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Crystal structure of PhoU from *Pseudomonas aeruginosa*, a negative regulator of the Pho regulon

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ABSTRACT

In *Escherichia coli*, seven genes (*pstS*, *pstC*, *pstA*, *pstB*, *phoU*, *phoR*, and *phoB*) are involved in sensing environmental phosphate (Pi) and controlling the expression of the Pho regulon. PhoU is a negative regulator of the Pi-signaling pathway and modulates Pi transport through P_i transporter proteins (PstS, PstC, PstA, and PstB) through the two-component system PhoR and PhoB. Inactivation of PhoY2, one of the two PhoU homologs in *Mycobacterium tuberculosis*, causes defects in persistence phenotypes and increased susceptibility to antibiotics and stresses. Despite the important biological role, the mechanism of PhoU function is still unknown. Here we have determined the crystal structure of PhoU from *Pseudomonas aeruginosa*. It exists as a dimer in the crystal, with each monomer consisting of two structurally similar three-helix bundles. Our equilibrium sedimentation measurements support the reversible monomer–dimer equilibrium model in which *P. aeruginosa* PhoU exists in solution predominantly as dimers, with monomers in a minor fraction, at low protein concentrations. The dissociation constant for PhoU dimerization is 3.2×10^{-6} M. The overall structure of *P. aeruginosa* PhoU dimer resembles those of *Aquifex aeolicus* PhoU and *Thermotoga maritima* PhoU2. However, it shows distinct structural features in some loops and the dimerization pattern.

1. Introduction

Inorganic phosphate (Pi) is required for cellular functions such as DNA/RNA and membrane phospho-lipid synthesis, generation of high-energy phosphate esters, and intracellular signaling (Bevington et al., 1992; Wanner, 1996; Bergwitz and Jüppner, 2011). The *Escherichia coli* phosphate regulon (Pho regulon), encoding a large number of genes related to phosphate metabolism, plays a key role in maintaining phosphate homeostasis under a Pi-limiting condition (Wanner, 1996; Han et al., 1999; Suziedeliene et al., 1999; Baek and Lee, 2006). The importance of the bacterial Pho regulon is not limited to phosphate homoeostasis but stretches to the virulence as well as resistance to various kinds of stresses such as the acid shock, nutritional deprivation, and antibiotics (Monds et al., 2001; Lamarche et al., 2005; Lamarche et al., 2008).

In *E. coli*, products of seven genes (*pstS*, *pstC*, *pstA*, *pstB*, *phoU*, *phoR*, and *phoB*) participate in sensing environmental phosphate and controlling the expression of the Pho regulon. The transmembrane Pi-signal transduction is believed to be regulated by the sensor histidine kinase PhoR, which can exist in three different states (Lamarche et al., 2005). PhoU is also required for Pi-signal transduction, but not for transport through the PstSCAB complex (Steed and Wanner, 1993). PhoU is a negative regulator of the Pi-signaling pathway; it modulates the Pi transport and Pho regulon through P_i transporter proteins (PstS, PstC, PstA, and PstB) and the PhoR–PhoB two-component regulatory system (Muda et al., 1992; Wanner, 1993; Rice et al., 2009; Hsieh and Wanner, 2010). Recently, PhoU was shown to interact with PhoR through the PAS domain of the latter and also with PstB to modulate the Pi transport into the cell by mediating the interaction between PhoR and PstB (Gardner et al., 2014). However, the specific mechanism of PhoU is still largely unknown.

Mycobacterium tuberculosis possesses two PhoU homologs (PhoY1 and PhoY2), with a sequence identity of 63.4% between

Abbreviations: Pa_PhoU, *P. aeruginosa* PhoU; Aa_PhoU, *Aquifex aeolicus* PhoU; Tm_PhoU2, *Thermotoga maritima* PhoU2; Pho regulon, phosphate regulon; Pi, inorganic phosphate; R.m.s., root mean square.

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them (Shi and Zhang, 2010). They show a sequence identity of 19.7% against *E. coli* PhoU. Disruption of the *phoY2* gene (*Rv0821c*), but not *phoY1*, resulted in increased sensitivity to environmental stress conditions, including nutrient deprivation and antibiotic treatments (Shi and Zhang, 2010; Wang et al., 2013). It was suggested that mycobacterial PhoY2 is required for maintaining intracellular Pi homeostasis and adaptation to stress conditions, which may provide an explanation for the suggested role of PhoY2 in drug tolerance (Shi and Zhang, 2010; Wang et al., 2013).

Until now, crystal structures of two PhoU proteins from nonpathogenic, hyperthermophilic bacteria (*Aquifex aeolicus* and *Thermotoga maritima*) have been published (Oganesyan et al., 2005; Liu et al., 2005). *Pseudomonas aeruginosa*, one of the major human bacterial pathogens, causes infectious diseases such as pneumonia and septic shock. PhoU from *P. aeruginosa* (Pa_PhoU) shares sequence identities of 24.9% and 26.2% with *M. tuberculosis* PhoY1 and PhoY2. A PhoU deletion mutant of *Pseudomonas putida* was reported to show high susceptibility to antibiotics and reduction of persisters (Lee et al., 2009). PhoU proteins from *P. aeruginosa* and *P. putida* share a sequence identity of 90%.

In this study, we have determined the crystal structure of Pa_PhoU by the single-wavelength anomalous diffraction (SAD) method to provide insights into its function and to reveal distinct structural features compared to other PhoU family members. Pa_PhoU exists as a dimer in the crystal, with each monomer consisting of two similar helical bundles. We have also performed equilibrium sedimentation experiments, which support the reversible monomer–dimer equilibrium model for Pa_PhoU in solution at low protein concentrations. The measured dissociation constant (K_d) for PhoU dimerization is 3.2×10^{-6} M. Pa_PhoU monomers dimerize in a slightly different manner from those of other PhoU proteins from *A. aeolicus* and *T. maritima*. Pa_PhoU also has distinct structural features in the α2–α3 and α3–α4 loops and the surface charge distribution of its dimer. Divalent metal ions are not bound to Pa_PhoU, although four E(D)xxxD sequence motifs are conserved. The present structure of Pa_PhoU from a bacterial pathogen may be useful for discovering inhibitors of PhoU as a potential antibacterial drug candidate.

2. Materials and methods

2.1. Gene cloning and overexpression

The *phoU* (*PA5365*) gene encoding the full-length PhoU protein of 242-residues was amplified by the polymerase chain reaction (PCR) using the genomic DNA of *P. aeruginosa* strain PAO1 as the template. The forward and reverse oligonucleotide primers were designed using the published genome sequence (Stover et al., 2000) as 5′-G GAA TTC CAT ATG ATG ATC AAC AAA GAC AGT CTC-3′ and 5′-CCG CCG CTC GAG TTA TCA CTC GCC G-3′, respectively. The underlined sequences are *Nde*I and *Xho*I digestion sites, respectively. The amplified DNA was inserted into the expression vector pET-21a(+) (Novagen) that was digested by both *Nde*I and *Xho*I. This construct adds an eight-residue tag (LEHHHHHH) to the C-terminus of the recombinant protein to facilitate protein purification. The resultant construct was confirmed by DNA sequencing.

2.2. Protein expression and purification

The recombinant protein was overexpressed in *E. coli* Rosetta2(DE3) cells. Cells were grown at 37 °C up to OD_{600} of 0.5 in LB culture medium containing 50 mg/ml ampicillin, and the protein expression was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells continued to be incubated at 18 °C for additional 20 h after IPTG induction and were harvested by centrifugation at 5600 g for 10 min at 4 °C. The cell pellet was lysed by sonication in buffer A (20 mM Tris–HCl at pH 7.9, 500 mM NaCl, and 50 mM imidazole) containing 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM tris-(2-carboxyethyl)phosphine-HCl (TCEP). The crude lysate was centrifuged at 36,000*g* for 1 h at 4 °C and the cell debris was discarded. The supernatant was applied to an affinity chromatography column of HiTrap chelating HP column (GE Healthcare), which was previously equilibrated by buffer A. The protein was eluted with a linear gradient of 0.05–1.0 M imidazole in buffer A. The peak fractions were collected and diluted fivefold with buffer B (20 mM Tris–HCl at pH 7.9 and 1 mM TCEP). The diluted protein sample was subject to ion exchange chromatography on a Hitrap Q column (GE Healthcare). The protein was eluted with a linear gradient of 0.0–1.0 M NaCl in buffer B. As a final step, gel filtration was performed on a Hiload 16/60 Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with 20 mM Tris–HCl at pH 8.0, 1 mM TCEP, and 150 mM NaCl. The purified protein was concentrated using YM10 ultrafiltration membrane (Amicon). The protein concentration was estimated by measuring the absorbance at 280 nm, employing a calculated extinction coefficient of 12,950 M^{-1} cm^{-1}.

2.3. Crystallization and X-ray diffraction data collection

Crystallization of both native and selenomethionine (SeMet)-substituted Pa_PhoU was performed at 24 °C by the sitting-drop vapor-diffusion method using 96-well crystallization plates. Initial crystallization conditions were established using screening kits from Hampton Research and Emerald Bio. Each sitting drop was prepared by mixing 2 μl each of the protein solution (5 mg/ml protein concentration in 20 mM Tris–HCl at pH 8.0, 1 mM TCEP, and 150 mM NaCl) and the reservoir solution (0.1 M sodium malonate at pH 4.0 and 12% (w/v) PEG3350), and was placed over 80 μl of the reservoir solution. Crystals grew to maximum dimensions of $0.5 \times 0.2 \times 0.5$ mm within a week.

The crystal was vitrified using the cryoprotectant solution consisting of the reservoir solution supplemented with 20% (v/v) glycerol. Crystals were soaked in the cryoprotectant solution for a few seconds before being frozen in liquid nitrogen. Sets of native and SAD data were collected at 100 K using a Quantum 210 CCD area detector (Area Detector Systems Corporation, Poway, California) at the BL-6C and BL-4A experimental stations of Pohang Light Source, Korea, from native and SeMet-substituted crystals of Pa_PhoU. Raw data were processed and scaled using the HKL2000 program package (Otwinowski and Minor, 1997). The native crystals belong to the tetragonal space group $P4_12_12$, with unit cell parameters of $a = b = 70.48$ Å, $c = 196.20$ Å, and $\alpha = \beta = \gamma = 90°$. The SeMet crystals belong to the orthorhombic space group $C222_1$, with unit cell parameters of $a = 100.24$ Å, $b = 100.29$ Å, $c = 196.54$ Å, and $\alpha = \beta = \gamma = 90°$. Despite nearly identical a and b, the SeMet SAD data gave a very high R_{merge} value (75.1%) when they were merged in the tetragonal space group $P4_12_12$. Apparently, essentially identical crystallization conditions yielded both tetragonal and orthorhombic crystals. Table 1 summarizes the data collection statistics.

2.4. Structure determination, refinement, and analysis

A set of SAD data collected from a crystal of the SeMet-substituted Pa_PhoU was used to solve the phase problem. Selenium atoms were located and the phases were improved with SOLVE and RESOLVE (Terwilliger and Berendzen, 1999; Terwilliger et al., 2007). The manual model building and addition of water molecules were conducted using the program Coot (Emsley et al., 2010).