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.

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**1. Introduction**

Cytoplasmic inorganic pyrophosphatase (PPiase) is a ubiquitous enzyme that plays a key role in phosphorus metabolism. PPiase hydrolyzes inorganic pyrophosphate (PPi) 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 PPi 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; Fabrichnii 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 an...
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$^{2+}$ ($K_M = 90–160$ $\mu$M) than that with Mn$^{2+}$ ($K_M = 90–160$ $\mu$M) (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 $\mu$g/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 attenuation 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 $\beta$-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 $\beta$-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% (w/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...
0.07 × 0.07 × 0.2 mm in size were grown in 6–7 days by vapor diffusion in hanging drops at 22 °C. In the drops, 1 μL of the concentrated protein was mixed with 1 μL of the reservoir solution (1.32 M ammonium citrate tris buffer) and equilibrated against 1 μL of the reservoir solution supplemented with 2 mM MnCl2. The crystals were then gradually transferred into the cryoprotectant solution (1.32 mM ammonium citrate tris buffer, 2 mM MnCl2, 15% glycerol), incubated for 30 min and rapidly frozen in liquid nitrogen. Similarly, co-crystallization was attempted with 20, 50 and 100 mM Na2PO4 as well as with 1 mM PNP. In addition, random factorial screening in the presence of PNP and fluoride was carried to crystallize SA PpaC–PNP complex in a crystal form different from that of apo-SA PpaC. These experiments as well as soaking the crystals in solutions containing phosphate or PNP did not yield a bound phosphate or PNP in the active site.

2.5. Data collection and crystal structure determination

The X-ray diffraction data were collected at synchrotron beamline 21-ID-G (sector LS-CAT) at the Advanced Photon Source at the Argonne National Laboratories (Argonne, IL). The data were indexed, integrated and scaled with HKL2000 (Otwinowski and Minor, 1997). The structure was determined by molecular replacement (MR) with program PHASER (McCoy et al., 2007) by using the structure of inorganic pyrophosphatase Bacillus subtilis (Ahn et al., 2001; PDB accession number: 1K23). The MR search was carried out in two steps: (1) with the N-terminal domain (up to residue 190), yielding a clear MR solution for this domain and (2) with the C-terminal domain (from residue 191 to the C-terminus), where the N-terminal domain was positioned as determined in step (1). The structure building and refinement was then carried out iteratively by using Coot (Emsley and Cowtan, 2004) and Refmac (Murshudov et al., 1997) programs, respectively. The refined structure contains a dimer in the asymmetric unit, with all annotated residues in the protein sequence present in the structure of each monomer, except for the first Met residue. The data collection and refinement statistics are given in Table 1. The crystal structure coordinates and structure factors for SA PpaC were deposited in the Protein Data Bank with the PDB accession number 4RP Am.

3. Results and discussion

3.1. The structure of PPhase II of S. aureus

A BLAST search (Altschul et al., 1997) yielded four structurally characterized homologs of SA PpaC from bacteria with 46–57% sequence identity to SA PpaC and one from archaea (Methanococcus jannaschii) with 41% sequence identity (Supplementary Fig. 1). The residues critical for Mn
2+ binding were observed in the active site of the enzyme. The structural sense (Mercel et al., 2001) and must be a consequence of structural changes that propagate from the active site to the dimerization interface. SA PpaC crystallized in the presence of Mn
2+, whereas no crystals were obtained at the same or other conditions without a divalent metal ion. This is only a second report of a crystal structure of a family II PPhase in the apo-form.

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<th>Table 1 X-ray diffraction data collection and refinement statistics for SA PpaC.</th>
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<td>Data collection</td>
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* Numbers in parentheses indicate the values in the highest–resolution shell.

** Indicates PROCHECK (Laskowski et al., 1993) statistics. 98% of residues are in favored regions and 1.8% are in the allowed regions according to RAMPAGE (Lovell et al., 2003).

Fig.1. Size-exclusion chromatograms of SA PpaC in the absence of a divalent metal ion (the red trace) and in the presence of 2 mM MnCl2 (the black trace) in the buffer. The dimer and the monomer peaks are labeled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The crystal structure of SA PpaC was determined by molecular replacement with individual domains of PPhase II of B. subtilis (57% identical in sequence) used sequentially as search models, and refined at 2.1 Å resolution (Table 1). SA PpaC is a homodimer of two 34 kDa subunits (Fig. 2A). The crystals contained a dimer of PpaC per asymmetric unit. Strong electron density was observed for the entire annotated polypeptide chain (residues 2–309) of PpaC. The space group of the crystals was determined by molecular replacement with individual domains of PPases (Ahn et al., 2001; Mercel et al., 2001), the N-terminal domain of SA PpaC (residues 1–190) consists of a six-stranded parallel β-sheet with extended loops on one side and helices on the other side. The C-terminal domain contains a six-stranded β-sheet with helices flanking it on both sides. The SA PpaC dimer is formed by adjoining of the two β-sheets of the N-terminal domains into one β-sheet, where β-strands β6 (Supplementary Fig. 1) from each monomer interact with each other in a symmetrical fashion. In addition, the dimer is stabilized by predominantly hydrophobic contacts between the loops of the two N-terminal domains and...
between a loop of the N-terminal domain of one monomer and the C-terminal helix of the other monomer. A similar dimeric interface is also observed in other family II PPiases and in horse liver alcohol dehydrogenase (Colonna-Cesari et al., 1986; Eklund et al., 1976).

Unexpectedly, even though no substrate or product is bound in the active site, the mutual disposition of the two domains of each monomers corresponds to the closed conformation of family II PPiases, as seen in the published structures of the homologs only in complexes with PNP, sulfate or chloride (Table 2). The closed conformation in these previously reported structures, thought to form upon substrate binding, ensures appropriate position of the residues of the N- and C-terminal domains for substrate binding and catalysis, as explained below.

3.2. The active site of SA PpaC

The active site of SA PpaC is located at the interface between the N- and C-terminal domains (Fig. 2A). In the omit Fo-Fc electron density map generated prior to including water or other ligands in the structure, two areas of strong density (>9σ) in the active site were observed that coincided with two strong density peaks in the anomalous difference Fourier map (>6σ; Fig. 2B). No significant peaks in the anomalous difference Fourier map were present elsewhere. These observations, together with the stabilizing effect of Mn2+ on the dimerization of SA PpaC indicate that the electron density peaks correspond to bound Mn2+ ions. However, the refinement clearly demonstrated that each site is half-occupied, indicating that a Mn2+ ion occupies one or the other site in each monomer in the crystal with equal probabilities. The half-occupancy reconciles the previous observations of the 1:1 stoichiometry of a tightly bound Mn2+ ion with the presence of two divalent metal ion binding sites in homologous apo-proteins (Halonen et al., 2005). These observations likely mean that in the absence of the substrate, binding of one Mn2+ ion to either of the two sites disfavors binding of the second ion to the other site. Substrate binding must stabilize simultaneous binding of both metal ions, since both M1 and M2 ions are coordinated to the substrate oxygens, as observed in the crystal structure of B. subtilis PPiase in complex with PNP (Fabrichniy et al., 2007).

The geometry of the active site (Fig. 2B) is similar to that of previous structures of PPiase family II enzymes. M1 and M2 metal ions are positioned the same way as in the structures from S. mutans, S. gordonii and B. subtilis. Ions M1 and M2 are coordinated to four and five ligands, respectively (Fig. 2B). Ion M1 is coordinated with Nε of His9, Oδ1 of Asp13, one of Oδ2 of Asp75 and a water molecule bridging M1 and M2. Ion M2 is coordinated with Oδ2 of Asp15, Nε of His97, Oδ2 of Asp149, the other Oδ2 of Asp75 and with the bridging water. Coordination with His residues has been noted as a property of Mn2+, whereas it is very rare for Mg2+ (Bock et al., 1999). In contrast to the closed conformation of substrate-bound PPiase of B. subtilis, M3 and M4 metal ions were not present because of the absence of a substrate or its analogue, which is normally involved in their coordination (Fabrichniy et al., 2007).

The conformations of Arg295, Lys296 and Lys297 differ dramatically from their counterparts in previously published structures of PpaC homologs (Fig. 3, Supplementary Fig. 3). This region is far away from any crystal packing interface (Supplementary Fig. 4) and, therefore, its conformation is not an artifact of dimer–dimer interactions in the crystal. This region of SA PpaC (residues 295–298) lacks secondary structure; its backbone and the side chains of Arg295 and Lys296 are flipped out of the active site and are solvent-exposed (Fig. 3A). Arg295 forms a surface salt bridge with Asp261, the residue which is conserved in B. subtilis (Supplementary Fig. 1), suggesting that this conformation may be relevant for the B. subtilis homolog as well. In contrast to SA PpaC, in the structure of B. subtilis PPiase in complex with PNP the respective region is α-helical, with the side chains of Arg295 and Lys296 pointing into the active site (Fabrichniy et al., 2007; Fig. 3B). The guanidine group of Arg295 and the amino group of Lys296 form salt bridges with the phosphate groups of the bound PPi, mimic, PNP. In other homologs, these two residues exhibit similar interactions with PNP or inorganic phosphate to those observed for the B. subtilis PPiase. Occasional exceptions from this rule is in that one of these two residues forms a hydrogen bond with other active site residues to ensure a proper active site conformation, instead of interacting

![Image](https://example.com/image.png)
with the substrate directly. For example, in PPiase from *S. agalactiae*, the side chain of Lys296 (SAPpaC residue numbering) points away from the substrate and its guanidinium group forms a hydrogen bond with the carbonyl oxygen of His97 (Rantanen et al., 2007). The DHH motif (residues 96–98) including His97 was demonstrated to be important for the function of phosphoesterase (Koonin and Tatusov, 1994; Yamagata et al., 2002) and is involved in organizing the active site for the catalysis. In the structures of *Streptococcus gordonii* and *S. mutans* PPiases in complex with SO$_4^{2-}$/CO$_3^{2-}$ (mimicking the enzyme bound to the product, PO$_4^{3-}$/CO$_3^{2-}$), Lys296 is pointing away from the substrate binding site forming hydrogen bonds with carbonyl oxygen atoms of Val116 and His97 (in *S. gordonii*; Ahn et al., 2001) or water (*S. mutans*; Fabrichnyi et al., 2004).

The structure of SA PPiase is the first example of uncoupling of the closed conformation of the enzyme and substrate binding (no substrate or analogue is present in the active site, despite the closed form). In all other structures of bacterial family II PPiases, the open conformation of the active site is observed only in the absence of a substrate, product or their analogue, and, conversely, the active site in the closed conformation invariably contains a ligand. The only structure of an archaeal representative, the putative family II PPiase from *M. jannaschii* (PDB ID: 2EB0, RIKEN Structural Genomics Initiative), which exhibits the closed active site, was refined with a water located at the position of a bridging oxygen atom of the PP$_i$ substrate. Upon close inspection of the electron density map, the electron density for this atom and short interatomic distances to the nearby water molecules ($\sim$2.4 Å) are more consistent with a chloride ion forming a salt bridge with Lys204, rather than a water molecule at this site, in agreement with the closed conformation (Supplementary Fig. 5). The chloride site coincides exactly with the location of the bridging oxygen atom of a bound PP$_i$, as predicted by superposing this structure and structures of PNP bound homologs. In addition, in place of Asp261, this homolog contains a glycine residue (bacterial homologs contain either an aspartate or a glutamine; Supplementary Fig. 1), which may disfavor the flipped out conformation of the RKK motif seen in SA PpaC. We tested by mutagenesis whether conformational flexibility of this substrate binding region provides this uncoupling. Indeed, mutation Arg295 to an alanine completely abolishes enzyme activity (Fig. 4A). This observation suggests that Arg295 and, through chemical constraints, Lys296, change their conformation to bind PP$_i$ substrate to position it for catalysis.

CD spectra of wild-type SA PpaC and Arg295A mutant (Fig. 4B) were consistent with mixed α/β fold of SA PpaC. The superimposed spectra of the wild-type and the mutant proteins indicate that the R295A mutation did not disturb the overall fold of the enzyme. Therefore, the observed loss of activity was not a result of protein unfolding.

4. Conclusions

A crystal structure of the inorganic pyrophosphatase PpaC from *S. aureus* reported herein revealed that the conformation of the enzyme and the occupancy of its active site by a substrate or a
product can be uncoupled through an unprecedented conformational change of the conserved substrate positioning motif RKK. Moreover, this structure strongly suggests that PPi binds to the closed state of SA PpaC, which is either fixed or is in equilibrium with the open state observed in the structure of *B. subtilis* PPiase in the apo-form (the only other structurally investigated apo-enzyme of this family), rather than binding to the open conformation and inducing its closing. Based on this and previously reported data, we propose that the RKK motif likely undergoes an induced-fit conformational change upon PPi binding. These mechanistic implications are yet to be tested. The observed conformation where this motif is disengaged from the active site may serve a target of inhibitors that would lock the enzyme in the inactive state.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.jsb.2014.12.003](http://dx.doi.org/10.1016/j.jsb.2014.12.003).

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