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Using directed evolution based on random mutagenesis and heat-treated selection, a thermostable His170Tyr mutant of *Geobacillus stearothermophilus* thermostable *p*-nitrophenylphosphatase (TpNPPase) was obtained. The temperature at which the His170Tyr mutant lost 50% of its activity ($T_{1/2}$) was found to be 4.40 K higher than that of wild-type TpNPPase, and the melting temperature of the His170Tyr mutant increased by 2.39 K. The crystal structure of the His170Tyr mutant was then determined at 2.0 Å resolution in the presence of a sodium ion and a sulfate ion in the active site. The cap domain of chain *B* shows a half-closed conformation. The hydrophobic side chain of the mutated residue, the hydroxyphenyl group, forms a hydrophobic contact with the methyl group of Ala166. This hydrophobic interaction was found using the *Protein Interactions Calculator (PIC)* web server with an interaction distance of 4.6 Å, and might be a key factor in the thermostabilization of the His170Tyr mutant. This study potentially offers a molecular basis for both investigation of the catalytic mechanism and thermostable protein engineering.

1. Introduction

Thermostable *p*-nitrophenylphosphatase (TpNPPase) is an Mg^{2+} -dependent enzyme involved in the hydrolysis of *p*-nitrophenylphosphate (pNPP). It has an optimum reaction temperature of 328 K and a half-activity retention temperature ($T_{1/2}$) of 325 K (Li *et al.*, 2003). The single ester bond of pNPP is cleaved by TpNPPase in the reaction, yielding phosphate and *p*-nitrophenol (pNP). pNP is yellow-coloured with an absorbance maximum at 405 nm. The enzyme is widely applied in nucleotide labelling and enzyme-linked immunosorbent assays. As a member of the haloalkanoate dehalogenase superfamily (HADSf) II-A subfamily, TpNPPase has a highly conserved α/β core domain consisting of four catalytic motifs: motifs I–IV. The nucleophilic Asp10 in motif I attacks the organophosphate and forms the aspartylphosphate intermediate, while another residue in motif I, Asp12 (the Asp+2 residue), is involved in general acid/base catalysis (Lu *et al.*, 2008). Thr43 in motif II assists in substrate binding through hydrogen bonding (Zhang *et al.*, 2004). Lys181 in motif III orients the nucleophile (Tremblay *et al.*, 2006), and motif IV (Asp206) functions in the coordination of Mg^{2+} (Zhang *et al.*, 2004). The subfamilies are also defined based on the presence and the location of a cap domain. The substrate-specificity loop, motif V, usually belongs to the cap domain (Tremblay *et al.*, 2006).

Enzymes from the same family commonly share a high level of structural similarity. However, they may exhibit totally different thermostabilities. Thermostable proteins usually have important biotechnological advantages in purification methods, reaction rates and resistance to chemical denaturants (Becker *et al.*, 1997). Additional intra-protein interactions usually contribute to protein thermostability, such as hydrophobic interactions and networks (You *et al.*, 2010; Gromiha *et al.*, 2013; Kim *et al.*, 2012), hydrogen bonds and ionic pairs (Vogt *et al.*, 1997; Matsui & Harata, 2007), disulfide bridges (Yang *et al.*, 2007), salt bridges (Chan *et al.*, 2011), aromatic clusters (Puchkaev *et al.*, 2003) and cation– π interactions (Matsumura *et al.*, 2007). An optimized conformational structure of proteins also plays an important role in enhancing thermostability, such as increased rigidity and decreased flexibility (Radestock & Gohlke, 2008, 2011), better packing (Fleming & Richards, 2000), more stable α -helices and

Identifying the molecular mechanisms involved in the stability of thermophilic proteins is valuable for protein engineering in both the scientific and industrial fields. Directed evolution based on random mutagenesis is a powerful and frequently used approach for investigation of the mechanisms of protein thermostability (Shionyu-Mitsuyama *et al.*, 2005; Shi *et al.*, 2008; Goto *et al.*, 2008; Stephens *et*

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data collection and processing	
X-ray source	BL17U-MX
Wavelength (Å)	0.9792
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	$a = 38.75$, $b = 83.25$, $c = 176.05$, $\alpha = \beta = \gamma = 90$
Resolution (Å)	40.5–2.0 (2.07–2.00)
Total reflections	206742
Unique reflections	39405
Multiplicity	5.2
Completeness (%)	97.86 (90.38)
Mean $I/\sigma(I)$	13.46 (4.56)
R_{merge} (%)	9.0 (47.6)
Solvent content (%)	49.79
Refinement	
No. of molecules in asymmetric unit	2
R_{work} (%)	17.10 (16.56)
R_{free} (%)	21.34 (23.33)
No. of atoms	
Protein	3978
Ligand	6
Water	258
R.m.s.d.	
Bonds (Å)	0.011
Angles (°)	1.23
Average B factor (Å ²)	22.60
Ramachandran statistics (%)	
Most favoured	94.4
Generously allowed	5.6
Disallowed	0
PDB code	4o8c

al., 2009; Ben Mabrouk *et al.*, 2013). Since a large number of mutants with either increased or decreased thermostabilities can be obtained and analyzed systematically, the moderate thermostability of TpnPPase makes it an ideal subject for study of the mechanism of protein thermostabilization. In this study, we report the X-ray structure of the His170Tyr mutant protein, which was obtained by directed evolution, with improved thermostability.

2. Materials and methods

2.1. Random mutagenesis and screening strategy of mutants

Random mutagenesis of TpNPPase was performed by error-prone PCR. The primers 5'-GGCGTATCACGAGGCCCTTTCG-3' and 5'-GATGGAGTTCTGAGGTCATTACTGG-3' flanked by *Bam*HI and *Hind*III restriction sites were used as the forward and reverse primers, respectively. PCR was carried out in a 50 µl reaction mixture containing dUTP, which was designed to increase the error frequency. The mixture was incubated at 356 K for 5 min followed by 30 cycles of 30 s at 367 K, 1 min at 323 K and 1 min at 345 K and a final extension at 345 K for 7 min. The mutagenic PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen) from a 1% TAE agarose gel. The gene was then cloned into the expression vector pQE 30a and expressed in the *Escherichia coli* M15 strain (Qiagen).

The mutants of TpNPPase were obtained based on the *in situ* colour screening method reported by Shu *et al.* (2002). *E. coli* M15 cells with a random mutagenesis DNA library were incubated on LB agar plates containing 50 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ kanamycin and 0.04 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 310 K. The bacterial colonies were transferred to filter papers wetted with 50 mM Tris-HCl pH 10.0 and incubated at 333 K for 10 min. After incubation, the filter papers were then moved to activity-indicator plates (containing 50 mM Tris-HCl pH 10.0, 1 mM MgCl₂ and 5 mM pNPP) and incubated at 328 K for a further 5 min.

Under these conditions, wild-type TpNPPase was mostly heat-inactivated and could not produce the yellow-coloured nitrophenolate. In this way, positive colonies with enhanced thermostability were selected by their colour. The mutants were identified by DNA sequencing.

2.2. Protein expression and purification

An approximately 10 ml LB culture of recombinant *E. coli* M15 was incubated overnight at 310 K in the presence of ampicillin (50 $\mu\text{g ml}^{-1}$) and kanamycin (50 $\mu\text{g ml}^{-1}$) and was then transferred into 1 l LB medium with ampicillin (50 $\mu\text{g ml}^{-1}$) and kanamycin (50 $\mu\text{g ml}^{-1}$). The bacterial culture was induced with 1 mM IPTG when the OD_{600} reached 0.6–0.8, followed by 4 h incubation at 301 K.

The induced cells were harvested by centrifugation and lysed by pressure (JN-3000 Plus, JNBio, People's Republic of China) at 277 K in the presence of 50 mM sodium phosphate pH 8.0, 300 mM NaCl and 10 mM imidazole. The mutant enzyme was harvested by centri-

fugation at 15 000 rev min^{-1} (Rotor No. R20A2, Hitachi) for 30 min at 277 K.

The six-His-tagged mutant was then purified on an Ni-NTA Superflow column (Qiagen) following a protocol with a linear imidazole elution gradient (10–250 mM) in 50 mM sodium phosphate, 300 mM NaCl pH 8.0. The obtained protein was further purified by gel filtration using a Superdex 75 column (GE Healthcare, USA) equilibrated with buffer consisting of 20 mM Tris-HCl pH 8.0, 200 mM NaCl. The pure His170Tyr mutant protein was dialyzed against Milli-Q water and concentrated to 30 mg ml^{-1} using a 10 kDa centrifugal filter (Millipore). The concentrated protein was flash-cooled in liquid nitrogen and maintained at 193 K until crystallization. The tag was not removed during the purification and crystallization steps.

2.3. Thermostability measurements

The thermostabilities of TpNPPase and the His170Tyr mutant were estimated by determining both the melting temperature (T_m) and the temperature at which 50% phosphatase activity was retained after 15 min incubation ($T_{1/2}$).

2.3.1. Residual phosphatase activities. The reaction buffer contained 50 mM Tris-HCl pH 10.0, 1 mM MgCl_2 and 5 mM pNPP. Enzymes were incubated for 15 min at various temperatures ranging from 293 to 338 K. After the enzymes had been cooled on ice, their residual phosphatase activities were examined according to the method described by Guo *et al.* (2013). Fitting of the collected data and calculation of $T_{1/2}$ were performed using *GraphPad Prism* 6.

2.3.2. Circular dichroism. The T_m values of native and mutant TpNPPase were compared by circular-dichroism spectroscopy (Jasco J-815). Proteins were diluted to 0.3 mg ml^{-1} in 10 mM PBS pH 8.0. Temperature-induced denaturation (313–353 K) was determined by stepped ramping in 2 K increments in a 0.1 cm path-length cuvette, and ellipticity data of the enzymes were collected in the UVCD band (200–230 nm). Thermal denaturation curves were fitted to a two-state model as described by Borgo & Havranek (2012). Calculation of T_m was performed using *Origin* 8.

Figure 2
(a) Residues 18–22 of the His170Tyr mutant shown in cartoon representation. The electron-density map of residues 18–22 is shown (contoured at 2.0σ). SO_4^{2-} , Asp12 and Gly18 are shown as sticks. Polar contact between Asp12 and Gly18 is shown as a dashed line. (b) Residues 18–22 are not visible in the structure of wild-type TpNPPase.

Figure 3
Superimposition of the active sites of wild-type TpNPPase (violet), the His170Tyr mutant (blue) and PLPP (green).

Figure 4

Conformational comparison of the cap domain. (a) The cap-open conformation of wild-type TpNPPase (PDB entry 4kn8; Guo *et al.*, 2013). (b) Complex structure of the His170Tyr mutant with Na⁺ and SO₄²⁻, showing a half-closed conformation. Na⁺ and SO₄²⁻ are represented by a red sphere and as sticks, respectively. (c) Complex structure of PLPP with substrate in the cap-closed conformation (PDB entry 2cft; B. S. Kang, H. J. Cho, K. J. Kim & O. S. Kwon, unpublished work). Ca²⁺ and PLP are shown as a red sphere and as sticks, respectively.

2.4. Crystallization and X-ray data collection

The preliminary crystallization conditions of the His170Tyr mutant were screened by sitting-drop and hanging-drop vapour diffusion at 293 K using Crystal Screen HT and Index HT (Hampton Research, USA). Crystals were obtained by mixing 1.2 µl protein solution with 1.2 µl reservoir solution, which consisted of 0.1 M HEPES pH 8.0, 1.9 M ammonium sulfate, 3% (w/v) polyethylene glycol 400 (PEG 400). The droplets were equilibrated against 500 µl reservoir solution.

Prior to data collection, the single crystal was soaked in mother liquor containing 30% (v/v) glycerol as a cryoprotectant solution and was flash-cooled directly in a liquid-nitrogen stream. The X-ray diffraction data were collected on beamline BL17U-MX at Shanghai Synchrotron Radiation Facility using a MAR DTB detector system. The X-ray wavelength was 0.979 Å. The exposure time was 0.8 s per frame with a crystal-to-detector distance of 280 mm. A complete data set was obtained by collecting 180 images with 1° oscillation. Integration and scaling of diffraction intensities were performed using the HKL-2000 software suite (Otwinowski & Minor, 1997).

2.5. Structure determination

The crystal structure of the mutant was determined by molecular replacement using the CCP4 suite of crystallographic programs (Winn *et al.*, 2011). The structure of wild-type TpNPPase (PDB entry 4kn8; Guo *et al.*, 2013) was used as the search model. Refinement was carried out using PHENIX v1.8.2-1309 (Terwilliger *et al.*, 2009) and manual fitting of side chains was performed in Coot (Emsley & Cowtan, 2004). The quality of the coordinates was validated using PROCHECK (Laskowski *et al.*, 1993). The structural figures were generated by PyMOL (DeLano, 2004).

3. Results and discussion

A thermostable mutant of TpNPPase, His170Tyr, was obtained through three rounds of random mutagenesis and one round of heat-treated selection. The $T_{1/2}$ and T_m of the mutant and the wild-type TpNPPase were then evaluated to determine their thermostabilities. The $T_{1/2}$ value of the His170Tyr mutant increased by 4.40 K (Supplementary Fig. S1[†]) and the T_m value increased by 2.39 K (Supplementary Fig. S2).

The His170Tyr mutant crystallized in space group $P2_12_12_1$ with two monomers in the asymmetric unit. The final model of the His170Tyr mutant was refined in the resolution range 40.5–2.0 Å to a crystallographic R_{work} value of 17.1% and an R_{free} value of 21.3%. Na⁺ and SO₄²⁻ ions were built into the structure with reasonable B factors (Fig. 1). After refinement, the final B factors of Na⁺ and SO₄²⁻ were 20.95 and 32.14 Å², respectively. A summary of the data-collection and refinement statistics is presented in Table 1. The overall structure of the His170Tyr mutant showed high similarity to that of wild-type TpNPPase (PDB entry 4kn8; Guo *et al.*, 2013), with a root-mean-square deviation of only 0.184 Å over all C $^{\alpha}$ atoms.

In wild-type TpNPPase residues 18–22 of chain B were not modelled owing to poor electron density, indicating that this loop might be quite flexible, whereas in the His170Tyr mutant this loop was successfully built with sufficient electron density (Fig. 2). Superimposition of the structures shows that the sulfate ion in the His170Tyr mutant occupies the position of the side chain of Asp12 in the wild type (Fig. 3). Owing to steric hindrance, the side chain of Asp12 is oriented away from the substrate-binding site. A polar contact is formed between Asp12 and Gly18 (2.3 Å) after side-chain

[†] Supporting information has been deposited in the IUCr electronic archive (Reference: HV5255).

rotation. This polar interaction probably helps to stabilize the conformation of the flexible loop.

Like most HADSF subclass II members, the core domain of TpNPPase lacks the ability to close off the active site from solvent. Residues 72–172 form a cap domain, the main functions of which may be substrate recognition and active-site desolvation. The cap and core domains move by a hinge-like motion to open/close the active site in response to substrate binding (Tremblay *et al.*, 2006). In the previous study, the structure of wild-type TpNPPase was only determined in a cap-open conformation (Guo *et al.*, 2013). As a result, this study uses human pyridoxal 5'-phosphate phosphatase (PLPP), a member of the HAD-like hydrolase superfamily, as an alternative for comparison. The structure of human PLPP was determined in a cap-closed conformation in complex with its substrate PLP (PDB entry 2cft; B. S. Kang, H. J. Cho, K. J. Kim & O. S. Kwon, unpublished work), and shares 26% sequence identity with TpNPPase in a homological comparison of amino acids (Supplementary Fig. S3). Compared with the wild-type TpNPPase and the cap-closed human PLPP, chain *B* of the His170Tyr mutant displays a half-closed conformation (Fig. 4). Superimposition of the active sites of these three structures was performed in PyMOL (Fig. 3). The active site of the His170Tyr mutant superimposed much better on the structure of PLPP (r.m.s.d.

of residues = 0.432 Å) than on that of wild-type TpNPPase (r.m.s.d. of residues = 1.211 Å). The half-closed conformation discovered in chain *B* is a new state which might be the transition state of the cap domain. The orientation of SO_4^{2-} is highly similar to that of PLP, indicating that chain *B* of the His170Tyr mutant may reflect the structure of TpNPPase in complex with coenzyme and substrate. The structure offers a molecular basis to deepen understanding of the catalytic mechanism of TpNPPase, and further investigation into the mechanism would require more experimental evidence.

In both the T_m and $T_{1/2}$ assays, the His170Tyr mutant showed an increased thermostability compared with the wild type. The mutated residue, His170/Tyr170, is positioned near the C-terminus on the seventh strand of the α -helix of TpNPPase with its side chain exposed to the solvent (Fig. 5*a*). The hydrophilic residue (His) was mutated to a hydrophobic aromatic residue (Tyr). The orientations of Tyr170 and His170 were similar (Fig. 5*b*). However, an additional hydrophobic interaction between Ala166 and Tyr170 was observed using the *Protein Interactions Calculator (PIC)* web server (Tina *et al.*, 2007). The average distance of 4.6 Å (within 5 Å) between the two hydrophobic side chains, the methyl group of Ala166 and the hydroxy-phenyl group of Tyr170, is close enough to form a hydrophobic contact which helps to stabilize the secondary structure of the α -helix, a possible indication of functional molecular folding and improved thermostability. Thus, the authors believe that the hydrophobic contact formed by Ala166 and Tyr170 is the main factor which enhances the thermostability of TpNPPase.

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Figure 5
(*a*) The mutation occurs near the seventh strand of the α -helix of TpNPPase with its side chain exposed to the solvent. (*b*) Crystal structures of His170Tyr mutant (blue) and wild-type TpNPPase (green) at the mutation position.

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