Phosphate-Binding Site of *Pseudomonas aeruginosa* Outer Membrane Protein P

ROBERT E. W. HANCOCK,[1] E. A. WOROBEC,[1] K. POOLE,[1] AND ROLAND BENZ[2]

Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1W5,[1] and Lehrstuhl für Biotechnogie, Universität Würzburg, D8700 Würzburg, Federal Republic of Germany[2]

When *Pseudomonas aeruginosa* cells are grown in phosphate-deficient medium, a high-affinity phosphate transport system is induced. Although some features of this transport system are quite similar to the well-studied phosphate-specific transport system of *Escherichia coli* (20), the outer membrane transport component, protein P, shows substantial differences from the equivalent component, the PhoE porin (2), of the phosphate-specific transport system. For example, the PhoE protein is a typical porin demonstrating large, weakly selective channels (although it has been suggested that PhoE is polyphosphate selective [12]). In contrast, protein P forms constricted, anion-specific channels (3, 9) which contain a strong phosphate-binding site (8). This paper reviews the attempts of our laboratories to relate the structure of protein P to its function in phosphate transport.

Coregulation of Protein P with a Phosphate Starvation-Inducible Regulon

The syntheses of a number of proteins are derepressed in *P. aeruginosa* upon growth in phosphate-limiting (0.2 mM P_i) medium. These include outer membrane protein P, a periplasmic phosphate-binding protein, and both periplasmic and excreted alkaline phosphatase and phospholipase C (16, 17). Coincidentally, a high-affinity phosphate transport system is derepressed (16, 17). A pleiotropic negative mutant strain was isolated which failed to respond to phosphate starvation by increasing levels of the above proteins. In addition, a mutant constitutive for alkaline phosphatase was additionally constitutive for phospholipase C, protein P, and the phosphate-binding protein (17). These data strongly support the existence of a phosphate regulon in *P. aeruginosa*, with protein P actively involved in phosphate transport across the outer membrane.

Involvement of Protein P and a Phosphate-Binding Protein in Phosphate Transport In Vivo

Mutants lacking protein P and a phosphate-binding protein were isolated. In the case of protein P, a Tn*501* insertion mutant was produced and confirmed as protein P deficient by its inability to interact with a protein P-specific antiserum (19). This mutant exhibited a K_m for high-affinity P_i transport 10 times greater than that of the parent strain (19).

Using the procedure of Brinkman and Beckwith (6), mutants constitutive for alkaline phosphatase and deficient in phosphate-binding protein were obtained (17). These mutants lacked a high-affinity phosphate transport system. Both of the above classes of mutants were deficient in their abilities to grow in phosphate-limiting medium. These data demonstrate that both protein P and the phosphate-binding protein are components of a high-affinity phosphate transport system.

Immunochemistry of Protein P

Protein P was demonstrated to form sodium dodecyl sulfate (SDS)-stable trimers (1) which dissociate into monomers of 47,000 daltons upon SDS solubilization at high temperatures (9). Immunoblot assays revealed that polyclonal antibodies raised against the native trimer form of protein P reacted exclusively with the trimer and not the monomer form. Conversely, P monomer-specific antiserum interacted only with the monomer (18; E. Worobec, unpublished data). This suggested that the major epitopes on the trimer are conformational.

Using a protein P trimer-specific polyclonal antiserum, it was possible to show by immunoblot assays a cross-reactivity of the oligomeric forms of phosphate starvation-inducible outer membrane proteins from bacteria representing 11 different genera of the families *Enterobacteriaceae* and *Pseudomonadaceae* (18). This antiserum did not react with the monomer form of these proteins, and P monomer-specific antiserum did not react with either the monomer or oligomer forms of any of these proteins (18). This demonstrated conserved conformational epitopes which may reflect the related porin functions of these proteins.

Protein P, in the native trimer conformation (purified by electroelution as described by Parr et al. [14]), was found to be resistant to all proteases tested except for trypsin, proteinase

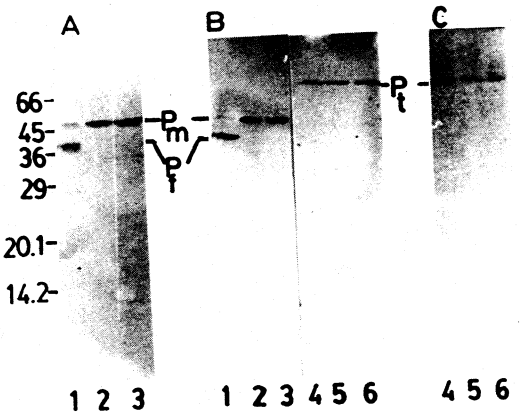

FIG. 1. SDS-PAGE and corresponding immunoblots of proteolytically digested protein P trimer. P_t, P trimer; P_m, P monomer; P_f, proteolytic fragment of P. Purified protein P trimer was mixed at a 50:1 weight ratio with a variety of proteases. In all cases digestion was for 24 h at 37°C. The digestion products were run on SDS-PAGE, either unheated (lanes 4–6) or heated at 100°C for 10 min prior to electrophoresis (lanes 1–3). SDS-PAGE and immunoblots were performed as previously described (13). (A) Immunoblot using polyclonal P monomer-specific antiserum at a 1:200 dilution. (B) Coomassie blue-stained SDS-PAGE. (C) Immunoblot using polyclonal P trimer-specific antiserum at a 1:200 dilution. Lanes 1 and 4, Protein P digested with pronase (similar data were obtained with trypsin and proteinase K); lanes 2 and 5, protein P digested with papain (similar data were obtained with chymotrypsin, carboxypeptidase A, and *Staphylococcus aureus* V8 protease); lanes 3 and 6, untreated protein P. Molecular weights of protein standards ($\times 10^3$) are indicated on the left.

K, and pronase. These enzymes did not alter the mobility of the trimer on SDS-polyacrylamide gel electrophoresis (PAGE), and proteolysis of protein P trimers with proteinase K had no effect on its pore-forming function as assayed by the black lipid model membrane system (Worobec, unpublished data). Heating the trimers in SDS resulted in the appearance of monomers with molecular weights of 37,000 (cf. 47,000 for the native protein) (Fig. 1B). P monomer-specific antiserum recognized these digestion products (Fig. 1A). Thus, there appears to be a protease-susceptible site on protein P which can be cleaved without grossly perturbing the functional trimer. This attests to the importance of tertiary and quarternary interactions in the formation of protein P trimers.

Black Lipid Bilayer Studies on Protein P

When purified and reconstituted into black lipid bilayer membranes (9), protein P trimers form well-defined channels with a considerably smaller single-channel conductance (0.25 nS in 1 M KCl) than other porin proteins (usually larger than 1.5 nS) (4, 5). Zero current potential measurements of ion selectivity demonstrated that this protein is more than 100-fold selective for anions over cations (3). This high selectivity for anions allowed an estimation of the effective diameter of the channel, 0.6 nm, based on the permeability of the channel for anions of different sizes (3). The basis of anion specificity is the presence of an anion-binding site within the channel with a binding constant, K_d, of, for example, 40 mM for Cl^-.

Lipid bilayer experiments in the presence of phosphate ions indicated that protein P channels were permeable to phosphate. Macroscopic conductance experiments revealed that phosphate was capable of inhibiting chloride flux in a dose- (Fig. 2) and pH-dependent fashion (8). From this information, I_{50} values (concentration of inhibitor giving 50% inhibition) were calculated (e.g., I_{50} = 0.38 mM P_i at pH 8.0). These values suggested that the affinity of protein P for phosphate was 60- to 100-fold greater than the affinity for other ions such as Cl^-. Phosphate analogs, such as pyrophosphate and arsenate, also inhibited the transport but not to the extent seen with phosphate (I_{50} at pH 7.0 = 4.9 mM PP_i and 1.3 mM AsO_4^{2-}, respectively).

Chemical Modification Studies on Protein P

To probe the nature of the phosphate-binding site of protein P, the ϵ-amino groups of the available lysine residues in the trimer were chem-

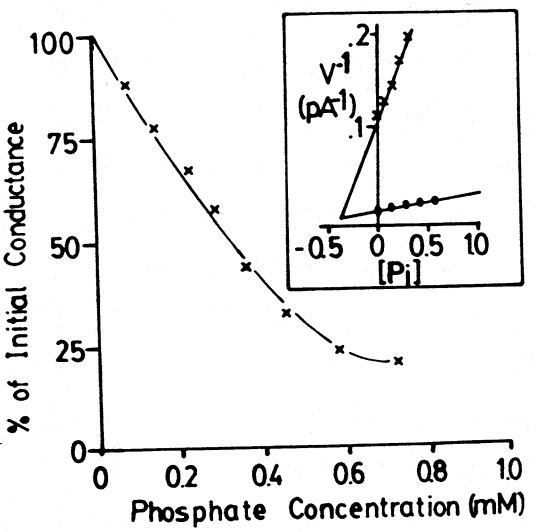

FIG. 2. Phosphate inhibition of chloride conductance at pH 8.0. In the insert, a Dixon plot is presented in which the inverse of the measured membrane current (at 20 mV applied voltage) was graphed against the cumulative phosphate concentration (millimolar) for two experiments in which different numbers of protein P channels were inserted into the membrane.

TABLE 1. Effect of chemical modification of the lysines of protein P on single-channel conductance and affinity for chloride and phosphate

Lysine modification	Single-channel conductance in 1 M KCl (pS)[a]	Affinity (mM) for:	
		Cl^-[b]	Phosphate[c]
None	260	40	0.46
Trinitrophenylation	290	ND[d]	ND
Methylation	140	1,000	1.25
Acetylation	25	>3,000	>90[e]
Carbamylation	43	>3,000	>90[e]

[a] Averaged for >200 single-channel events.
[b] Estimated as the K_d for Cl^- from experiments testing the effect of Cl^- concentration on single-channel conductance.
[c] Estimated as the I_{50} from experiments like that depicted in Fig. 2.
[d] ND, Not determined, but the channel remained strongly anion selective.
[e] These data were extrapolated from experiments like that in Fig. 2 but performed in the presence of 1 M KCl.

ically modified, and the protein was then studied for its ability to transport anions in the lipid bilayer system (Table 1). Modification of all available lysines with the bulky reagent trinitrobenzene sulfonate, by the method of Fields (7), had little effect on protein P channel properties, indicating that the critical lysine groups are located in a constriction of the channel which was not available for modification (10a). To confirm this hypothesis, the lysine ϵ-amino groups were modified by use of less bulky reagents (8). Acetylation (10) and carbamylation (15, 22) produced uncharged residues and destroyed the anion- and phosphate-binding sites (Table 1). Methylation (11) resulted in the modification of lysines but still allowed the retention of the positive charge. Methylated protein P channels were still anion specific but had an altered affinity for chloride and phosphate ions (Table 1). In all cases, the modified proteins retained their trimer conformation as determined by SDS-PAGE. These data strongly suggest that lysines are the key residues involved in both the anion- and phosphate-binding sites of protein P, and suggest that these two sites involve the same lysine residues.

Other Phosphate Starvation-Induced Membrane Proteins

A number of bacterial strains were shown to synthesize phosphate starvation-inducible outer membrane proteins (18). Four members of the family *Pseudomonadaceae* (*Pseudomonas fluorescens* branch) produced proteins which had several functional characteristics in common with protein P. Those examined were found to form small (180–297 pS) anion-selective channels which possessed phosphate-binding sites (K. Poole, T. R. Parr, and R. E. W. Hancock, submitted for publication).

Physical Association between Protein P and the Periplasmic Phosphate-Binding Protein

An association between the maltose-binding protein and LamB porin protein of *E. coli* has been suggested to be necessary for the transport of maltose and maltodextrins across the *E. coli* outer membrane (23). To determine whether the same was true for the high-affinity transport system involving protein P and the periplasmic phosphate-binding protein, a variety of methods were used to examine the association in vitro. By use of a modified enzyme-linked immunosorbent assay, the phosphate-binding protein (17) was immobilized in the bottom of wells of microtiter plates and examined for its ability to interact with protein P. The association of protein P with the immobilized phosphate-binding protein was indicated by a 56% increase over background of an enzyme-linked immunosorbent assay using protein P-specific antibodies (K. Poole, Ph.D. thesis, University of British Columbia, Vancouver, British Columbia, Canada, 1986). This assay was reproducible but of low sensitivity. Numerous attempts to demonstrate interaction, using either protein P or phosphate-binding protein immobilized on an affinity column matrix, were unsuccessful.

Black lipid bilayer experiments were performed in which protein P and a 300-fold molar excess of phosphate-binding protein were mixed prior to the measurement of macroscopic conductance in the presence of 1 M KCl (Fig. 3). The presence of the phosphate-binding protein caused a 10-fold decrease in the final conductance level achieved. However, we were unable to distinguish between the possibilities that the binding protein caused blockage of channels or that it prevented incorporation of protein P into the membrane. These data, while by no means conclusive, indicate a physical association of these proteins.

Discussion

Protein P provides an excellent model protein for examination of structure-function relationships in facilitated diffusion proteins. The presence within the channel of a defined binding site(s) for phosphate (K_d, approximately 0.3 mM P_i) and other anions (e.g., K_d = 40 mM Cl^-) provides this protein with a set of easily studied functional properties including anion specificity, saturation at higher salt concentrations, and competitive blockage of anion (e.g., Cl^-) movement by the preferred anionic substrate phos-

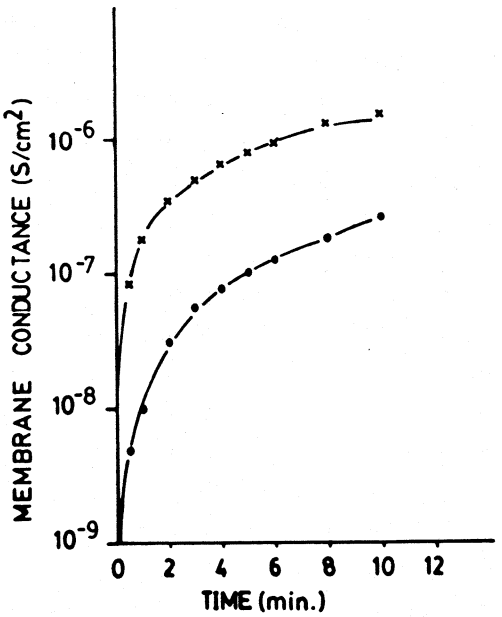

FIG. 3. Inhibition of chloride conduction of protein P by the periplasmic phosphate-binding protein. Protein P was incubated with a 300-fold molar excess of the phosphate-binding protein prior to lipid bilayer experiments. Both proteins were present in soluble forms in 20 mM Tris hydrochloride (pH 6.8) plus 0.05% (vol/vol) Triton X-100. The experiments were carried out in 1 M KCl and 20 mV was the applied voltage. Results from an average of five to eight separate experiments are presented. A conductance of 10^{-7} S/cm^2 is equivalent to 400 protein P channels per cm^2. Symbols: ×, protein P alone; ●, protein P plus the phosphate-binding protein.

phate. This has allowed us to provide a mathematical model for anion movement through the protein P channel (R. Benz and R. E. W. Hancock, submitted for publication). The simplest form of this model is a two-barrier, one-site model. In this model there is a binding site within the channel. Binding of an anion to this site is preceded by an energy barrier (presumably partial dehydration of the anion) and is followed by a second energy barrier (probably due to dissociation of the anion from the binding site). The net energy level of the anion approaching the site and departing from the site would be identical since the anion would almost certainly be hydrated at both times. Nevertheless, these energy barriers must be overcome, and presumably the "driving force" for this would be the concentration gradient. In this regard, the presence of a stronger binding site in the periplasm, like the high-affinity binding site (K_d = 0.3 μM P_i) of the phosphate-binding protein, would be valuable in maintaining the concentration gradient. The possible interaction of protein P and the phosphate-binding protein may favor this mechanism (i.e., maintenance of the concentration gradient), although it is not obligatory.

Protein P, like other porins, has certain disadvantages in structural studies. The apparent lack of linear epitopes on the native trimer, and the maintenance of a stable trimer form even after protease digestion, suggest that protein P has extensive tertiary and quaternary structure (possibly β-sheet folding like other porins [21]). Therefore, we anticipate some difficulty in determining its three-dimensional configuration. Nevertheless, its production in large amounts, ease of purification, and extreme stability to detergents and chemical reagents have allowed us to provide a realistic model for the phosphate/anion-binding site. Given the data described above showing that the protein is a trimer (1), contains its phosphate/anion-binding site in a channel constriction of approximately 0.6 nm (3), and contains lysines as critical residues in the binding site (10), we have suggested (8) that three lysines (one per monomer subunit) are symmetrically arranged in a circle of approximately 0.6 nm diameter within the channel. The HPO_4^{2-} anion is about 0.58 nm in diameter and contains three symmetrically arranged charges represented by two negatively charged oxygens and a partial negative charge on the double-bonded oxygen. Thus, in this model the symmetrical positive charges on the lysine ϵ-amino groups would "coordinate" the symmetrical negative charges on these oxygens. Our current research is directed at determining the protein structure in the neighborhood of this binding site with a view to testing and expanding on this model.

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Medical Research Council (for the immunochemistry experiments) to R.H., the Deutsche Forschungsgemeinschaft (Be865/1-2 and Be865/3-1) to R.B., and the Alberta Heritage Foundation for Medical Research in the form of a postdoctoral fellowship to E.W.

LITERATURE CITED

1. **Angus, B. L., and R. E. W. Hancock.** 1983. Outer membrane porin proteins F, P and D1 of *Pseudomonas aeruginosa* and PhoE of *Escherichia coli*: chemical cross-linking to reveal native oligomers. J. Bacteriol. **155**:1042–1051.
2. **Benz, R., R. P. Darveau, and R. E. W. Hancock.** 1984. Outer membrane protein PhoE from *Escherichia coli* forms anion selective pores in lipid bilayer membrane. Eur. J. Biochem. **140**:319–324.
3. **Benz, R., M. Gimple, K. Poole, and R. E. W. Hancock.** 1983. An anion-selective channel from the *Pseudomonas aeruginosa* outer membrane. Biochim. Biophys. Acta **730**: 387–390.
4. **Benz, R., and R. E. W. Hancock.** 1981. Properties of the large ion-permeable pores formed from protein F of *Pseudomonas aeruginosa* in lipid bilayer membranes. Bio-

chim. Biophys. Acta **646**:298–308.

5. **Benz, R., A. Schmid, and R. E. W. Hancock.** 1985. Ion selectivity of gram-negative bacterial pores. J. Bacteriol. **162**:722–727.
6. **Brinkman, E., and J. Beckwith.** 1975. Analysis of regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and φ80-transducing phages. J. Mol. Biol. **96**:307–316.
7. **Fields, R.** 1972. The rapid determination of amino groups with TNBS. Methods Enzymol. **25**:464–469.
8. **Hancock, R. E. W., and R. Benz.** 1986. Demonstration and chemical modification of a specific phosphate binding site in the phosphate-starvation inducible outer membrane porin protein P of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **860**:699–707.
9. **Hancock, R. E. W., K. Poole, and R. Benz.** 1982. Outer membrane protein P of *Pseudomonas aeruginosa*: formation of small anion specific channels in lipid bilayer membrane. J. Bacteriol. **150**:730–738.
10. **Hancock, R. E. W., K. Poole, M. Gimple, and R. Benz.** 1983. Modification of the conductance, selectivity and concentration-dependent-saturation of *Pseudomonas aeruginosa* protein P channels by chemical acetylation. Biochim. Biophys. Acta **735**:137–144.

10a. **Hancock, R. E. W., A. Schmid, K. Bauer, and R. Benz.** 1986. Role of lysines in ion selectivity of bacterial outer membrane porins. Biochim. Biophys. Acta **860**:263–267.

11. **Jentoft, N., and D. G. Dearborn.** 1980. Protein labeling by reductive methylation with sodium cyanoborohydride: effect of cyanide and metal ions on the reaction. Anal. Biochem. **106**:186–190.
12. **Korteland, J., J. Tommassen, and B. Lugtenberg.** 1982. PhoE protein pore of the outer membrane of *Escherichia coli* K12 is a particularly efficient channel for organic and inorganic phosphate. Biochim. Biophys. Acta **690**:282–289.
13. **Mutharia, L. M., and R. E. W. Hancock.** 1983. Surface localization of *Pseudomonas aeruginosa* porin protein F by using monoclonal antibodies. Infect. Immun. **42**:1027–1033.
14. **Parr, T. B., Jr., K. Poole, G. W. K. Crockford, and R. E. W. Hancock.** 1986. Lipopolysaccharide-free *Escherichia coli* OmpF and *Pseudomonas aeruginosa* protein P porins are functionally active in lipid bilayer membranes. J. Bacteriol. **165**:523–526.
15. **Plapp, B. V., S. Moore, and W. H. Stein.** 1971. Activity of bovine pancreatic deoxyribonuclease A with modified amino groups. J. Biol. Chem. **246**:939–945.
16. **Poole, K., and R. E. W. Hancock.** 1983. Secretion of alkaline phosphatase and phospholipase C in *Pseudomonas aeruginosa* is specific and does not involve an increase in outer membrane permeability. FEMS Microbiol. Lett. **16**:25–29.
17. **Poole, K., and R. E. W. Hancock.** 1984. Phosphate transport in *Pseudomonas aeruginosa*: involvement of a periplasmic phosphate-binding protein. Eur. J. Biochem. **144**: 607–612.
18. **Poole, K., and R. E. W. Hancock.** 1986. Phosphate-starvation-induced outer membrane proteins of members of the families *Enterobacteriaceae* and *Pseudomonadaceae*: demonstration of immunological cross-reactivity with an antiserum specific for porin protein P of *Pseudomonas aeruginosa*. J. Bacteriol. **165**:987–993.
19. **Poole, K., and R. E. W. Hancock.** 1986. Isolation of a Tn*501* insertion mutant lacking porin protein P of *Pseudomonas aeruginosa*. Mol. Gen. Genet. **202**:403–409.
20. **Rosenberg, H., R. G. Gerdes, and K. Chegwidden.** 1977. Two systems for the uptake of phosphate in *Escherichia coli*. J. Bacteriol. **131**:505–511.
21. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*. Regular arrangement on the peptidoglycan and unusual dodecylsulfate binding. J. Biol. Chem. **249**:8019–8029.
22. **Stark, G.** 1972. Use of cyanate for determining NH_2-terminal residues in protein. Methods Enzymol. **25**:579–584.
23. **Wandersmann, C., M. Schwartz, and F. Ferenci.** 1979. *Escherichia coli* mutants impaired in maltodextrin transport. J. Bacteriol. **140**:1–13.

Phosphate Metabolism and Cellular Regulation in Microorganisms

Edited by

Annamaria Torriani-Gorini
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts

Frank G. Rothman
Division of Biology and Medicine
Brown University
Providence, Rhode Island

Simon Silver
Department of Microbiology and Immunology
The University of Illinois College of Medicine
Chicago, Illinois

Andrew Wright
Department of Molecular Biology and Microbiology
Tufts University
Boston, Massachusetts

Ezra Yagil
Department of Biochemistry
The George S. Wise Faculty of Life Sciences
Tel Aviv University
Tel Aviv, Israel

AMERICAN SOCIETY FOR MICROBIOLOGY
Washington, D.C.