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Structure of inorganic pyrophosphatase from *Staphylococcus aureus* reveals conformational flexibility of the active site

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ABSTRACT

Cytoplasmic inorganic pyrophosphatase (PPiase) is an enzyme essential for survival of organisms, from bacteria to human. PPiases are divided into two structurally distinct families: family I PPiases are Mg^{2+}-dependent and present in most archaea, eukaryotes and prokaryotes, whereas the relatively less understood family II PPiases are Mn^{2+}-dependent and present only in some archaea, bacteria and primitive eukaryotes. *Staphylococcus aureus* (SA), a dangerous pathogen and a frequent cause of hospital infections, contains a family II PPiase (PpaC), which is an attractive potential target for development of novel antibacterial agents. We determined a crystal structure of SA PpaC in complex with catalytic Mn^{2+} at 2.1 Å resolution. The active site contains two catalytic Mn^{2+} binding sites, each half-occupied, reconciling the previously observed 1:1 Mn^{2+}:enzyme stoichiometry with the presence of two divalent metal ion sites in the apo-enzyme. Unexpectedly, despite the absence of the substrate or products in the active site, the two domains of SA PpaC form a closed active site, a conformation observed in structures of other family II PPiases only in complex with substrate or product mimics. A region spanning residues 295–298, which contains a conserved substrate binding RKK motif, is flipped out of the active site, an unprecedented conformation for a PPiase. Because the mutant of Arg295 to an alanine is devoid of activity, this loop likely undergoes an induced-fit conformational change upon substrate binding and product dissociation. This closed conformation of SA PPiase may serve as an attractive target for rational design of inhibitors of this enzyme.

1. Introduction

Cytoplasmic inorganic pyrophosphatase (PPiase) is a ubiquitous enzyme that plays a key role in phosphorus metabolism. PPiase hydrolyzes inorganic pyrophosphate (PP_i) generated upon nucleic acid synthesis and other numerous essential nucleotidyl transfer reactions into two molecules of inorganic phosphate (P_i), thereby providing a thermodynamic sink as well as eliminating inhibitory PP_i product (Chen et al., 1990; Kornberg, 1962; Lundin et al., 1991; Peller, 1976; Sonnewald, 1992). PPiases are divided into two families based on their amino acid residue sequences (Shintani et al., 1998; Young et al., 1998) and structures (Ahn et al., 2001; Merckel et al., 2001). Family I includes hexameric archaeal and bacterial enzymes and dimeric eukaryotic PPiases that share a common catalytic fold (Baykov et al., 1999), whereas family II are dimeric PPiases with a distinct fold present only in some bacteria, archaea and primitive eukaryotes. Understanding the structure and function of family II PPiases is biomedically important because of their occurrence and essentiality in human pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mutans* and *Bacillus anthracis*. Expression of family II PPiases is regulated depending on culture conditions (Lahti, 1983). The essentiality, the regulation and the uniqueness of family II PPiases to specific bacteria make them attractive potential targets for discovery and development of novel selective antibacterial agents.

Family II PPiases are two-domain proteins with the active site located at the domain interface. Based on the crystal structures of PPiase II of *Bacillus subtilis* (Ahn et al., 2001; Fabrichniy et al., 2007), this interface is thought to be formed by a pivoting rotation of one domain relative to the other from a so-called open to a

Abbreviations: SA, *Staphylococcus aureus*; PPiase, inorganic pyrophosphatase; PNP, imidodiphosphate.

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closed, active, conformation. This is inferred from the observation that a substrate analogue imidodiphosphate (PNP) is present in the active site only in the closed conformation of this enzyme (Ahn et al., 2001; Fabrichniy et al., 2007; Merckel et al., 2001; Rantanen et al., 2007). The only reported structure of PPiase II that is in the open conformations does not contain a bound substrate or a product (Fabrichniy et al., 2004). The catalytic mechanism of family II PPiases involves activation of a nucleophilic water by metal ions, where three metal ions likely participate directly or indirectly in the catalysis. The maximum catalytic turnover rate in the presence of Mn^{2+} or Co^{2+} (k_{cat} = 1700–3300 s^{-1}) is much higher than that in the presence of Mg^{2+}, the preferred catalytic ion for family I PPiases (k_{cat} = 110–330 s^{-1}) (Fabrichniy et al., 2004; Kuhn et al., 2000; Parfenyev et al., 2001). This rate difference is offset by the lower K_m in the presence of Mg^{2+} (K_m = 10–30 μM) than that with Mn^{2+} (K_m = 90–160 μM) (Parfenyev et al., 2001), making it unclear what physiological ion drives catalysis *in vivo*. However, it is thought that family II PPiases use Mn^{2+}, because they contain 1 nM-affinity Mn^{2+} binding site and because bacteria containing family II PPiases tend to accumulate Mn^{2+} (Charney et al., 1951; Martin et al., 1986), through action of dedicated Mn^{2+} transporters. Upon substrate binding, a high affinity metal site changes its geometry from five-coordinated square pyramidal or trigonal bipyramidal to six-coordinated octahedral (Fabrichniy et al., 2007). Returning from the six-coordinated substrate-bound state to the five-coordinated substrate-free state facilitates product release (Fabrichniy et al., 2004). Transition metal ions (Mn^{2+} or Co^{2+}), but not Mg^{2+}, which is nearly always six-coordinated (Harding, 2001), tolerate flexible coordination geometry.

First structures of family II PPiases from *B. subtilis* and *S. mutans* showed two metal ions (M1 and M2) coordinating with protein ligands and another one (M3) that did not directly interact with the protein (Ahn et al., 2001; Merckel et al., 2001). A recent structural study of PPiase II from *B. subtilis* in complex with PNP revealed a fourth metal ion site occupied by protein-coordinated metal ion M4 (Fabrichniy et al., 2007). The trimetal center created by M1, M2 and M4 allows proper substrate binding and positioning of the nucleophilic water for a catalytic attack. The nucleophilic water is positioned above the trimetal plane in the absence of substrate, and it crosses the plane upon substrate binding. Protein conformational changes that occur in concert with binding and dissociation of metal ions, the substrate and the products in family II PPiases remain a substantial area of interest.

Herein, we determine a crystal structure of the family II PPiase from an important pathogen *S. aureus* (SA PpaC), and compare it to other family II PPiases.

2. Materials and methods

2.1. Cloning, protein expression and purification

For construction of expression vector pET22-PpaC, the *ppaC* gene (locus tag: SAV1919) was amplified from the genomic DNA of SA strain Mu50 (ATCC #700699) by polymerase chain reaction and inserted between the NdeI and XhoI restriction sites in vector pET22b. As a result, SA PpaC bears a C-terminal hexahistidine tag. Site-directed mutagenesis was carried out by using the QuikChange mutagenesis kit (Agilent Technologies) according to the kit manual. The construct sequences were confirmed by DNA sequencing at the University of Kentucky DNA Sequencing Core.

For protein purification, the pET22-PpaC plasmid was transformed into BL21 (DE3) chemically competent cells. A fresh colony from the transformation plate (Luria Bertani (LB) agar containing 100 μg/mL ampicillin) was grown in 5 mL of LB/ampicillin to the mid-log phase at 37 °C with shaking at 200 rpm and then used to inoculate 4 L of LB/ampicillin medium. The culture was incubated with shaking at 200 rpm, at 37 °C to attenuance of 0.2–0.3 at 600 nm, and then incubated at 16 °C for 1.5 h before induction with IPTG (final concentration of 0.5 mM). The induced culture was grown for an additional 16–18 h at 16 °C, 200 rpm. All purification steps were carried out at 4 °C. The cells were harvested by centrifugation at 5000×*g* for 10 min. The cell pellet was resuspended in lysis buffer (300 mM NaCl, 40 mM Tris–HCl pH 8.0, adjusted at room temperature, and 2 mM β-mercaptoethanol). The cells were disrupted by sonication on ice and the lysate was clarified by centrifugation at 40,000×*g* for 45 min. The supernatant was filtered through a 0.45 μm Millex-HV PVDF filter (Millipore) and applied to a 1 mL Ni-IMAC HisTrap column (GE Healthcare) equilibrated with lysis buffer. The column was washed with 20 mL of lysis buffer containing 50 mM imidazole. Then the protein was eluted with 9 mL of lysis buffer containing 500 mM imidazole and 2 mM $MnCl_2$ in 9 fractions of 1 mL each. The presence of $MnCl_2$ was critical for obtaining a homogeneous dimerization state of SA PpaC. The fractions containing more than 95% pure desired protein, as determined by SDS–PAGE, were pooled. The protein was further purified on a size-exclusion S-200 column (GE Healthcare) equilibrated with the gel filtration buffer (40 mM Tris–HCl pH 8.0, 100 mM NaCl, 2 mM $MnCl_2$ and 2 mM β-mercaptoethanol). The protein-containing fractions were pooled and the protein was concentrated using an Amicon Ultra (5000 MWCO) centrifugal filter device (Millipore) to 12 mg/mL.

2.2. Pyrophosphatase activity assay

The SA PpaC activity was measured with sodium pyrophosphate as a substrate, similarly to the previously described protocol (Baykov et al., 1988). A malachite green stock solution (0.12%, w/v) was made by dissolving the dye in 3 M sulfuric acid. Fresh malachite green reagent was prepared prior to the assays by adding one volume of 7.5% (w/v) ammonium molybdate into four volumes of the malachite green stock solution followed by the addition of Tween 20 to a final concentration of 0.2% (v/v). For activity measurements, purified SA PpaC (0.3 nM) was added into a freshly prepared reaction mixture containing 1 mM sodium pyrophosphate and 0.5 mM $MnCl_2$, in a reaction buffer (25 mM Tris–HCl, 50 mM NaCl, pH 7.0) at 22 °C for 4 min. To stop the enzymatic reaction and determine the phosphate concentration of a sample, one volume of the malachite green reagent was mixed with four volumes of the enzymatic reaction mixture to be analyzed. The mixture was incubated for 2 min, and the absorbance at 630 nm was measured with a BIOMATE 3″ UV/vis spectrophotometer (Thermo Scientific). We confirmed that under these conditions, the enzyme kinetics were linear over time. To obtain relative activities, the absorbance of the sample was corrected for non-enzymatic degradation of pyrophosphate and normalized against the maximum absorbance of the sample containing wild-type PpaC and 0.8 mM sodium pyrophosphate.

2.3. Circular dichroism spectroscopy (CD)

CD experiments were performed using a JASCO J-815 CD spectrometer equipped with a Peltier temperature controller. Blank scans were collected from dialysis buffer and subtracted from the spectra containing enzyme. Cuvettes of 1 mm or 1 cm pathlength were used for far UV and near UV scans, respectively.

2.4. Protein crystallization

The initial crystallization conditions for SA PpaC were identified by the high-throughput screening service at the Hauptman-Woodward Institute, NY (Luft et al., 2003), by a microbatch method. After optimization, single rod-shaped crystals of SA PpaC