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Inorganic pyrophosphatase crystals from *Thermococcus thio还原ens* for X-ray and neutron diffraction

Inorganic pyrophosphatase (IPPase) from the archaeon *Thermococcus thio还原ens* was cloned, overexpressed in *Escherichia coli*, purified and crystallized in restricted geometry, resulting in large crystal volumes exceeding 5 mm³. IPPase is thermally stable and is able to resist denaturation at temperatures above 348 K. Owing to the high temperature tolerance of the enzyme, the protein was amenable to room-temperature manipulation at the level of protein preparation, crystallization and X-ray and neutron diffraction analyses. A complete synchrotron X-ray diffraction data set to 1.85 Å resolution was collected at room temperature from a single crystal of IPPase (monoclinic space group C2, unit-cell parameters $a = 106.11$, $b = 95.46$, $c = 113.68$ Å, $\alpha = \gamma = 90.0$, $\beta = 98.12^\circ$). As large-volume crystals of IPPase can be obtained, preliminary neutron diffraction tests were undertaken. Consequently, Laue diffraction images were obtained, with reflections observed to 2.1 Å resolution with $I/\sigma(I)$ greater than 2.5. The preliminary crystallographic results reported here set in place future structure–function and mechanism studies of IPPase.

1. Introduction

Inorganic pyrophosphatase (IPPase; EC 3.6.1.1) catalyzes the hydrolysis of inorganic pyrophosphate (PP_i) to form orthophosphate (P_i). The action of this enzyme shifts the overall equilibrium in favor of synthesis in the polymerization of nucleic acids and the production of coenzymes and proteins. IPPases from a diversity of sources have been studied at the biochemical and structural levels. Cytosolic IPPases have been divided into two subfamilies, family I and family II, based on their binding preferences to Mg²⁺ and Mn²⁺ cofactors, respectively, sequence conservation and activation efficiency. Family I IPPases are single-domain OB-fold proteins, whereas family II enzymes have two domains. The catalytic mechanisms are quite similar for both families despite no or very little sequence homology. The IPPases from *Saccharomyces cerevisiae* (yeast; Oksanen *et al.*, 2007; Heikinheimo *et al.*, 2001) and *Escherichia coli* (Kankare *et al.*, 1996; Salminen *et al.*, 1995) both belong to family I and are the most commonly studied and well known IPPases.

Prokaryotic and archaeal IPPase structures are predominantly homohexamers with a subunit molecular mass of approximately 20 kDa (Harutyunyan *et al.*, 1997; Chao *et al.*, 2006; Liu *et al.*, 2004; Leppänen *et al.*, 1999). Eukaryotic IPPases predominantly exist as dimers consisting of two 30 kDa monomers. While family I IPPases can be found in all kingdoms of life, family II IPPases occur almost exclusively in bacterial species, many of which are human pathogens. An example of a family II monomeric IPPase is that from *Streptococcus agalactiae*, a prokaryote that infects neonates and immunocompromised adults (Rantanen *et al.*, 2007). Overall, the two families are structurally dissimilar; however, the active-site structures in the two families converge (Merckel *et al.*, 2001).

IPPases derived from thermostable microorganisms are of great interest because of their inherent chemical and thermal stability for long-duration biophysical studies. Examples of extremely thermostable IPPases that have been characterized with known structures are predominantly of the bacterial and archaeal types. They include enzymes from *Pyrococcus horikoshii* (Liu *et al.*, 2004), *P. furiosus* (Zhou *et al.*, 2005), *Sulfolobus acidocaldarius* (Leppänen *et al.*, 1999)

and *Thermus thermophilus* (Tepljakov *et al.*, 1994). The active sites of the thermally resistant IPPases are similar to those of their mesophilic equivalents in having an elaborate network of noncovalent interactions consisting of hydrogen bonds and ionic interaction between 3–4 divalent metal cations, water molecules, protein groups and the pyrophosphate substrate.

A homologous IPPase gene for a family I IPPase was identified in the genome sequence of *Thermococcus thio-reducens*, an obligate sulfur-reducing hyperthermophilic archaeon (Pikuta *et al.*, 2007). Here, we report the cloning, recombinant expression and crystallization of *T. thio-reducens* IPPase. The crystals of IPPase were observed to have extreme thermal and chemical stability, such that a long crystallization process was achievable at room temperature allowing the possibility of large-volume crystal growth. Consequently, IPPase crystals were amenable to room-temperature X-ray synchrotron and neutron diffraction analyses. Using these crystals, future X-ray and neutron crystallographic studies of IPPase will provide insight into the catalytic mechanism of the enzyme and will allow direct structural comparison with other members of the IPPase families.

2. Materials and methods

2.1. Cloning

The coding region for IPPase was selectively amplified by the polymerase chain reaction (PCR; Mullis & Faloona, 1987) against the genomic DNA of *T. thio-reducens* using the PCR primer pairs 5'-tttgttaactttaagaaggagatatacatATGAACCGTTCCACGAGCT-3' (Operon, USA) and 5'-tccttctgggcttctgttagcagccgatccTCACTCCTCCTTGCCGAAC-3'. The primers were designed from the exact alignment of the first and last 20 nucleotides of the IPPase open reading frame (upper-case letters). The oligonucleotide primers were also preceded by linker sequences corresponding to 30 nucleotides of the expression plasmid vector pET3a (Novagen) containing *NdeI*/*Bam*HI insertion sites (lower-case letters). The amplification reaction was performed in a total volume of 50 µl containing 20 pmol of each primer, 20 ng genomic template, 2 U Rapid Fidelity DNA polymerase (iXpressGenes, USA), 200 µM of each dNTP, 1 mM MgCl₂ and standard PCR buffer (Mullis *et al.*, 1986) in sterile PCR-grade water. PCR was carried out using a Perkin Elmer GeneAmp PCR System 9600 thermal cycler for 30 s initial denaturation at 368 K, followed by 30 cycles of 30 s at 368 K, 30 s at 328 K and 1 min at 347 K. A final extension step was performed for 2 min at 347 K.

The amplified IPPase open reading frame was inserted into a linearized plasmid vector of pET3a digested with *NdeI*/*Bam*HI by *in vivo* homologous recombination as described by Marsic *et al.* (2008). The recombinant plasmid was propagated in *E. coli* DH5α cells and purified by standard molecular-biology protocols (Sambrook *et al.*, 1989) using a Qiagen Plasmid Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Purified plasmids were sequenced by the MWG DNA-sequencing service to verify the IPPase-coding sequence. The translated protein sequence from the IPPase open reading frame was calculated to have a molecular weight of 20.85 kDa as computed using the ExPASy Bioinformatics Resource Portal.

2.2. Expression and purification

An error-free recombinant plasmid construct was transformed into *E. coli* Rosetta 2 cells (Novagen) and cultures were typically grown in 2 l Luria–Bertani (LB) broth containing 100 µg ml⁻¹ carbenicillin and 35 µg ml⁻¹ chloramphenicol. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a

final concentration of 1 mM when the optical density of the culture reached an OD_{600 nm} of 0.6. The culture was shaken overnight at 250 rev min⁻¹ at 291 K. After 16 h, cells were harvested by centrifugation at 8000g using a JLA-9.1000 rotor in a Beckman Avanti J-25 centrifuge. Protein purification was accomplished by resuspending the pellet (~10 g wet weight) in 100 ml buffer A (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA). The cells were disrupted by eight cycles of sonication using a Branson Sonifier 250 cell disruptor (VWR Scientific, USA). The resulting lysate was centrifuged for 20 min at 10 000g in a JA-25.50 rotor and the supernatant was subjected to heat treatment for 30 min in an Erlenmeyer flask submerged in a 348 K water bath. Denatured protein was removed by centrifugation at 10 000g for 20 min using the same rotor and the supernatant was loaded onto a 5 ml HiTrap Q Sepharose XL cation-exchange column (GE Healthcare, USA) that had been pre-equilibrated with buffer A. The protein was eluted with a 0.05–1.0 M NaCl linear gradient in buffer A using an ÄKTAexplorer FPLC system (Amersham Pharmacia, USA). Fractions containing IPPase were pooled and the final protein was judged to be substantially free of any major protein contaminant as determined by SDS–PAGE analysis. The purified protein was applied onto a Centricon-10 ultrafiltration device (Millipore) and centrifuged at 4000g to reduce the volume to 2 ml prior to loading onto a S-200 Sephadex size-exclusion column (GE Healthcare) equilibrated with 100 mM HEPES pH 7.5, 50 mM NaCl. Fractions corresponding to the major peak were pooled and analyzed by SDS–PAGE. The final protein sample was concentrated using a Centricon 10 ultrafiltration device to 25 mg ml⁻¹ as determined by Bradford analysis (Bradford, 1976).

2.3. Crystallization

Crystallization trials were performed at 293 K using the sitting-drop vapor-diffusion method in a 96-well Intelli-Plate (Art Robbins) with commercial sparse-matrix screens (Jancarik & Kim, 1991) from Hampton Research. Crystal Screen (48 conditions) and Crystal Screen 2 (48 conditions) were used as reservoir solutions in the initial trial. Protein and precipitant in 1:1 and 2:1 ratios were placed in the small and large drop wells, respectively. The plate was allowed to equilibrate undisturbed for one week at 293 K in a low-temperature incubator. Small IPPase crystals were observed when the protein was equilibrated against 30% 2-methyl-2,4-pentanediol (MPD), 100 mM sodium acetate pH 4.6, 20 mM calcium chloride.

Crystallization was initially optimized using sitting-drop vapor diffusion. The largest and best diffracting crystals were obtained in the condition 25% 2-methyl-2,4-pentanediol (MPD), 100 mM sodium acetate pH 4.6, 10 mM calcium chloride, 2 mM ammonium sulfate. This optimized condition was subsequently used for setups similar to counter-diffusion crystallization in restricted geometry (García-Ruiz, 2003; Ng *et al.*, 2003; Gavira *et al.*, 2002). With the aim of obtaining large-volume crystals, 10 cm quartz capillaries (Vitrocom) with diameters of 1.0, 1.5 and 2 mm were used. The precipitating agent and protein solutions were arranged in juxtaposition to one another inside a volume-constrained channel. In this case, half of the volume of the capillary was filled with purified IPPase at a concentration of 35 mg ml⁻¹ and was allowed to diffuse against an equal volume of the same precipitating solution as described above. The two solutions were separated by a 100 µl 1% low-melting agarose plug to minimize shock on mixing. The capillary tube was sealed at both ends with soft wax and coated with two layers of fast-drying enamel. The crystallization process took place at 293 K and the capillaries were kept in a horizontal arrangement throughout the equilibration process.

2.4. X-ray data collection

A single IPPase crystal obtained in a 1 mm diameter quartz capillary was used for *in situ* X-ray data analysis at 298 K. The entire capillary containing the targeted crystal was mounted horizontally and centered directly in front of the X-ray source. The IPPase crystal filled up the diameter of the capillary and was immobilized by its attachment to the capillary wall. X-ray diffraction data were recorded using a MAR 225 CCD (charge-coupled device) detector on the SER-CAT beamline 22-BM (Argonne National Laboratory, Chicago, Illinois, USA) using 0.92 Å wavelength X-rays. The beam was attenuated to 20% intensity using a narrow slit collimator. The exposure time for each image was 1 s per 1.0° oscillation angle recorded by the detector. Data were processed and reduced with *HKL-2000* and the relevant statistics are shown in Table 1.

2.5. Neutron diffraction analysis

A IPPase crystal grown in a quartz capillary tube (inner diameter 2.0 mm) was measured to be have a volume of greater than 5.0 mm³ and was targeted for neutron diffraction analysis. Labile H atoms were exchanged for deuterium by removing the surrounding liquid around the targeted crystal and allowing it to equilibrate against a D₂O mother-liquor solution containing 78–91% deuterated MPD in D₂O (CDN Isotopes) in the capillary. The exchange was performed by vapor diffusion against the new deuterated solution for a period of one month with bi-weekly exchanges of new deuterated solutions.

A wavelength-resolved time-of-flight neutron Laue diffraction image of a single IPPase crystal setting was recorded at the Protein Crystallography Station (PCS) at the Los Alamos Neutron Science Center (LANSCE; Langan *et al.*, 2004). The crystal used was more than 5 mm³ in volume and was exposed for 16 h. The crystal-to-detector distance was 730 mm, giving 120° detector coverage in the horizontal plane with 16° detector coverage in the vertical plane. The neutrons were generated from a tungsten target in pulses with a frequency of 20 Hz and their energy was moderated by a specially designed partially coupled water moderator. The primary neutron flight path was 28 m in length and the wavelength range and angular

Table 1

Data-collection statistics.

Values in parentheses are for the outer shell.

X-ray source	SER-CAT 22-BM
Crystal volume (mm ³)	0.5
Crystallization method	Counter- and free-interface diffusion
Data-collection temperature (K)	298
Wavelength (Å)	0.92
No. of frames	180
Oscillation range (°)	1
Exposure time (s)	1
Beam attenuation (%)	20
Space group	C2
Unit-cell parameters (Å, °)	$a = 106.11$, $b = 95.46$, $c = 113.68$, $\alpha = \gamma = 90.0$, $\beta = 98.12$
Molecules per asymmetric unit	6
Solvent content (%)	46.1
Resolution (Å)	50.0–1.85
No. of reflections	296646
No. of unique reflections	95841
Multiplicity	3.1
Crystal mosaicity (°)	0.14
Completeness (%)	99.0 (98.4)
$R_{\text{merge}}^{\dagger}$ (%)	7.8 (39.6)
$\langle I/\sigma(I) \rangle$	13.5 (2.63)
Wilson plot B factor (Å ²)	18.2

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

divergence of the incident neutron beam were shaped by a composite T0/T1 chopper and a series of collimating inserts to give a wavelength range of 0.6–6 Å with a beam divergence of 0.12° (FWHM) at the sample position. Diffraction data were processed using a modified version of *d*TREK* (Pflugrath, 1999).

Large-volume IPPase crystals were also tested using the quasi-Laue neutron diffractometer LADI-III at the Institut Laue–Langevin (ILL), Grenoble, France. The cylindrical area detector of LADI-III provides a large coverage of reciprocal space ($>2\pi$ sr) and thereby allows a large numbers of Bragg reflections to be recorded simultaneously. Neutron diffraction data were collected using quasi-Laue methods *via* the use of Ni/Ti multilayer band-pass filters. Several neutron quasi-Laue diffraction images were recorded at room temperature (exposure times from 6 to 12 h) for the deuterium-exchanged IPPase crystals using a neutron wavelength range from 3.1 to 4.2 Å for data collection.

3. Results and discussion

The recombinant IPPase could easily be purified to homogeneity in four major steps with a high yield. 75 mg purified protein could usually be obtained from 4 l culture and the molecular mass of the protein was estimated to be about 21 kDa by SDS–PAGE (Fig. 1). This value is consistent with the calculated value of 20.85 kDa derived from the protein sequence. No sequence tags were necessary for the initial purification step, as more than 75% purity could be obtained after heat treatment based on SDS–PAGE analysis. By the same analysis, purified IPPase could be obtained with more than 95% homogeneity after two chromatography steps (Fig. 1).

A single prismatic crystal of IPPase grew to a volume exceeding 5 mm³ in a capillary tube with an inner diameter of between 1 and 2 mm (Fig. 2). Despite the large diameter, a transient supersaturation gradient was still achieved owing to the increase in viscosity of the precipitating agent containing 30% MPD. This was evident from the formation of slight precipitation near the gel–plug interface followed by the progressive growth of many small crystals into a single protein crystal along the length of the capillary. This pattern is commonly observed in counter-diffusion equilibration (Ng *et al.*, 2003; García-

Figure 1

SDS–PAGE analysis of IPPase. The production of recombinant IPPase was evaluated on 12% SDS–PAGE at various purification steps. Crude lysate (lane 1) showed strong expression of the IPPase, corresponding to a molecular size of about 21 kDa when compared with molecular-mass standards (lane M; labeled in kDa). Heat treatment at 34 K removed most temperature-labile protein contaminants (lane 2). Anion-exchange (lane 3) and size-exclusion chromatography (lane 4) resulted in further purification of IPPase to greater than 95% homogeneity. Approximately 15 µg protein was loaded in each lane, stained with Coomassie Brilliant Blue and visualized by white-light illumination.

Ruiz, 2003). Within three weeks, there was notable slow mixing and consequently a single crystal within the capillary filled the diameter of the capillary at the expense of other crystals (Otálora *et al.*, 1996). Consistently, the largest crystal was physically robust and immobilized to the capillary wall.

The sequence and mechanism of IPPase crystal growth occurring in the capillary is more complex than in a purely diffusive counter-diffusion experiment. Detailed observation and quantitative analysis of the crystallization process is presently in progress. However, a general observation that is consistent with every preparation is the

notable formation of many small crystals and their dissolution to favor the formation of single large crystals along the length of the capillary during the early equilibrium process. The agarose plug that divides the precipitant and protein chambers is necessary to prevent convective mixing and to allow the diffusion of the precipitant and protein molecules, thus producing a supersaturation gradient during the equilibration process. In the absence of an agarose plug, protein and precipitant solution rapidly mix, resulting in immediate precipitation; consequently, no supersaturation gradient is formed (Howard *et al.*, 2009). The agarose plug plays an important role in preventing

Figure 2

Single crystals of IPPase for X-ray and neutron diffraction. (a) A prismatic crystal of IPPase is shown to fill up the inner diameter (1 mm) of the capillary. The crystal dimensions were approximately $1.0 \times 1.0 \times 0.5$ mm and it continued to grow at the expense of the adjacent smaller crystal seen at the bottom left. The principal crystal was completely attached to the capillary walls and was used directly for *in situ* X-ray data collection. (b) An IPPase single crystal grown in a 2 mm inner diameter capillary tube was used for preliminary neutron diffraction tests. The crystal dimensions were measured to be $2.0 \times 1.5 \times 2.5$ mm. Both crystals were obtained within 3–4 weeks at 293 K and showed no evidence of dissolution or change of habit after six months of storage at the same temperature.

Figure 3

Synchrotron X-ray diffraction images of IPPase at room temperature. 1° oscillation images are shown during the initial data collection (a), after 90 frames (b) and after 180 frames (c). The edges of the diffraction images correspond to 1.85 Å resolution. Outer edge reflections are magnified in the bottom panels corresponding to the images.

mixing and sustaining controlled diffusion. As the crystals started to form in the free protein solution, convective mixing proceeds with the capillary remaining in a horizontal position to minimize the effect of gravity. This convective flow eventually helps to speed up the growth of large crystal volumes at the expense of the dissolution of smaller crystals because solubility is a function of crystal size. Other hyperthermophilic proteins that we have purified (data not shown) also demonstrate the same crystallization phenomena in the same geometry as long as the chemical and thermal stability of the protein are sustained throughout the equilibration process. The approach described here to obtain large-volume crystal growth for IPPase may be an effective strategy to obtain crystals of other proteins that are suitable for neutron crystallography.

X-ray diffraction analysis was performed *in situ* at room temperature on the best crystal of IPPase using synchrotron radiation without any cryogenic treatment. The IPPase crystal showed no evidence of disorder and displayed little radiation damage after 180 images. Thus, it was possible to obtain a high-quality complete diffraction data set from a single crystal (Fig. 3). The reflection spots were compact, had low mosaic spread and were easily indexed and scaled.

The data-processing statistics are shown in Table 1. The diffraction limit of the same crystals could be extended to beyond 1.5 Å when the X-ray beam was not attenuated and the exposure time was not restrained to 1 s. IPPase crystallized in the presence of MPD and calcium was always identified to belong to a monoclinic space group. Higher symmetry IPPase crystals have been observed to form in the presence of pyrophosphate substrate and other divalent cations. With the aim of utilizing less neutron beam time, an effort is being made to obtain a higher symmetry large-volume crystal form of IPPase.

Six molecules were assumed to be present in the asymmetric unit, resulting in a Matthews coefficient of 2.28 Å³ Da⁻¹ corresponding to a solvent content of 46.10% (Matthews, 1968). A preliminary structure solution of IPPase was obtained by molecular-replacement calculations in *MOLREP* (Vagin & Teplyakov, 2010) using the crystal structure of *P. furiosus* IPPase (PDB entry 1twl; Southeast Collaboratory for Structural Genomics, unpublished work) as the search model. *T. thio-reducens* IPPase is 88% identical in sequence to

P. furiosus IPPase. The correlation coefficient (CC) and *R* factor for the correct unrefined solution were 0.77 and 0.45, respectively, using 47.73–2.00 Å resolution data. Examination of the best solution revealed good crystal packing and no clashes between symmetry-related molecules. Refinement and analysis of the complete structure will be reported elsewhere. Interestingly, after six months of storage at 293 K the same crystal showed little decay; the diffraction quality was about the same as the original X-ray image obtained using the same synchrotron source (data not shown).

It was also evaluated whether IPPase crystals would be suitable for neutron diffraction. The capillary geometry allowed facile hydrogen exchange against deuterated solutions. Surrounding solutions and neighboring crystals were removed without disturbing the targeted IPPase crystal, leaving only a trace of mother liquor at the base of the crystal. Since the IPPase crystal filled up the diameter of the capillary, it was completely immobilized and thus solutions could be filled and removed without any disturbance to the principal IPPase crystal. Exchanging solutions were filled from both sides of the capillary up to about 1 cm away from the IPPase crystal, leaving an air space for vapor diffusion. Bi-weekly exchanges for a period of one month were sufficient to prepare the crystal for an adequate neutron diffraction image. Preliminary neutron Laue diffraction at 293 K using the PCS at Los Alamos showed that with an exposure of 16 h Bragg reflections could be measured to 2.1 Å resolution with no spatial overlaps (Fig. 4*a*). $I/\sigma(I)$ was greater than 2.5 at the highest resolution. The crystals were indexed as *C*-centered monoclinic, with unit-cell parameters that were nearly identical to those determined by room-temperature X-ray diffraction. Similarly, different crystals prepared in the same manner were analyzed using the LADI-III instrument at the ILL. Reflection spots could be observed to beyond 2.1 Å resolution after an exposure time of 12 h. Neutron quasi-Laue data were analyzed at 2.5 Å resolution with $I/\sigma(I)$ greater than 5.0 at the highest resolution (Fig. 4*b*). Owing to limited synchrotron and neutron beam time, as well as having to transport the crystals to different locations around the world, we were not able to analyze exactly the same crystal using both X-ray and neutron analysis.

IPPase crystals can be consistently grown to large volumes and are shown to be suitable for neutron diffraction at different neutron

Figure 4 Initial neutron Laue diffraction images from single IPPase crystals. (*a*) One complete image from a 120° curved (arc length of 1.5 m) ³He gas neutron detector collected at the PCS instrument is shown. Reflections are measured to 2.1 Å resolution with $I/\sigma(I)$ greater than 2.5. (*b*) IPPase crystals soaked with different metals were tested using the LADI-III instrument. A quasi-Laue diffraction pattern using a 12 h exposure is shown. Reflections were measured to 2.5 Å resolution with an $I/\sigma(I)$ greater than 5.0. The crystals used in preliminary neutron diffraction tests were measured to be greater than 5 mm³ in volume.

sources. Interestingly, perdeuterated IPPase has been observed to crystallize in the capillary in a similar manner, except that the rate of crystal growth is almost twice as slow and the protein crystals often do not fill up the diameter of the capillary like those of non-perdeuterated IPPase. This isotope effect on this crystallization process merits further study.

The apparent chemical and thermal stability of IPPase and its propensity to crystallize in capillary geometry makes this protein an ideal target for X-ray and neutron crystallographic studies. Since the protein crystal equilibration time is long (several months at room temperature), large-volume crystals can be obtained. Room-temperature data collection using synchrotron X-ray sources was exploited to eliminate the need for cryogenic preparation. Finally, IPPase crystals are readily amenable to neutron crystallographic analysis as crystals with large volumes ($>5\text{ mm}^3$) can be grown and they undergo hydrogen exchange readily without any compromise to crystal quality. It has recently been shown that combining X-ray and neutron data in a joint structure-refinement procedure provides more accurate and more complete structures of biological macromolecules (Adams *et al.*, 2009). The ability to obtain both X-ray and neutron crystallographic data for IPPase will be a considerable advantage to support structure–function and mechanism studies of this important enzyme.

4. Conclusion

Soluble IPPase is an essential enzyme in phosphorus metabolism. To understand the mechanism of its catalytic action requires the accurate structure of the topological features and active site of the enzyme, especially with regard to the protonation states of the protein and proton transfer. We believe that IPPase may be an important protein to provide information regarding intermolecular and intramolecular interactions and the catalytic mechanism of family I IPPases. Because IPPase can be produced in high yield, can be easily purified, is chemically and thermally stable and can be crystallized with large volumes, the protein is suitable for both X-ray and neutron crystallography. Thus, the important atomic features of this enzyme and its biological functions can be further understood. One important point of interest is the effects of divalent metal ions on the pK_a values of its essential general acids and bases. This can be investigated by forming metal complexes using the protein crystals reported here. Future studies will focus on these areas.

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