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Expression, purification and preliminary X-ray characterization of histidinol phosphate phosphatase

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Histidinol phosphate phosphatase (HisPPase) catalyzes the eighth step of histidine biosynthesis, in which L-histidinol phosphate undergoes dephosphorylation to give histidinol. A recombinant form of the histidinol phosphate phosphatase from *Thermus thermophilus* HB8 has been expressed in *Escherichia coli*, purified and crystallized in two crystal forms by the hanging-drop vapour-diffusion technique. Crystal form I belongs to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 84.8, b = 97.2, c = 74.9 Å, and crystal form II belongs to the orthorhombic space group $C222_1$, with unit-cell parameters a = 76.9, b = 157.6, c = 116.7 Å. The crystals probably contain two monomers in the asymmetric unit, with V_M values of 2.57 $Å^3$ Da^{-1} for form I and 2.96 $Å^3$ Da^{-1} for form II. X-ray data have been collected to 1.70 and 1.75 Å resolution for crystal forms I and II, respectively.

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1. Introduction

Histidinol phosphate phosphatase (HisPPase) catalyzes the eighth step of histidine biosynthesis, in which L-histidinol phosphate, the product of the seventh step catalyzed by histidinol phosphate aminotransferase, undergoes dephosphorylation to give histidinol, which is converted to histidine by histidinol dehydrogenase (Voet & Voet, 1994). The HisPPases are divided into *Escherichia coli* and *Bacillus subtilis* types (le Coq *et al.*, 1999). The *E. coli*-type HisPPases are bifunctional enzymes in which the N-terminal domain has HisPPase activity and the C-terminal domain has imidazoleglycerol phosphate dehydratase activity (Chiariotti *et al.*, 1986; Carlomagno *et al.*, 1988; Winkler *et al.*, 1996). The enzymes belong to the 'DDDD' superfamily named after the presence of four invariant aspartate residues (Thaller *et al.*, 1998). Non-specific acid phosphatases, phosphoglycolate phosphatases, phosphoserine phosphatases and trehalose-6-phosphatases also belong to the DDDD superfamily. The *B. subtilis*-type HisPPases are monofunctional enzymes with HisPPase activity. The enzymes belong to the PHP superfamily named after polymerase and histidinol phosphatase, the catalytic site of which has four motifs with conserved histidine residues (Aravind & Koonin, 1998). The PHP superfamily includes histidinol phosphatases, the α-subunits of bacterial DNA polymerase III and family X DNA polymerases.

During the *Thermus thermophilus* HB8 structural genomics project conducted by the RIKEN Structural Genomics Initiative (Yokoyama *et al.*, 2000), a gene homologous to the *B. subtilis*-type HisPPases belonging to the PHP superfamily was identified (tHisPPase). A homology search using *FASTA* (Pearson & Lipman, 1988) and *CLUSTALW* (Thompson *et al.*, 1994) indicated that the sequence of the gene was highly or significantly identical to those of probable HisPPases from *Deinococcus radiodurans* and *Helicobacter heptaticus*, the HisPPases from *B. cereus*, *B. anthracis* and *B. subtilis*, and the small domain of the DNA polymerase X family from *B. cereus*, with sequence identities of 52, 38, 29, 29, 27 and 21%, respectively. tHisPPase and HisPPases that show high sequence identities (>24%) with tHisPPase have the four conserved sequence motifs [His-*h*-His, *h*-*x*-*x*-His, Asp-Phe (or Tyr)-*h*-Ile-*x*-Ser-*h*-His and *h*-*h*-*h*-*x*-*x*-Asp-*x*-His, where *h* is a hydrophobic residue and *x* is any residue] mentioned above and two further conserved motifs Gly-*h*-Glu and *h*-Gly-His-*h*-Asp. The histidine and aspartate residues in these motifs might be involved in active-site formation, interacting with metal ions.

We expressed the gene in *E. coli*, purified the product protein and confirmed that the recombinant enzyme has HisPPase activity. tHisPPase has 267 residues per subunit and a molecular weight of 29 976 Da. Structure determination of tHisPPase should help to clarify the role of the active-site residues and the mechanism of substrate recognition, since the three-dimensional structure of HisPPase is not available. Moreover, a comparison of the structure of tHisPPase with those of other phosphatases, such as acid and alkaline phosphatases, fructose-1,6-bisphosphatase and pyrophosphatases (Metzler, 2001), will provide

insight into the catalytic mechanism of the hydrolysis of phosphate esters. In this communication, we report the crystallization and preliminary X-ray diffraction studies of tHisPPase.

2. Experimental

2.1. Expression and purification

The tHisPPase gene was amplified by PCR using a set of primers (5′-ATATCATATGGTAGATAGCCACGTCCACACTCCCCTCT-3′ and 5′-ATATAGATCTTTATTACGAGGCCCTGGACAAGGGGTAGGCC-3′) and cloned into pT7Blue (Novagen). After confirmation of the nucleotide sequence, the tHisPPase gene was ligated into the expression vector pET-11a (Novagen) at the *Nde*I/*Bam*HI sites. The resulting expression plasmid was used to transform *E. coli* strain BL21(DE3) (Novagen). The transformant was cultured at 310 K in 6 l of Luria broth supplemented with ampicillin (50 µg ml^{-1}). When the OD_{600} of the medium reached 0.6, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 m*M*. Cells were harvested by centrifugation after 16 h and stored at 193 K until use.

The cells were suspended in 20 m*M* Tris–HCl, 5 m*M* 2-mercaptoethanol and 50 m*M* NaCl pH 8.0 and then disrupted by sonication. The cell lysate was incubated at 343 K for 10 min and then ultracentrifuged (200 000*g*) for 60 min at 277 K. Solid ammonium sulfate was added to the resulting supernatant to a final concentration of 1.5 *M* and the solution was applied onto a hydrophobic column (Resource PHE, Amersham Biosciences) equilibrated with 50 m*M* sodium phosphate pH 7.0 containing 1.05 *M* ammonium sulfate. The enzyme was eluted with a linear gradient of 1.5–0 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.0. Fractions containing tHisPPase were desalted and applied onto an anion-exchange column (Resource Q, Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0. tHisPPase was eluted with a linear gradient of 0–0.5 *M* NaCl in 20 m*M* Tris–HCl pH 8.0. Fractions containing tHisPPase were desalted and applied onto a hydroxyapatite CHT2-I column (Bio-Rad) equilibrated with 10 m*M* sodium phosphate pH 7.0. The flowthrough fractions containing tHisPPase were pooled and concentrated by ultrafiltration. The concentrated solution was applied onto a gel-filtration column (HiLoad 16/60 Superdex 200 pg, Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl and 150 m*M* NaCl pH 8.0 to determine the apparent molecular weight of tHisPPase based on the elution time of the protein peak. The peak fractions were concentrated and stored at 277 K.

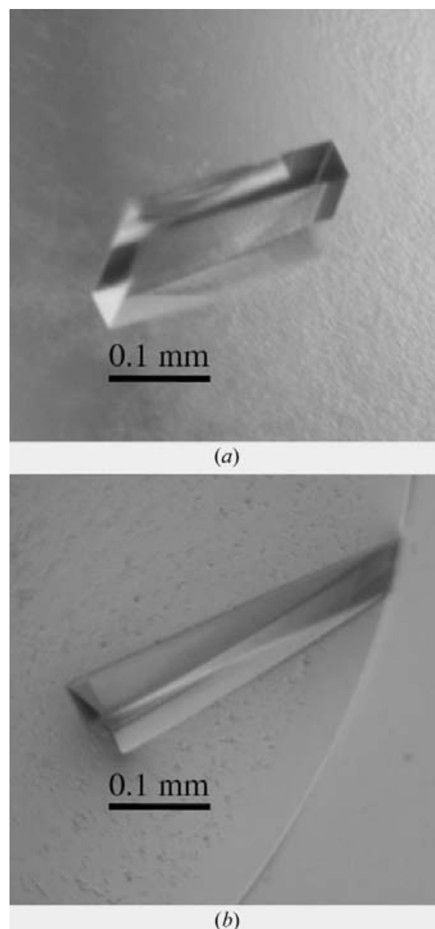

Figure 1
Crystal forms I (*a*) and II (*b*) of histidinol phosphate phosphatase from *T. themophilus* HB8.

2.2. Enzyme assay

Enzymatic activity was measured by analyzing the inorganic phosphate released from histidinol phosphate using a previously reported procedure (Lanzetta *et al.*, 1979). Measurements were performed in 60 m*M* Tris–HCl buffer pH 7.5 containing 0.024 µ*M* (per subunit) tHisPPase at 343 K. The reaction was initiated by adding histidinol phosphate and terminated by the addition of malachite green reagent solution. The protein concentration was estimated by the method of Bradford (1976). The specific activity was 5.36 (1) µmol min^{-1} mg^{-1} at 343 K. Measurement of the molecular weight was performed using dynamic light scattering (DynaPro-801, Protein Solutions) by injecting 20 µl of a 1 mg ml^{-1} protein in Tris–HCl pH 8.0.

2.3. Crystallization

tHisPPase was crystallized by the hanging-drop vapour-diffusion method at 293 K. The initial screening for the crystallization conditions was performed using the sparse-matrix screen Crystal Screen I from Hampton Research (Jancarik & Kim, 1991). Minute crystals of tHisPPase appeared from solutions No. 6 [100 m*M* Tris–HCl pH 8.5, 30%(*w*/*v*) PEG 4000 and 0.2 *M* $MgCl_2$] and No. 39 [100 m*M* Na HEPES pH 7.5, 2.0 *M* ammonium sulfate and 2%(*v*/*v*) PEG 400]. The crystallization conditions were optimized by changing the concentration of the precipitants and additives, replacing $MgCl_2$ by $CaCl_2$ (solution No. 6) and adding MPD (solution No. 39). Two crystals forms (form I and form II) of tHisPPase were obtained from two different crystallization conditions. Form I crystals were obtained by equilibration of a mixture containing 3 µl protein solution (6.0 mg ml^{-1} protein and 100 m*M* Na HEPES pH 7.5) and 3 µl reservoir solution [100 m*M* Na HEPES pH 7.5, 1.5 *M* ammonium sulfate, 2%(*v*/*v*) PEG 400 and 1%(*v*/*v*) MPD] against the reservoir solution. Form II crystals were obtained by equilibration of a mixture containing 3 µl protein solution (6.0 mg ml^{-1} protein and 10 m*M* Tris–HCl pH 8.0) and 3 µl reservoir solution [100 m*M* Tris–HCl pH 8.5, 17%(*w*/*v*) PEG 4000 and 0.2 *M* $CaCl_2$] against the reservoir solution.

2.4. Data collection

For the preliminary characterization, intensity data for unit-cell parameter and space-group determination were collected at 100 K on a Rigaku R-AXIS IV^{++} image-plate detector. Prior to flash-freezing, the crystals were soaked for a few seconds in a reservoir solution containing 25% glycerol (form I) and 30% glycerol (form II). The crystals were then mounted in a 0.5 mm cryoloop (Hampton Research) and flash-frozen in a cold nitrogen stream at 100 K. Data collection for crystal forms I and II was performed at 100 K using a wavelength of 1.00 Å from the synchrotron-radiation source at SPring-8 BL44B2 and a MAR CCD165 detector system (Hyogo Japan). The data were processed using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Crystal forms I and II appeared within a week of incubation and grew to maximum

Table 1
Data-collection and processing statistics.

Values for the highest resolution shell are given in parentheses.

Data set	Form I	Form II
Space group	$P2_12_12$	$C222_1$
Unit-cell parameters		
a (Å)	84.8	76.9
b (Å)	97.2	157.6
c (Å)	74.9	116.7
Temperature (K)	100	100
Wavelength (Å)	1.0	1.0
Resolution (Å)	20.0–1.70 (1.76–1.70)	20.0–1.75 (1.81–1.75)
Total No. reflections	670639	602224
No. unique reflections	68779	71065
Completeness (%)	100.0 (100.0)	99.5 (99.2)
Mean $I/\sigma(I)$	26.2 (2.9)	19.1 (2.0)
$R_{\rm merge}$ (%)	4.8 (29.2)	6.1 (30.9)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i I_{hkl,i}$, where I is the observed intensity and $\langle I\rangle$ is the mean intensity for multiple measurements.

dimensions of 0.2 × 0.2 × 0.05 and 0.3 × 0.1 × 0.03 mm, respectively (Fig. 1). Crystal form I belongs to the orthorhombic space group $P2_12_12$, with a unit-cell volume of 6.17×10^5 Å^3. Crystal form II belongs to the orthorhombic space group $C222_1$, with a unit-cell volume of 1.42×10^6 Å^3. Assuming the presence of two monomers in the asymmetric unit, the Matthews coefficient ($V_{\rm M}$) values were calculated to be 2.57 and 2.96 Å^3 Da^{-1}, respectively, indicating estimated solvent contents of 45 and 52% in the unit cell for crystal forms I and II (Matthews, 1968). These values are within the range for typical protein crystals. Dynamic light-scattering measurements showed that the purified protein was monodisperse, with an estimated molecular weight of 118 kDa. Gel-filtration chromatography estimated the molecular weight to be 92 kDa. Taken together, these results suggest that tHisPPase with a subunit molecular weight of 29 976 Da exists as a homotetramer rather than a homodimer in solution and has a crystallographic twofold axis in both crystal forms. An X-ray diffraction data set for crystal form I has been collected with 68 779 unique reflections, giving a data-set completeness of 100.0% in the resolution range 20.0–1.7 Å (Table 1). A data set for crystal form II has been collected with 71 065 unique reflections, giving a data-set completeness of 99.2% in the resolution range 20.0–1.75 Å (Table 1). These data indicated that the crystals were of good quality. The crystal showed no significant decay upon exposure. The X-ray structure determination of tHisPPase based on a multiple isomorphous replacement method is currently under way.

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