

The single-channel conductance of the Lys$^{74, 121, 126}$ → Glu triple mutant was somewhat lower than any of the single mutants in both 0.1 M and 1 M KCl; however, the phosphate-

TABLE III
P_i binding of OprP Lys→Glu mutants

The phosphate-induced inhibition of chloride conductance of the mutant channels was determined by measuring the single-channel conductance in 0.1 M KCl containing increasing concentrations of potassium phosphate.

OprP mutation	Phosphate inhibition of chloride conductance	
	Maximum inhibition[a]	I_{50}[b]
	%	mM
Wild-type	74	0.96
Lys^{13}→Glu	89	0.62
Lys^{15}→Glu	87	1.20
Lys^{25}→Glu	79	1.00
Lys^{30}→Glu	81	0.73
Lys^{74}→Glu	58	1.95
Lys^{109}→Glu	79	0.84
Lys^{121}→Glu	30	>10
Lys^{126}→Glu	84	0.81
Lys^{181}→Glu	80	0.90

[a] Maximum conductance inhibition after addition of 3.3 mM potassium phosphate.

[b] Concentration of phosphate ions resulting in a 50% decrease in chloride ion conductance as assessed by the single-channel conductance at different phosphate ion concentrations.

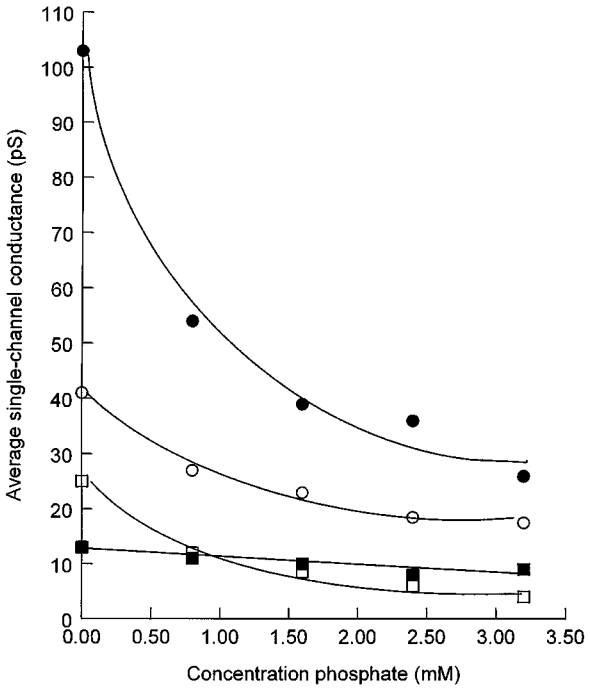

FIG. 3. **Phosphate-induced inhibition of chloride conductance.** The ability of selected OprP Lys → Glu mutants to bind P_i was assessed by measuring the inhibition of single-channel conductance (in 0.1 M KCl) induced by the addition of increasing concentrations of phosphate ions. Single-channel conductance was plotted as a function of phosphate ion concentration. ●, OprP; ○, Lys^{74} → Glu; ■, Lys^{121} → Glu; □, Lys^{126} → Glu.

TABLE IV
Channel characteristics of OprP Lys→Gly and Lys→Gln mutants

The average single-channel conductance of the Lys^{74}, Lys^{121}, and Lys^{126} substitution mutant proteins in 0.1 and 1 M potassium chloride was determined using the planar bilayer method. The salt solutions were used unbuffered (pH approximately 6). The phosphate-induced inhibition of chloride conductance was determined by measuring the single-channel conductance of the mutant proteins in 0.1 M potassium chloride containing increasing concentrations of potassium phosphate.

OprP mutation	Average single-channel conductance[a]		Maximum inhibition[b]
	0.1 M KCl	1 M KCl	
			%
Wild-type	103 ± 45	230 ± 42	74
Lys^{74}→Glu	41 ± 14	116 ± 34	58
Lys^{74}→Gly	39 ± 9	205 ± 35	72
Lys^{121}→Glu	10 ± 2	74 ± 11	30
Lys^{121}→Gly	8 ± 2	84 ± 26	16
Lys^{121}→Gln	10 ± 3	67 ± 18	24
Lys^{126}→Glu	25 ± 6	99 ± 17	79
Lys^{126}→Gly	13 ± 3	52 ± 8	ND[c]
Lys^{126}→Gln	13 ± 2	87 ± 28	ND[c]
$Lys^{74,121,126}$	4 ± 1	47 ± 9	20

[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.

induced inhibition of conductance of this mutant channel was in the range of the Lys^{121} 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 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^{121} → 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. Alternately, 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 KCl 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 porins. 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^{121} 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 constricting the interior of the channels formed by several bacterial porins (19–21). The equivalent lysine residue in PhoE (Lys^{125}) 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.

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