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Expression, purification and preliminary diffraction studies of PhnP

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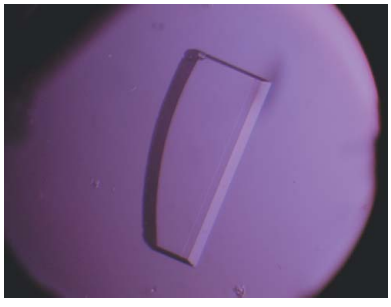

PhnP belongs to a 14-gene operon that supports the growth of *Escherichia coli* on alkylphosphonates as a sole source of phosphorus; however, the exact biochemistry of phosphonate degradation by this pathway is poorly understood. The protein was recombinantly expressed in *Escherichia coli* and purified to homogeneity. Sitting-drop vapour diffusion in combination with microseeding was used to obtain crystals that were suitable for X-ray diffraction. Data were collected to 1.3 Å and the crystals belonged to space group *C*2, with unit-cell parameters $a = 111.65$, $b = 75.41$, $c = 83.23$ Å, $\alpha = \gamma = 90$, $\beta = 126.3^\circ$.

1. Introduction

The availability of free phosphate in nature can be a limiting factor for bacterial growth. Bacteria have adapted by obtaining phosphate from phosphate esters and organophosphonates, the latter of which are characterized by a direct carbon–phosphorus (C—P) bond that is exceptionally stable compared with the carbon–oxygen–phosphorus (C—O—P) bond of phosphate esters. During phosphate limitation, *Escherichia coli* exhibits a many-hundred-fold upregulation of a C—P lyase pathway. The pathway has broad substrate specificity and is the only means of phosphonate breakdown in the K-12 strain (Wanner & Boline, 1990).

The C—P lyase operon consists of 14 genes (*phnCDEFGHIJKLMNOP*). Based on mutational and sequence analysis, the gene products PhnC–PhnE are thought to be involved in transport of substrates (Rizk *et al.*, 2006), PhnF is a transcription repressor protein (Gebhard & Cook, 2008), PhnO is an aminoalkylphosphonate *N*-acetyltransferase (Errey & Blanchard, 2006), PhnG–PhnM are enzymes involved in C—P bond cleavage and PhnN is a ribose 1,5-bisphosphokinase (Hove-Jensen *et al.*, 2003). Although the *phnP* gene is conserved in the prokaryotic C—P lyase operon, its precise role has not been determined. Mutational studies have suggested that PhnP is required in the presence of a functional *phnN* product for phosphonate utilization (Metcalf & Wanner, 1993). Attempts to detect C—P lyase activity in a cell-free system have been inconsistent, possibly owing to the fact that the enzyme complex is predicted to be membrane-associated (Kononova *et al.*, 2007; Ternan *et al.*, 1998). Consequently, the reaction mechanism of enzymatic C—P bond cleavage is poorly understood.

In order to determine the role of PhnP in the C—P lyase pathway, structural studies have been undertaken. Here, we present the expression, purification, crystallization and preliminary X-ray diffraction analysis of PhnP.

2. Materials and methods

2.1. Cloning, expression and purification

The *phnP* gene was amplified directly from *E. coli* HO764 (Post *et al.*, 1996) using Vent DNA polymerase (New England Biolabs) and the forward and reverse primers 5′-GA*GAATTC*ATTAAAGAGGAGAAATTAACT**ATG**AGCCTGACCCTCACGCTCACCGGCACCGGCGG-3′ and 5′-TGGTTG*GGATCC*CGAGCCATGGTTA-

TTA**ATGGTGATGGTGATGGTG**CGCCACCCCAATCTCCATC-CCATCAAACCCC-3′, respectively. *Eco*RI and *Bam*HI restriction-endonuclease sites are italicized and the starting ATG and hexa-histidine codons are shown in bold. The PCR product was digested by *Eco*RI and *Bam*HI and ligated into similarly digested DNA of pUHE23-2 (H. Bujard, unpublished work). Results were confirmed by DNA sequencing. The plasmid was designated pHO520. The NCBI accession number for the *phnP* nucleotide and protein sequences is 948600.

PhnP was expressed by co-transformation of pHO520 and p*LacI* (Novagen) into *E. coli* BL21 (DE3) cells (Novagen). The p*LacI* vector was necessary to provide sufficient lactose repressor to control transcription from pHO520, which lacks a *lacI* gene. Cells were then grown in Luria–Bertani medium with ampicillin (100 µg ml^{-1}) and chloramphenicol (30 µg ml^{-1}) until the A_{600} of the culture reached 0.6 at 303 K. At this point, the culture was induced with 0.5 m*M* IPTG. After further incubation at 288 K for 20 h, the cells were harvested by centrifugation at 3000*g* at 277 K for 20 min and stored at 253 K until purification.

Cell pellets were resuspended in 50 m*M* phosphate pH 7.2, 10 m*M* imidazole, 300 m*M* NaCl and lysed with an Emulsiflex-C5 homogenizer (Avestin). The lysed cells were then centrifuged at 40 000*g* and 277 K for 30 min. The soluble fraction of the lysate was passed through a nickel–nitrilotriacetic acid (Ni–NTA) agarose column (Qiagen) and the captured PhnP was eluted with an imidazole gradient from 10 to 500 m*M* over ten column volumes at a flow rate of 5 ml min^{-1} using an ÄKTA FPLC system (GE Healthcare). Fractions containing >95% pure PhnP, as demonstrated by SDS–PAGE analysis, were pooled, concentrated to 1 ml and further purified by gel filtration using a Superdex 200 column (1 × 30 cm, prep-grade; GE Healthcare) pre-equilibrated with 50 m*M* HEPES pH 7.2, 150 m*M* NaCl. The pooled fractions from the gel-filtration column were concentrated using Millipore Amicon Ultra centrifugal filters (10 000 Da molecular-weight cutoff); this was followed by passage through a 0.22 µm syringe filter. The concentration of purified protein was determined from the absorbance at 280 nm using the calculated extinction coefficient $\varepsilon_{280}^{1\%} = 1.18$ and a molecular weight of 28.67 kDa (Gasteiger *et al.*, 2005). The protein was flash-frozen in liquid nitrogen and stored at 193 K.

The selenomethionine derivative of PhnP was produced in the methionine-auxotroph strain DL41 (DE3) grown in M9 SeMET High Yield medium (Medicilon). The selenomethionine-labelled PhnP was purified using the same procedure as described above for the native protein.

2.2. Crystallization

All crystallization experiments were performed at room temperature. For initial screening, the protein sample was diluted to 360 µ*M* (∼10 mg ml^{-1}) in protein buffer (20 m*M* HEPES pH 7.5, 150 m*M* NaCl). Sitting-drop vapour-diffusion trials were carried out in 96-well plates (Greiner): 1 µl protein solution was mixed with 1 µl crystallization solution and equilibrated against 100 µl well solution. Initial hits were obtained in condition Nos. 38–41 of the PACT Suite

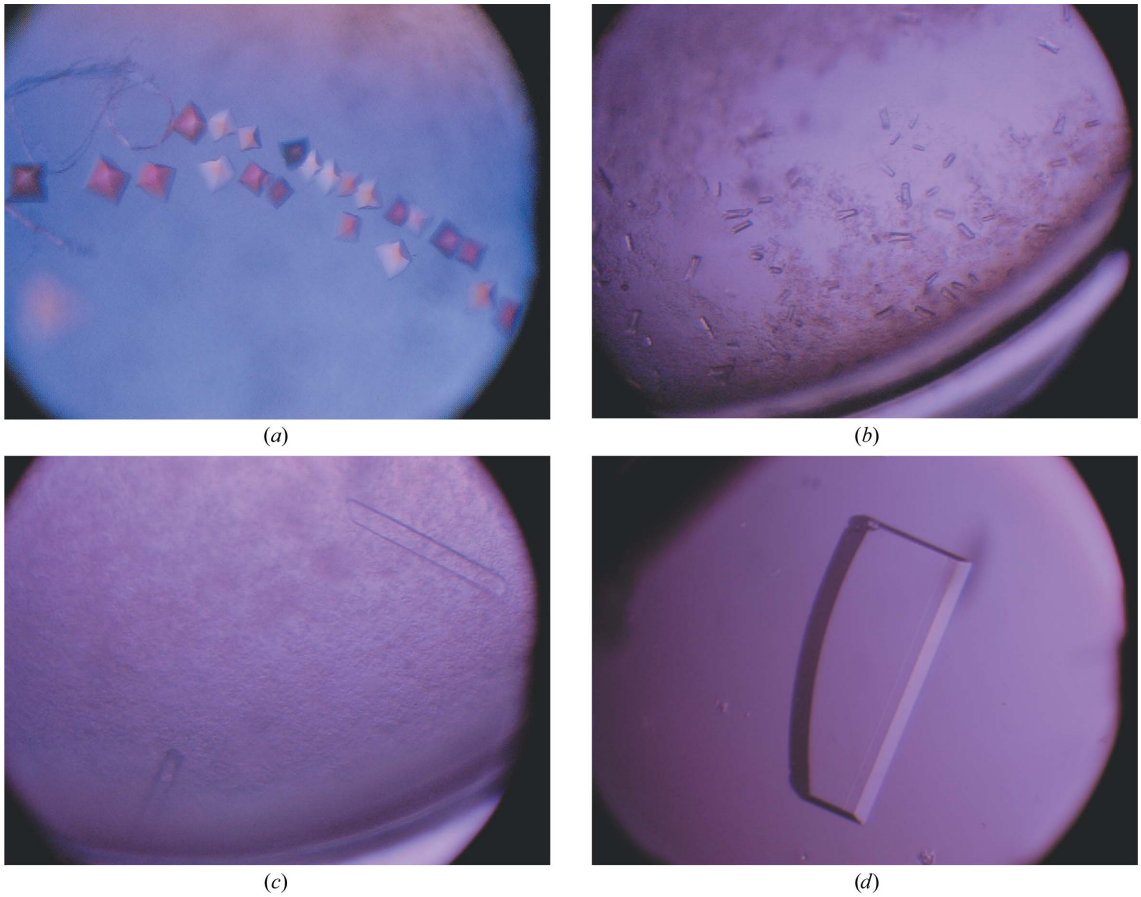

Figure 1
(*a*) Initial crystals obtained from PACT Suite condition Nos. 38–41: 0.1 *M* MMT buffer pH 5.0–8.0 and 25%(*w*/*v*) PEG 1500. (*b*, *c*) Crystals produced during the optimization process in (*b*) the absence and (*c*) the presence of 50 m*M* L-Asp and L-Glu in the protein buffer. (*d*) Optimized crystals were obtained in 0.1 *M* MMT pH 5.2–5.8, 8% PEG 8000 and 6% PEG 400.

(Qiagen) containing 0.1 *M* MMT buffer pH 5.0–8.0 and 25%(*w*/*v*) PEG 1500. MMT is a composite buffer that consists of 37 m*M* MES, 20 m*M* L-malic acid and 43 m*M* Tris.

To further improve the homogeneity of the PhnP preparation, the purified protein was incubated at 315 K for 30 min and precipitate was removed by centrifugation. The protein concentration was adjusted to 280 µ*M* (~8 mg ml^{-1}); 50 m*M* L-arginine and 50 m*M* L-glutamate pH 7.6 were then added to the protein sample 1 h prior to crystallization. The protein sample was mixed with crystallization solution in a 1:1 ratio (2 + 2 µl) and placed on a microbridge (Hampton) containing 20 µl Fluorinert (Hampton). The microbridge was placed in a well of a VDX plate containing 1 ml crystallization solution and sealed with a glass cover slip.

The crystallization conditions were optimized using a grid screen of various molecular-weight PEGs *versus* the pH of the MMT buffer. Addition of PEG 400 was found to prevent crystal cracking upon immersion in cryoprotectant solution prior to flash-freezing. The final crystallization conditions contained 0.1 *M* MMT pH 5.2–5.8, 8% PEG 8000 and 6% PEG 400. Crystals appeared overnight and reached their maximum size within 48 h. Crystal quality was further improved by the standard streak-seeding technique using a horse hair and a 1/10 dilution of microseed stock. Selenomethionine-derivative crystals of PhnP were obtained using the same procedure as for the native crystals.

2.3. Data collection and diffraction measurements

Single-wavelength anomalous dispersion data were collected from SeMet PhnP crystals on the X12B beamline at Brookhaven National Laboratory National Synchrotron Light Source using an ADSC Quantum-4 CCD detector. All data were collected at 100 K. Prior to flash-freezing in liquid nitrogen, the crystals were sequentially immersed in crystallization solution containing 5%, 15% and 25% 2-methyl-2,4-pentanediol (MPD) as a cryoprotectant. Oscillations of 1°, with an exposure time of 6 s per image, and a crystal-to-detector distance of 200 mm were used. The data were indexed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

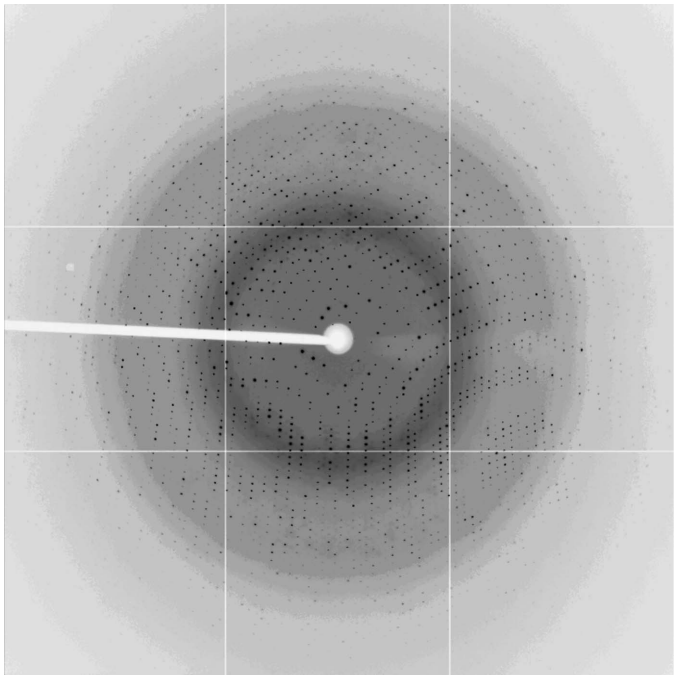

Figure 2
Diffraction of SeMet PhnP crystals. The edge of the detector is 1.3 Å; data were processed to 1.4 Å.

Table 1
Diffraction data for SeMet PhnP crystals.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 111.65, b = 75.41, c = 83.23, β = 126.3
Wavelength (Å)	0.97916
Temperature (K)	100
Resolution range (Å)	67.1–1.4 (1.46–1.40)
Observed reflections	1628375
Unique reflections	109380
Data completeness (%)	94.5 (70.4)
Redundancy	7.1 (5.1)
$R_{\rm merge}$† (%)	8.3 (25.7)
$\langle I/\sigma(I)\rangle$	30.5 (5.5)
Matthews coefficient (Å^3 Da^{-1})	2.469
Solvent content (%)	50.1

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values. The summation is over all measurements.

3. Results and discussion

The recombinant PhnP protein was purified to homogeneity using a two-step purification protocol. After size-exclusion chromatography, PhnP was found to be approximately 95% pure, as confirmed by SDS–PAGE (data not shown).

PhnP initially crystallized overnight in condition Nos. 38–41 from the PACT Suite (Qiagen; Fig. 1*a*). Expansion of these conditions using the hanging-drop vapour-diffusion method in 24-well screw-cap plates (Qiagen) produced crystals that had split ends and were too small for diffraction studies (less than 0.05 mm in the longest axis; Fig. 1*b*). An exhaustive search for compounds that would improve the crystal size was conducted using Additive and Detergent Screens 1, 2 and 3 (Hampton), but no consistent change in crystal size and quality was observed. It has been reported that the addition of 50 m*M* L-glutamate and L-arginine greatly improves protein solubility and long-term stability (Golovanov *et al.*, 2004). The effect of these amino acids on protein crystallization was assessed by adding them to PhnP samples immediately after thawing and at least 1 h prior to crystallization trials. Surprisingly, the crystal size increased to about 0.2–0.35 mm along the longest axis (Fig. 1*c*). Most crystals still possessed internal defects, however, and produced only a few low-resolution spots in X-ray diffraction patterns.

In an attempt to rid the PhnP crystals of internal defects and prevent nucleation along the contact area of the drop with the cover slip, which made it difficult to harvest the crystals, the floating-drop method utilizing microbridges with Fluorinert was used. The crystallization drops did not float on top of the Fluorinert, but sank to the bottom of the microbridge, resembling a microbatch experiment. The use of Fluorinert did not remedy the nucleation problem, but dramatically improved the crystal size and quality (Fig. 1*d*). This was attributed to slower equilibration of the drop with the reservoir and hence slower crystal growth. The standard streak-seeding technique was implemented in order to minimize spontaneous nucleation from the contact area between the drop and the microbridge.

Short heat treatments can improve protein homogeneity by denaturing partially unfolded molecules (Pusey *et al.*, 2005). To further improve the quality of the crystals, purified PhnP was incubated at 315 K for 30 min prior to crystallization in order to precipitate less stable protein. The resulting large and flawless crystals

produced diffraction to 1.3 Å resolution (Fig. 2). The typical crystal had dimensions of 0.3 × 0.2 × 0.2 mm.

The crystals belonged to the *C*-centred monoclinic space group *C*2, with unit-cell parameters $a = 111.65$, $b = 75.41$, $c = 83.23$ Å, $\alpha = \gamma = 90$, $\beta = 126.3^\circ$. Diffraction data for PhnP were processed in the resolution range 60–1.4 Å. A Matthews coefficient of 2.469 $Å^3\,Da^{-1}$ was obtained (Matthews, 1968), with a solvent content of 50.1%, representing a dimer in the asymmetric unit. This is consistent with size-exclusion results, which indicate that the protein is dimeric in solution (data not shown). A summary of the crystal parameters and the statistics of the diffraction data are presented in Table 1. Structure determination of PhnP is currently under way.

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