

## Phosphate transport in *Pseudomonas aeruginosa* Involvement of a periplasmic phosphate-binding protein

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A binding protein for inorganic phosphate was purified to apparent homogeneity from the shock fluids of phosphate-limited *Pseudomonas aeruginosa*. The purified protein bound one molecule of phosphate per molecule of binding protein with an average  $K_d$  of 0.34  $\mu\text{M}$ . Arsenate, pyrophosphate and polyphosphates up to 15 units long could inhibit the binding of phosphate to the binding protein, although organic phosphates, such as glucose 6-phosphate, glycerol 3-phosphate and adenosine 5'-monophosphate could not. Mutants lacking the phosphate-binding protein were isolated and shown to be deficient in phosphate transport compared with wild-type cells. Two kinetically distinct systems for phosphate uptake could be observed in wild-type cells, with apparent  $K_m$  values of  $0.46 \pm 0.10 \mu\text{M}$  (high affinity) and  $12.0 \pm 1.6 \mu\text{M}$  (low affinity). In contrast, only a single low-affinity transport system was observable in mutants lacking the binding protein ( $K_m$  apparent =  $19.3 \pm 1.4 \mu\text{M P}_i$ ), suggesting the involvement of the binding protein in the inducible high-affinity phosphate-uptake system of *P. aeruginosa*.

The transport of inorganic phosphate has been documented in a number of bacteria including *Staphylococcus aureus* [1], *Streptococcus faecalis* [2], *Bacillus cereus* [3], *Micrococcus lysodeikticus* [4], *Escherichia coli* [5–7], and most recently *Pseudomonas aeruginosa* [8]. Phosphate transport in *P. aeruginosa* occurs with biphasic kinetics [8] suggesting the involvement of both a high-affinity and a low-affinity component. Thus, phosphate uptake in *P. aeruginosa* may resemble that of *E. coli*, where a high-affinity and low-affinity system have been identified [6, 7].

The high-affinity system in *E. coli* requires a phosphate-binding protein (the *phoS* gene product) for maximal activity [6] and for maintenance of its high affinity [9]. Similarly, the ability of osmotic shock (known to disrupt binding-protein-dependent transport processes [10]) to reduce phosphate transport dramatically in *P. aeruginosa* at low phosphate concentrations [8] favours the involvement of a binding protein in high-affinity phosphate transport in *P. aeruginosa*. Furthermore, we have previously observed that phosphate limitation, which derepresses the phosphate-binding protein and induces high-affinity phosphate uptake in *E. coli* [6], also results in the synthesis of several protein species in *P. aeruginosa*. These include alkaline phosphatase and phospholipase C [11], an outer-membrane channel-forming protein (protein P) [12] and a 37-kDa protein, which exists as the major protein in the periplasm [12]. The observed anion-selectivity of protein P channels in black lipid bilayer membranes [12] argues for a role in phosphate movement across the outer membrane for

this protein. However, the model system studies suggest that this protein channel is not unidirectional. Thus, the uptake of phosphate into cells would probably require the existence of an extrinsic high-affinity phosphate-binding site, for example a periplasmic phosphate-binding protein. The derepression of the 37-kDa protein by phosphate limitation and the magnitude of its production in the periplasm make this protein a good candidate for the phosphate-binding protein in *P. aeruginosa*. We, therefore, undertook to purify and characterize the 37-kDa protein *in vitro*, as well as to isolate mutants lacking this protein and to observe the effect on phosphate transport *in vivo*.

## MATERIALS AND METHODS

### Bacterial strains and media

*Pseudomonas aeruginosa* PAO1 strain H103 [13] was employed in the isolation of the phosphate-binding protein. *Pseudomonas aeruginosa* PAO1 strain H242 (a threonine auxotroph) was used as the parental strain in the isolation of the alkaline phosphatase constitutive mutant KAPC1. The phosphate-sufficient and phosphate-deficient minimal sodium-Hepes-buffered media used throughout have been described previously [12]. Threonine was added, as required, at a final concentration of 1  $\mu\text{M}$ . All strains were routinely maintained on 1% proteose/peptone no. 2 agar plates.

### Osmotic shock procedure and purification of phosphate-binding protein

*P. aeruginosa* PAO1 strain H103 was grown in phosphate-deficient minimal Hepes medium to induce the synthesis of the periplasmic 37-kDa protein [12]. Induced cells (6 l cell

**Abbreviations.** SDS, sodium dodecyl sulfate; XP, 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine; PAGE, polyacrylamide gel electrophoresis; PBP, phosphate-binding protein; Me<sub>2</sub>SO, dimethylsulfoxide.

**Enzymes.** Alkaline phosphatase (EC 3.1.2.1); phospholipase C (EC 3.1.4.3).

culture at  $A_{600} \approx 0.50$ ) were harvested by centrifugation at  $4^\circ\text{C}$  ( $10000 \times g$  for 10 min) and resuspended in 500 ml 10 mM Tris buffer pH 8.5 containing 0.2 M  $\text{MgCl}_2$ . Subsequently the cells were subjected to two rounds of cold shock according to the procedure described by Hoshino and Kageyama [14]. The shocked cells were removed by centrifugation ( $10000 \times g$  for 10 min) and the supernatant concentrated approximately 50 times to a final volume of 10 ml via Amicon pressure filtration using a PM10 microfilter (Amicon Corp., Danvers, MA, USA). Remaining whole cells and debris were removed by centrifugation using a clinical, table-top centrifuge. The concentrated shock fluids were then desalted by passage over a Biogel P-10 (Bio-Rad, Richmond, CA, USA) column ( $20 \times 1.5$  cm) equilibrated with 20 mM Tris buffer pH 7.4. The eluted protein peak (total volume of 15–20 ml) was applied to a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column ( $8.0 \times 1.5$  cm) also equilibrated with 20 mM Tris buffer pH 7.4. The binding protein did not bind to DEAE-Sephacel at this pH and was collected in the flow-through fraction. Binding-protein-containing fractions were pooled (total volume 25–30 ml) and the pH of the binding protein solution decreased to 5.0 with 1 M sodium acetate/acetic acid buffer pH 5.0. This solution was then applied to a CM-Sephacel (Pharmacia) column ( $4.0 \times 0.7$  cm) equilibrated with 20 mM sodium acetate/acetic acid buffer pH 5.0. At this pH the binding protein bound to the column and was eluted with a 20-ml NaCl gradient of 0.1–0.4 M. The binding protein eluted at between 0.1 M and 0.2 M NaCl as a single peak of homogeneous protein. Using this procedure, 6 l cell culture (at  $A_{600} = 0.50$ ) typically yielded approximately 4 mg pure phosphate-binding protein. ( $^{32}\text{P}$ )Orthophosphate-binding activity was monitored at all stages of the purification.)

#### Gel electrophoresis and whole-cell protein extracts

Sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis was performed as described previously [13] using a 12% (w/v) acrylamide running gel. Whole cell protein extracts were obtained as described previously [15].

#### Filter-binding assays

Periplasmic extracts (shock fluids) and purified phosphate-binding protein were screened for their ability to bind phosphate utilizing a nitrocellulose-filter-binding assay based on the method described by Lever for the histidine-binding protein [16]. Briefly, protein extracts were added to Eppendorf tubes in a final volume of 250  $\mu\text{l}$  containing 1  $\mu\text{M}$  [ $^{32}\text{P}$ ]orthophosphate (specific activity = 1 mCi/ $\mu\text{mol}$  phosphate, Amersham Corp., Oakville, Ont., Canada) and 10 mM Tris buffer pH 8.0. After 5 min at  $23^\circ\text{C}$  100- $\mu\text{l}$  aliquots were removed and filtered on nitrocellulose membrane filters (Millipore Corp., Bedford, MA, USA, type HA, 0.45  $\mu\text{m}$  pore size). After washing once with 600  $\mu\text{l}$  10 mM LiCl, the filters were removed, dried and counted in 5 ml PCS aqueous scintillation cocktail (Amersham Corp.). To determine the specificity of phosphate binding, various inhibitors, as indicated, were included in the reaction mixture.

#### Equilibrium dialysis

To determine the  $K_d$  for the phosphate-binding protein the equilibrium dialysis technique was employed. Dialysis bags (Spectrapor, 6.4 mm diameter, Spectrum Medical Industries, Inc., Los Angeles, CA, USA) were filled with 7  $\mu\text{g}$  purified

phosphate-binding protein in a final volume of 300  $\mu\text{l}$ . The binding protein solutions were dialysed against 40 ml radioactively labeled orthophosphate in 50-ml conical tubes. The concentration of phosphate ranged from 0.1  $\mu\text{M}$  to 5.0  $\mu\text{M}$ . After dialysis for 24 h at  $4^\circ\text{C}$ , 25- $\mu\text{l}$  aliquots (in duplicate) were removed from the dialysis bags and from the solutions surrounding the bags and counted in 3 ml PCS aqueous scintillation cocktail (Amersham) using a Beckman model LS7500 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA, USA). (Although dialysis bag volumes can change over the course of dialysis, reproducible volume changes of  $\leq 10\%$  were experienced using this method.)

#### Mutagenesis

Diethyl sulfate mutagenesis of *P. aeruginosa* PAO1 strain H242 was carried out as described [16a] with modifications. An overnight culture (0.1 ml) was suspended in 5 ml saturated solution of diethyl sulfate in 50 mM Hepes buffer pH 7.0 for 30 min at  $25^\circ\text{C}$ . Cells were then diluted 1:49 into proteose/peptone no. 2 broth and allowed to grow overnight at  $37^\circ\text{C}$ .

#### Selection of mutants lacking the phosphate-binding protein

After overnight growth, mutagenized cells were harvested by centrifugation and washed three times in a minimal Hepes medium without phosphate. The washed cells were resuspended in the same medium and dilutions were plated onto a minimal Hepes medium containing 1 mM phosphate (phosphate-sufficient medium) and 20  $\mu\text{g}/\text{ml}$  5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine (XP) (Bachem) [17]. [It was necessary to dissolve the XP in dimethylsulfoxide ( $\text{Me}_2\text{SO}$ ) prior to its addition to plates but the final concentration of  $\text{Me}_2\text{SO}$  was  $< 1\%$ .] This medium identified alkaline-phosphatase-constitutive mutants, which upon producing alkaline phosphatase at 1 mM phosphate (at which concentration it is normally repressed) yielded a blue-green colour due to hydrolysis of the XP by the alkaline phosphatase. After overnight growth at  $37^\circ\text{C}$ , blue-green pigmented colonies were picked and cultured overnight in phosphate-deficient minimal Hepes medium (to induce the phosphate-binding protein). Shock fluids and whole-cell protein extracts were obtained and screened using SDS/polyacrylamide gel electrophoresis for the absence of the phosphate-binding protein under inducing conditions.

#### Transport assays

Overnight cultures of *P. aeruginosa* H242 and its mutant strain KAPC1, grown in phosphate-deficient minimal Hepes medium (to induce the phosphate-binding protein in strains where it was present), were harvested by filtration and washed with three volumes of minimal Hepes medium without phosphate. Washed cells were resuspended by vortexing in the same phosphate-less medium at a final absorbance at 600 nm of 0.2–0.3 and stored on ice until needed. Prior to assaying phosphate accumulation, cells were shaken at  $37^\circ\text{C}$  for 10 min. To assay phosphate uptake, 1-ml samples of pre-warmed cells were added to 10-ml culture tubes containing radioactively labeled orthophosphate. The cells were vortexed to ensure adequate aeration, and 200- $\mu\text{l}$  aliquots were removed at various times and filtered on nitrocellulose membrane filter cups (0.45  $\mu\text{m}$  diameter, Amicon Corp) in an Amicon vacuum manifold. Filtered cells were washed twice with 1.5 ml minimal Hepes medium containing 1 mM cold

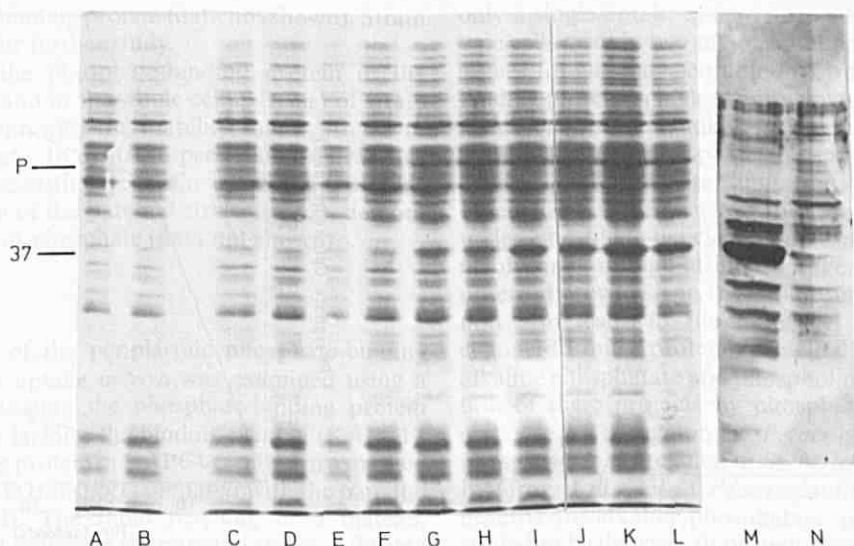


Fig. 1. Induction of the 37-kDa periplasmic protein by phosphate limitation. Cells grown under phosphate-sufficient conditions were harvested, washed in phosphate-free minimal Hepes medium and resuspended in phosphate-deficient minimal Hepes medium at an absorbance of 0.20 at 600 nm. Cells were shaken at 37°C, and at 15-min intervals cell samples were removed and whole cell protein extracted and run on SDS/polyacrylamide gels (lanes A–L). Lanes M and N represent an SDS/PAGE of periplasmic extracts derived from phosphate-sufficient and phosphate-deficient grown cells respectively. Periplasmic proteins were visualized using a silver stain procedure [20]. All samples were solubilized at 88°C for 10 min prior to electrophoresis. P, Protein P; 37, 37-kDa protein. (Because the periplasmic extracts were extremely dilute, it was necessary to 'overstain' in order to visualize minor protein components, resulting in some background staining and smearing)

phosphate. The filters were then removed, dried and counted in 3 ml PCS scintillation cocktail (Amersham). For strain H242 it was necessary to dilute the cells 1:4 in prewarmed minimal Hepes medium at the time of assay because of the rapid rate of uptake of undiluted cells, which precluded accurate rates of phosphate uptake. For the determination of kinetic constants, initial rates of uptake only were employed using three to four time points taken over the first 40 s of transport, during which time the uptake rate was linear (see Fig. 4).

#### Other assays

Protein determinations were made using either the method of Lowry et al. [18], using bovine serum albumin as the standard, or the method of Warburg et al. [19] using absorbance at 260 nm and 280 nm.

#### Chemicals

Pyrophosphate, polyphosphates P3, P5 and P15 and the organic phosphates, glucose 6-phosphate, glycerol 3-phosphate and adenosine 5'-monophosphoric acid (AMP) were obtained from the Sigma Chemical Co. (St Louis, MO, USA).

## RESULTS

#### Purification and properties of the periplasmic phosphate-binding protein

Phosphate limitation of *Pseudomonas aeruginosa* H103 cells resulted in the induction of a major periplasmic protein of 37 kDa (Fig. 1). Using the procedure outlined in Materials and Methods it was possible to obtain a highly purified preparation of this protein (Fig. 2, lane B). Equilibrium dialysis studies (Fig. 3) revealed that the purified protein

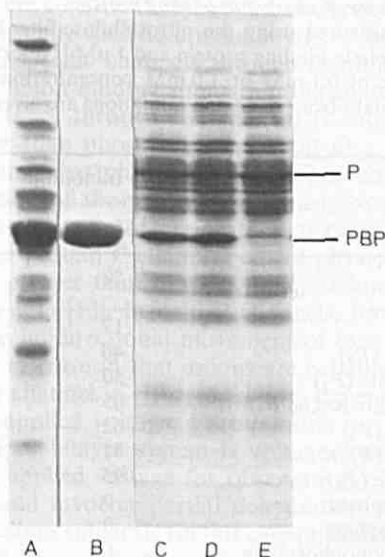


Fig. 2. SDS/polyacrylamide gel electrophoretogram of purified phosphate-binding protein (lane B) and whole-cell protein extracts of a phosphate-binding protein-deficient mutant (KAPC1) (lane E) and the parental strain (H242) (lane D) grown under inducing (i.e. phosphate-deficient) conditions. An alkaline-phosphatase-constitutive strain (KAPC3), retaining the phosphate-binding protein, is represented in lane C. Lane A represents the shock fluid (concentrated 50×) of a phosphate-limited wild-type strain, from which the phosphate-binding protein was purified. P, Protein P; PBP, phosphate-binding protein

bound 1 molecule of phosphate/molecule of protein ( $n = 0.91$  from the Scatchard plot) with an average  $K_d$  of 0.34  $\mu$ M. (From three independent experiments,  $K_d$  values of 0.29, 0.35 and 0.39  $\mu$ M were derived for the binding protein.) The speci-



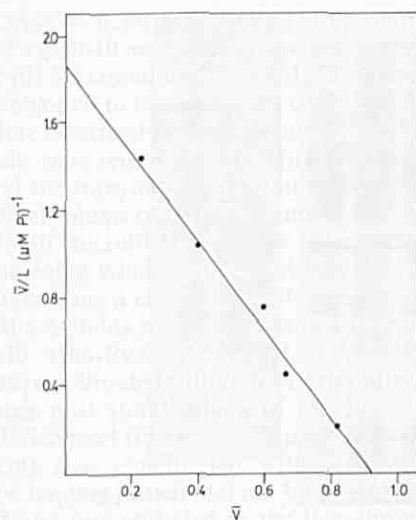


Fig. 3. Scatchard plot of phosphate-binding activity. Equilibrium dialysis binding assays were performed with 7  $\mu$ g binding protein and varying amounts of phosphate.  $\bar{V}$  represents nmol phosphate bound/nmol phosphate-binding protein.  $L$  represents the concentration of phosphate. Binding constants were derived by linear regression analysis of the data which yielded correlation coefficients ( $r$ ) of 0.96–0.99

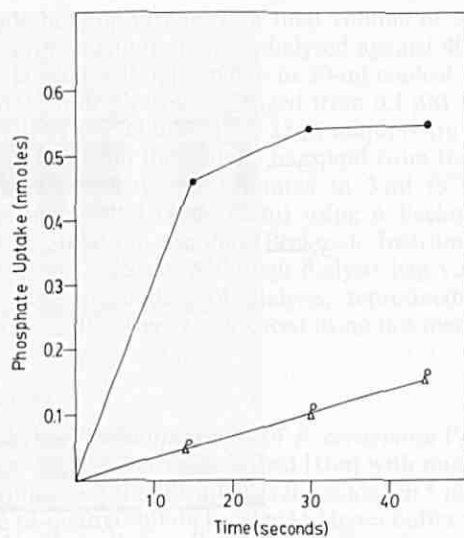


Fig. 4. Phosphate uptake in *P. aeruginosa*. The procedure for uptake assays is described in Materials and Methods. The concentration of phosphate was 2.5  $\mu$ M. All cells were assayed at an absorbance at 600 nm of 0.30 except as indicated. Strain H242 ( $\bullet$ — $\bullet$ ); strain KAPC1 ( $\Delta$ — $\Delta$ ); strain H242 diluted 1:4 (final  $A_{600}$  = 0.06) ( $\circ$ — $\circ$ )

Table 1. Specificity of phosphate-binding protein

Activity was measured using the nitrocellulose-filter-binding assay with 5  $\mu$ g phosphate-binding protein and 1  $\mu$ M [ $^{32}$ P]orthophosphate in the presence of 0.1 mM or 1.0 mM concentration of inhibitor. Representative data from three determinations are given; n.d. = not determined

Inhibitor	Inhibition	
	0.1 mM	1.0 mM
	%	
Arsenate	17	61
Pyrophosphate ( $P_2$ )	40	75
Triphosphate ( $P_3$ )	50	80
Trimetaphosphate (cyclic $P_3$ )	65	93
Polyphosphate ( $P_5$ )	62	n.d.
Polyphosphate ( $P_{15}$ )	41	n.d.
Orthophosphate ( $P_1$ )	95	99
Glucose 6-phosphate	0	0
Glycerol 3-phosphate	0	n.d.
Adenosine 5'-monophosphate	0	0

ficity of the phosphate-binding protein was tested using a number of potential inhibitors of phosphate binding (Table 1). The organic phosphates glucose 6-phosphate, glycerol 3-phosphate and adenosine 5'-monophosphate did not compete with orthophosphate for binding, even at a 1000-fold excess over orthophosphate. In contrast, polymers of phosphate from  $P_2$  (pyrophosphate) to  $P_{15}$ , as well as arsenate, inhibited the binding of orthophosphate to the binding protein.

#### Isolation of mutants lacking the phosphate-binding protein

Mutants lacking the phosphate-binding protein of *Escherichia coli* (designated *phoS*) are constitutive for alkaline

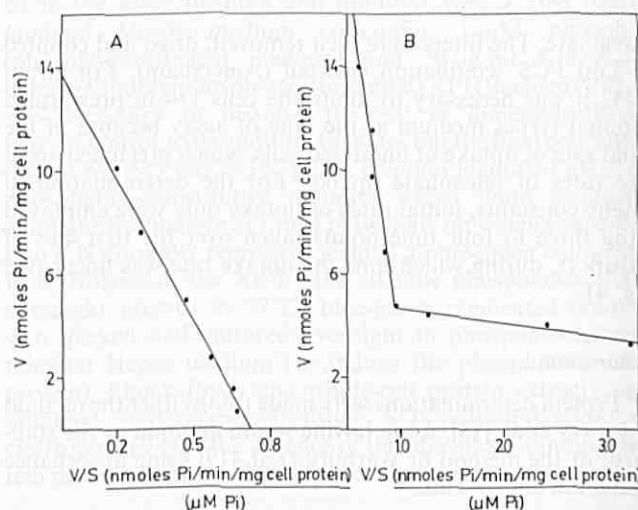


Fig. 5. Kinetics of phosphate uptake in *P. aeruginosa*. Data from uptake assays, performed at various concentrations of phosphate, were plotted as an Eadie-Hofstee plot with kinetic constants derived by least-squares analysis. (A) Mutant strain KAPC1 containing only a single phosphate-uptake system. (B) Wild-type strain H242 containing two phosphate-uptake systems. (Representative results from a single experiment)

phosphatase [21]. Therefore, to obtain mutants lacking the phosphate-binding protein in *P. aeruginosa* we selected mutants constitutive for alkaline phosphatase using the procedure of Brickman and Beckwith [18]. Of nine alkaline-phosphatase-constitutive mutants obtained, four lacked the phosphate-binding protein on SDS/polyacrylamide gels (e.g. Fig. 2, lane E). The remainder were pleiotropically positive for a number of phosphate-regulated constituents, in addition to alkaline phosphatase, including protein P, phospholipase

C and the phosphate-binding protein (data not shown). Strain KAPC1 was chosen for further study.

The absence of the phosphate-binding protein in the periplasmic extracts (and in the whole-cell extracts) of strain KAPC1 was consistent with the inability of these extracts to bind labelled phosphate. In contrast, periplasmic extracts of alkaline-phosphatase-constitutive strains retaining the binding protein, as well as of the parental strain (H242), demonstrated good binding of phosphate (data not shown).

### Phosphate transport

The involvement of the periplasmic phosphate-binding protein in phosphate uptake *in vivo* was examined using a wild-type strain containing the phosphate-binding protein (H242) and a mutant lacking the binding protein (KAPC1). The loss of the binding protein in KAPC1 resulted in a marked deficiency in phosphate transport compared with the parental strain (H242) (Fig. 4). The rapid reaching of a plateau, observed for the uptake curve of the parental strain, indicated that the available phosphate was being depleted, precluding the determination of an accurate rate of transport at the concentration of phosphate used in Fig. 4. Indeed, it was necessary to dilute the wild-type parental cells 1:4 compared with mutant cells in order to obtain a comparable rate of phosphate uptake.

### Kinetics of phosphate transport

In wild-type cells of *P. aeruginosa*, two major components of phosphate uptake were observable (Fig. 5B), confirming preliminary results presented previously [8]. The high-affinity component of uptake was characterized by an apparent  $K_m$  of  $0.46 \pm 0.10 \mu\text{M}$  phosphate and a  $V_{\max}$  of  $5.4 \pm 0.2 \text{ nmol phosphate taken up min}^{-1} \text{ mg cell protein}^{-1}$  while the low-affinity component was characterized by an apparent  $K_m$  of  $12.0 \pm 1.6 \mu\text{M}$  phosphate. The extrapolated  $V_{\max}$  value for the 'low-affinity' curve ( $16 \pm 1.5 \text{ nmol phosphate taken up min}^{-1} \text{ mg cell protein}^{-1}$ ) actually represents the sum of both the high and low-affinity parameters. Given that the extrapolated  $V_{\max}$  of the high-affinity system in wild-type is  $5.4 \mu\text{M}$ , the actual  $V_{\max}$  for the low-affinity system can be estimated to be approximately  $11 \mu\text{M}$ . This is in good agreement with the value derived from the mutant strain containing only the low-affinity transport system (approximately  $12 \mu\text{M}$ ). In the phosphate-binding-protein-deficient mutant KAPC1 only a single phosphate uptake component with a  $K_m$  of  $19.3 \pm 1.4 \mu\text{M}$  phosphate and a  $V_{\max}$  of  $12.1 \pm 0.5 \text{ nmol phosphate taken up min}^{-1} \text{ mg cell protein}^{-1}$  was observable (Fig. 5A). (Kinetic constants represent the mean of at least three determinations  $\pm$  standard deviation.) Thus, the loss of the phosphate-binding protein by mutation in KAPC1 correlated with the loss of high-affinity phosphate uptake.

### DISCUSSION

This report describes the involvement of a periplasmic phosphate-binding protein in phosphate transport in *Pseudomonas aeruginosa*. Preliminary reports [8] indicated that two kinetically distinct systems of phosphate uptake were operable in *P. aeruginosa*, although owing to the complexity of the data the existence of more than two uptake components could not be ruled out. The demonstration here that the mutational removal of the phosphate-binding protein leaves

only a single uptake system argues for the existence in wild-type cells of only two major uptake systems. Furthermore, it argues for the indispensable involvement of the phosphate-binding protein in high-affinity uptake, which is eliminated in the absence of the binding protein.

High-affinity phosphate transport in *P. aeruginosa* is inducible by phosphate limitation (not shown), and the observed derepression of the phosphate-binding protein under phosphate-deficient conditions is consistent with its involvement in high-affinity uptake. In addition, it has been observed previously in this laboratory that phosphate limitation also leads to the induction of an outer-membrane channel-forming protein (protein P) [12] and the enzymes alkaline phosphatase and phospholipase C [11]. The co-induction of these proteins by phosphate limitation implies the existence of a regulon in *P. aeruginosa* analogous to the phosphate or *pho* regulon in *Escherichia coli* [22]. The isolation by Gray et al. [23] of *P. aeruginosa* mutants pleiotropically negative for alkaline phosphatase, phospholipase C, demonstrated to be deficient in protein P and the phosphate-binding protein as well (Keith Poole, unpublished results), and our isolation in this paper of single mutants constitutive for these components, further support the existence of such a regulon in *P. aeruginosa*. This co-regulation of constituents physiologically induced by phosphate deprivation suggests a common function in phosphate acquisition *in vivo*.

Although protein P is induced under conditions of phosphate limitation, its properties in planar lipid bilayer membranes are consistent with, but do not prove, its involvement in phosphate-specific uptake *in vivo*. While the protein forms anion-specific channels through membranes [12] and contains an anion-binding site within the channel [24], chloride conductance through the channel is substantially (25-fold) greater than phosphate movement at a given voltage [12], suggesting that phosphate ions approach the size limit for permeation of the protein P channel. Nevertheless, the maximal possible rate of permeation of phosphate through reconstituted protein P channels is still physiologically relevant, being greater than the  $V_{\max}$  of most known transport processes *in vivo* [12]. Protein P channels, however, do not facilitate the unidirectional movement of ions across model membranes *in vitro*, in that anions are capable of migrating through the channel in either direction, dependent upon the sign of the applied voltage. Furthermore, ion movement in the black lipid bilayer system is voltage-dependent (i.e. it requires an applied voltage for observation of anion movement) [12] and involves partial dehydration of the moving anions [25]. Since substrate (in this case phosphate) movement *in vivo* must be unidirectional from the exterior of the cell across the outer membrane and ultimately to the cell interior, in order to satisfy cellular nutritional requirements, an additional component is necessary to facilitate this unidirectional uptake across the outer membrane. The presence of a phosphate-binding protein existing exclusively in the periplasm [11] could facilitate phosphate movement into the cell by providing a high-affinity binding site at the inner surface of the outer membrane. Furthermore, if the phosphate-binding protein was physically associated with protein P channels *in vivo* this would create a highly specific binding site at or near the periplasmic opening of the protein P channel, thus increasing the unidirectional movement of phosphate through the anion-selective protein P channel. Phosphate could bind to the weak anion-binding site within the protein P channel ( $K_d = 34 \text{ mM}$  for  $\text{Cl}^-$ , unpublished results) and then dissociate and bind to the strong phosphate-

binding site on the periplasmic phosphate-binding protein ( $K_d = 0.34 \mu\text{M}$ ).

Precedent for the association of outer membrane porins and periplasmic binding proteins exists in the literature. In the maltose/maltodextrin uptake system of *E. coli*, it was shown that the periplasmic maltose-binding protein (*malE* gene product) and an outer-membrane pore-forming protein (*lamB* gene product) could physically associate [26]. It was suggested that this association was necessary for high-affinity maltose uptake and for the uptake of maltodextrins, such uptake being absent in *lamB* mutants and in certain *malE* mutants [27]. Indeed, one *malE* mutant isolated was defective in maltodextrin uptake although the MalE protein present in this mutation could still bind maltodextrins, and a functional LamB protein was present [27]. The implications were that the mutant was defective in the ability of the maltose-binding protein and LamB pore to interact, thus preventing access of the binding protein to maltodextrins present in the LamB channel. Furthermore, it was demonstrated that the LamB protein could function as a non-specific pore in the uptake of small molecules [28–30] and, although isolated LamB porin exhibited some specificity toward maltose and maltodextrins [30, 31], its affinities were not in agreement with respective overall transport parameters *in vivo* [27].

Further analogy with the maltose system of *E. coli* is revealed by the potential ability of polyphosphates to be transported by the high-affinity phosphate uptake system in *P. aeruginosa*. If the observed polyphosphate inhibition of phosphate binding by the phosphate-binding protein is competitive (as yet undetermined) then polyphosphates (up to 15-mers) may be transported intact *in vivo*, without prior hydrolysis by alkaline phosphatase or other phosphatases. Growth of *P. aeruginosa* on polyphosphates as the sole phosphate source has been demonstrated [32], although the features of its utilization were not addressed. Since phosphate approaches the exclusion limit of protein P channels, the ability of polyphosphates up to 15 phosphate units to enter the cell via the high-affinity uptake components would depend upon the proper orientation of the polyphosphate at the mouth of the protein P channel. This could be carried out both by the anion-binding site of the protein P channel and by the association of the high-affinity phosphate-binding protein with the protein P channel. Similarly, the ability of *E. coli* to transport maltodextrins above the exclusion limit of the LamB pore through this porin has been postulated to depend upon binding sites both in the channel and on the maltose-binding protein in association with the porin [27, 30].

Presently an association between protein P and the phosphate-binding protein is being examined, and further experiments are underway to determine the role of protein P in phosphate and polyphosphate uptake *in vivo*.

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