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Threonine 138 is crucial for the Quaternary Structure and the Thermostability of *Thermus thermophilus* Inorganic Pyrophosphatase

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ABSTRACT

Inorganic pyrophosphatase (EC. 3.6.1.1) from *Thermus thermophilus* (*Tth* PPase) forms the thermostable hexamer, and it was suggested from X-ray studies that its intersubunit interactions stabilize the whole molecule. However, the contribution of Thr138 at the intertrimer interface to quaternary structure and thermostability was unknown functionally. Therefore, we prepared four Thr138-substituted variants (T138A, V, N, and H) by site-directed mutagenesis. Then, thermostabilities of the enzyme activity and the quaternary structure for T138V and A were decreased relative to those of the wild type *Tth* PPase, whereas T138H and N variants remained much hexamer contents and the enzyme activity than T138V and A. Therefore, we suggest that the polar group in Thr138 of *Tth* PPase is more crucial than the methyl group for thermostability and quaternary structure, and it may contribute to the formation of stable trimer-trimer interface.

Keywords: Inorganic pyrophosphatase; Site-directed mutagenesis; Subunit interaction; Thermostability; Thermus thermophilus.

INTRODUCTION

Inorganic pyrophosphatase (PPase, EC 3.6.1.1) catalyzes the hydrolysis of inorganic pyrophosphate, and divalent cations such as Mg^{2+} are essential for the enzymatic activity [1]. To date, so many PPases had been isolated from various source, and categorized into Family I and Family II PPases on the basis of characteristics such as catalytic features, primary and oligomeric structures [2-4]. Family I PPases are more divided into three subfamilies, prokaryotic PPases are one of subfamilies, which are comprised of 162-220 amino acids per monomer and their quaternary structures are tetramer or hexamer. Other subfamilies in Family I PPase are plant and animal/fungal PPases, which formed monomer or dimmer [4]. On the other hand, Family II PPases are new group of PPases, which are not similar to Family I PPases in amino acid sequence and catalytic properties [2,3].

PPase from *Thermus thermophilus* HB8 (*Tth* PPase) belongs to Family I PPase, which is consists of 174 amino acids per monomer and forms thermostable homohexamer [5]. The three-dimensional structure of *Tth* PPase was already determined by X-ray crystallography at 2.0Å resolution, and then it was reported that the hexamer of *Tth* PPase was comprised of two trimers, in which the trimer-trimer interface were formed from α-helices A of molecule. Moreover, this trimer-trimer interface (intertrimer interface) is mainly formed by interactions of Gln130, His134, Thr138 and Leu142 in *Tth* PPase [6]. On the other hand, some prokaryotic and archaeal PPases were also involved in Family I, and comprised of homohexamer or homotrimer, *i.e.* PPases from *Geobacillus stearothermophilus* [7], thermophilic bacterium PS-3 [8], *Escherichia coli* (*E.coli* PPase) [9], and *Sulfolobus acidocardarius* (*Sac* PPase) [10]. In these PPases, it was reported that *Tth* and *Sac* PPases exhibited higher thermostabilities than *E.coli* PPase [5,11,12], and the three-dimensional structures of *Tth*, *E.coli* and *Sac* PPase had already elucidated [13,14]. From the comparison with three-dimensional structures between *Tth* and *E.coli* PPases, it was deduced that the difference in their thermostabilities would caused by oligomeric interactions, and then this intertrimer interface might

be the one of candidates, which is formed by His136, His140, Asp143 and Leu144 in *E.coli* PPase [15]. Meanwhile, Site-directed mutagenesis studies on the intertrimer amino acids had been performed only in *E.coli* and PS-3 PPase thus far [16-18]. In the case of PS-3 PPase, His125 is corresponded to His134 in *Tth* PPase, and His136 in *Sac* and *E.coli* PPases, and then Aoki *et al.* suggested that the effect of replacement of His125 by Ala (H125A) induced the drastic reduction of enzyme activity, accompanying with the destabilization of quaternary structure [16]. In the case of *E. coli* PPase, Baykov *et al.* suggested that the substitution of intertrimeric residues like His136 and His140 to Gln destabilized the hexamer in *E.coli* PPase, and decreased the rate constant for the substrate binding. Therefore, it was considered that two His residues play a key role in the intertrimer interface in *E.coli* PPase [17,18]. On the contrary, the contribution of amino acid residues at intertrimer interface to quaternary structure and thermostability was unknown in *Tth* PPase functionally. Here we focused on non-homologous Thr138 in *Tth* PPase, which corresponds to above His140 in *E.coli* PPase, and explore the consequences for characteristics of the thermostablity of Thr138-substituted variants, and also described the significance of Thr138 in hexamer formation of *Tth* PPase.

MATERIALS AND METHODS

Materials and Chemicals

T4 DNA ligation Kit ver.2 and Pyrobest DNA polymerase were obtained from Takara Shuzo. Restriction endonucleases were purchased from promega, New England Bio Labs and Takara shuzo. DEAE-cellulose was purchased from Wako pure chemical, and Sephacryl S-200HR was obtained from Pharmacia. *E. coli* JM109 and BL21 (DE3) strain were used for the site-directed mutagenesis and for the expression of wild type *Tth* PPase and its variants. The plasmid harboring *Tth* PPase gene was constructed as reported previously [5].

Site-Directed Mutagenesis by Polymerase Chain Reaction

Site-directed mutagenesis was performed by means of polymerase chain reaction. The genes of Thr138-substituted variants were constructed as described below. The sense-primer for T138A, V, H and N was 5'- GTGTGGAATTGTGAGCGGATAAC - 3' (23mer, corresponding to pUC118 plasmid). The antisense-primer for T138A was 5'-TTGGCCTCGAG GGCCTTGTAGGCTTCGAAGA -3' (31mer, corresponding to Phe135-Lys145), whereas antisense-primer for T138V was 5'- TTGGCCTCG AGGGCCTTGTAGACTTCGAAGA-3' (31mer, corresponding to Phe135-Lys145). On the other hand, anti-sense primer for T138H/N was 5'-TTGGCCTC GAGGGCCTTGTAGTKTTCGAAGA -3' (31mer, corresponding to Phe135- Lys145). The amplified fragment was digested with *Xho* I and *Nco* I, and then inserted into pETTP after digestion with the same endonucleases. Then, it was sequenced, and the resultant expression vectors for variants were named as pETTPT138A, V, H and N, respectively.

Preparation of Wild type Tth PPase and its variants

Purification of wild type *Tth* PPase and its variants was performed according to the previous paper [5]. Briefly, *E. coli* BL21 (DE3) cells transformed with each expression vector for wild type *Tth* PPase and its variants were cultured in 1 liter of LB/Amp medium at 37 ℃ for 20 h. Cells were lysed by sonication, and soluble fraction was collected. And the fraction was applied on DEAE-cellulose anion-exchange column chromatography followed by Sephacryl S-200HR gel filtration chromatography. Fractions which showed a single band on polyacrylamide gel electrophoresis were collected, and used as the purified enzyme.

Enzyme Assay

The activity of PPase was assayed at 37°C essentially according to the method described previously [19], the liberation of inorganic phosphate being determined by the method of Peel and Loughman [20]. Protein concentrations were determined by the method of Bradford [21], using bovine serum albumin as the standard.

Thermostability

Enzyme (0.1mg/ml) was incubated at different temperatures for 1 h at pH 7.8, followed by rapid cooling on ice for 10 min. The remaining activity was measured as described above.

Circular Dichroism (CD) Spectra Measurements

CD spectra were recorded with a J-720 automatic recording dichrograph (JASCO) at room temperature with protein concentration of 0.1 mg/ml. The far-UV CD spectra were measured between 200 and 250 nm in a 1mm optical path cuvette. CD data are expressed in terms of mean residue ellipticity, [θ], using the mean residue molecular weight from the primary structure.

Fluorescence Spectra Measurements

Fluorescence measurements were made with an F-2500 spectrofluorometer (Hitachi) at room temperature, using a 1 cm path length quartz cuvette. The protein concentration was always adjusted to 0.05 mg/ml in 20mM Tris-HCl buffer (pH 7.8). Tryptophan excitation was performed at 295 nm. The emission spectra were set between 320 and 400 nm. Samples after heating were centrifuged, and each supernatant was measured.

Analysis for Quaternary Structure

Each molecular weight of the wild type *Tth* PPase and its variants was determined by TSKgel G3000SW column (Tosoh) with bed dimensions of 7.5mm I.D. × 30cm. The column was run at room temperature with 50mM Tris-HCl buffer (pH 7.8) as the eluent (flow rate, 0.3 ml/min.). Protein concentration of applied samples was adjusted to 0.1 mg/ml. Samples were heated at 40, 50, 60, 70, 75, 80, 85 and 90 °C for 1 h, followed by cooling, filtration with Millex filter (pore size 0.45 mm, Millipore) and injected to the column. Reversibility of hexamer formation was performed as follows: Each sample diluted to 0.1mg/ml was incubated at 4°C for 25days, and then concentrated by microcon-10 (Millipore). Protein concentration was adjusted to 0.5 mg/ml and 1.0 mg/ml, and analyzed by TSKgel as described above. The ratio of hexamer/trimer was determined by estimating each peak area of the gel filtration with a Shimadzu Chromatopac C-R6A.

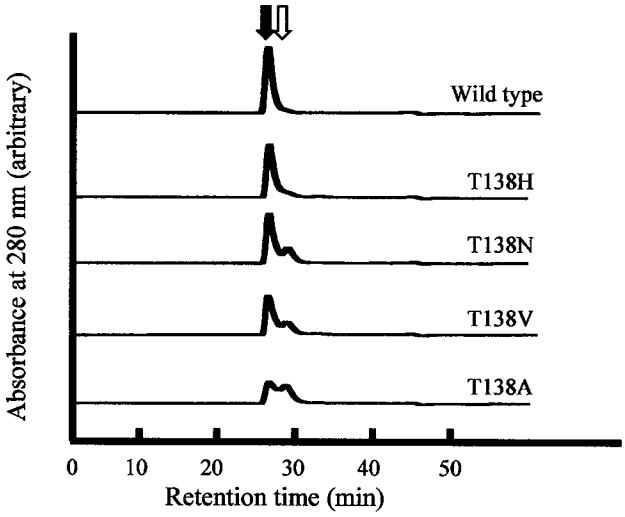

Fig. 1 The elution profiles of TSKgel G3000SW gel filtration chromatography (HPLC) for wild type *Tth* PPase and Thr138-substituted variants.

The black and white arrows indicate the retention times for the hexamer and trimer, respectively. The flow rate was 0.3 ml/min, eluted with 50mM Tris-HCl (pH 7.8). 50μl of each protein (0.1mg/ml) was injected to column, followed by eluted and analyzed as described in MATERIALS AND METHODS.

RESULTS

As described above, *Tth* PPase forms stable hexamer, and it was predicted that Thr138 plays a key role in the formation of trimer-trimer interface. In order to confirm the role of Thr138 in hexamer formation of *Tth* PPase, Thr138 was substituted to hydrophobic amino acids (Ala and Val), and polar amino acids (Asn and His). According to the protocol as described in Materials and Methods, we prepared four Thr138-substituted variants (T138A, V, N and H).

Firstly, we measured the specific activities of Thr138-substituted variants (T138A, V, N and H) after the dilution to a concentration of 0.1mg/ml (Table 1). The specific activities of T138A and V showed about 14.5% and 29.7% of the activity of the wild type *Tth* PPase, respectively, whereas those of T138N and H were 68.4% and 58.0%. These results indicate that variants which Threonine is replaced by the above hydrophilic residues still retain considerable enzyme activity, whereas variants with small hydrophobic residue substitutions show

Table 1 The characteristics of wild type *Tth* PPase and Thr138-substituted variants.

	Enzymatic activity		Fluorescence spectra		CD spectra	
	Specific activity (units/mg)	Relative activity [a] (%)	λ_{max} [b] (nm)	F_{max} [c]	$[\theta]_{222nm}$ (deg· cm²/dmol)	α-helix content [d] (%)
Wild type	890	100	335.0	2770	-8480	20.3
T138N	609	68.4	336.0	3000	-8330	19.8
T138H	516	58.0	336.0	3080	-8110	19.0
T138V	264	29.7	335.5	2430	-7860	18.2
T138A	129	14.5	335.0	2220	-7550	17.2

[a] The specific activity of wild type *Tth* PPase was taken as 100%. [b] λmax is the maximum wavelength of tryptophan fluorescence spectra. [c] Fmax is the fluorescence intensity (arbitray unit) at the emission maximum of tryptophan fluorescence spectra. [d] α - helix content was calculated by the following equation [25]; α - helix content (%) = $-([\theta]_{222nm} + 2{,}340) \times 100 / 30{,}300$.

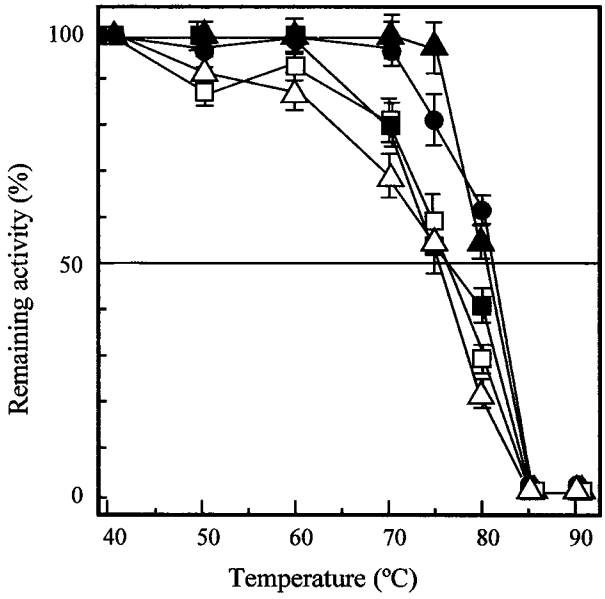

Fig.2 Thermostability of enzyme activity for wild type *Tth* PPase and Thr138-substituted variants.
The enzyme (0.1mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at the indicated temperatures for 1 h. Then the enzyme activity was measured at 37℃ after rapid cooling, and the activity at 40℃ was taken as 100%. The error bars indicate standard errors of the mean. Symbols: ●, wild type; ■, T138H; ▲, T138N; Δ, T138V, □, T138A.

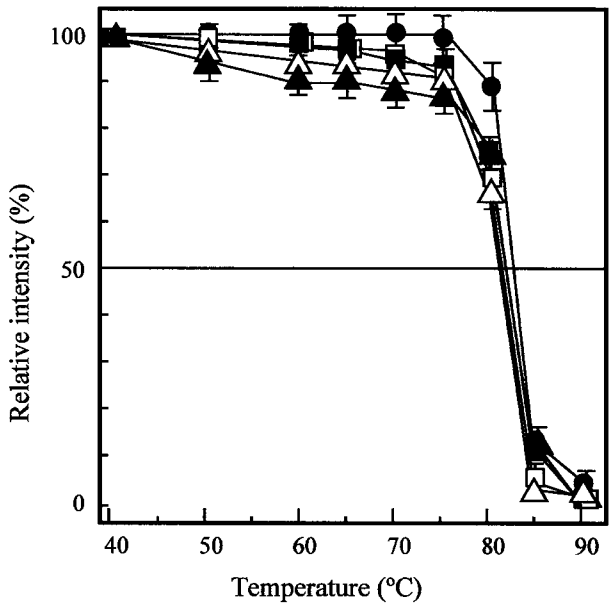

Fig.3 Thermostability of Tryptophan fluorescence spectra for wild type *Tth* PPase and Thr138-substituted variants.
. The enzyme (0.05mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at the indicated temperatures for 1 h. Then, the emission spectrum of each sample was measured with excitation at 295 nm after rapid cooling. On heating above 85 ℃, each sample was centrifuged after heating, and then its supernatant was measured. The fluorescence intensity at maximum wavelength was plotted as relative value to that at 40 °C. The error bars indicate standard errors of the mean. Symbols: ●, wild type; ■, T138H; ▲, T138N; Δ, T138V, □, T138A.

drastically decreased activity. Thus, the hydrophilic environment around Thr138 is crucial for maintaining enzyme activity. Therefore, we examined whether structural changes of Thr-substituted variants occurred or not. CD spectra in the far-UV region and fluorescence spectra were measured (Table 1). Consequently, we observed slightly conformational changes in the CD spectra of Thr-substituted variants, whereas the environment in the vicinity of Trp residues of T138V and A became more hydrophilic than that in the wild type. Furthermore, we investigated quaternary structure of Thr-substituted variants, T138H, N, V and A, at the concentration of 0.1mg/ml. As shown in Fig.1, T138N, V and A were dissociated into trimer, and it was suggested that substitution to Histidine at 138 position is the most suitable among variants for the stable intertrimer interface of *Tth* PPase. Furthermore, it was proved that the enzyme activity and conformation of T138N was remained to some extent in spite of dissociation into trimers, while variants substituted with hydrophobic amino acid (T138V and A) exhibited the dissociation into trimers and subsequent slight conformational changes, and then their enzyme activities were lower than those of wild type *Tth* PPase, T138N and H variants.

Secondly, the thermostabilities of their enzyme activities, Trp-excited fluorescence spectra and quaternary structure were examined as criteria of the effects of substitution and dissociation on the active site and conformational thermostability of *Tth* PPase. In the thermostability of enzymatic activity (Fig.2), those of T138H, V and A variants were decreased relative to wild type and T138N. Tm values of wild type and T138N were estimated to be approximately 82°C, whereas those of T138H, V, and A were 77°C. These results suggested that the hydrophilicity and volume at this 138 position might be effective on the formation of thermostable active site in *Tth* PPase.

As to Tryptophan fluorescence spectra (Fig.3), though thermostabilities of all variants were slightly less than that of wild type, remarkable perturbation by the thermal aggregation was also observed in variants as same as wild type after heating above 85 ℃. Then, it was suggested that the environment around Trp residues (Trp149 and 155) left unaltered drastically

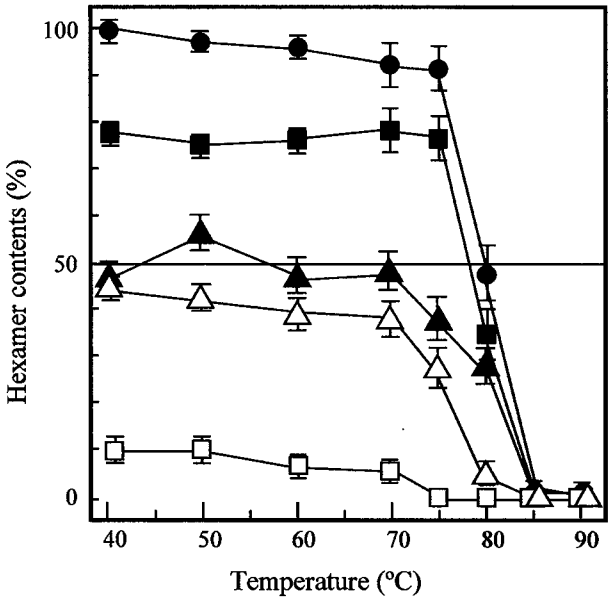

Fig. 4 Oligomeric thermostability for wild type *Tth* PPase and Thr138-substituted variants.

The enzyme (0.1mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at the indicated temperatures for 1 h. After rapid cooling, the gel filtration chromatography was performed under the same conditions as Fig. 1. Hexamer and trimers contents were estimated from the peak area corresponding to hexamer and trimer in the HPLC elution profiles, and total peak area of each enzyme at 40°C was taken as 100%. The error bars indicate standard errors of the mean. Symbols: ●, wild type; ■, T138H; ▲, T138N; Δ, T138V, □, T138A.

by substitution, and Thr138 might be almost irrespective of formation of the thermal aggregation that causes inactivation of enzyme.

As described above, T138N and V were slightly dissociated into trimer in the native state, whereas remarkable dissociation into trimer was observed in the T138A variant. Wild type and T138H were not dissociated under this condition. Therefore, the thermostabilities of quaternary structure for Thr-substituted variants were investigated, as shown in Fig.4. As results, the wild type enzyme showed the highest thermostability of hexamer, whereas those of four variants were decreased. In particular, hexamer of T138A was only 10% after heating even at 40°C, and then its trimer was prominent (89.3% at 40°C). Temperatures for the half of hexamer at 40°C were estimated to be approximately 79-80°C in wild type, T138H and N, whereas 76°C and 71°C in T138V and T138A, respectively. Hence, the results suggested that Threonine was the most suitable for the formation of stable hexamer at the 138 position of *Tth* PPase, and the hexameric thermostability exhibited much higher in order of His, Asn, Val and Ala at this position.

Moreover, in order to examine the cold lability and reversibility of hexamer formation by concentration, 0.1mg/ml of wild type and Thr-substituted variants incubated at 4°C for 25days were concentrated up to 0.5 and 1.0 mg/ml, respectively, and then we analyzed quaternary structure by gel filtration (Fig.5). As results, hexamer in all variants was more dissociated into trimers after 25days, as compared with no incubation (Fig.1), whereas the hexamer of wild type enzyme was retained. Additionally, when we examined their reformation of hexamer by concentration, wild type was independent of the enzyme concentration, and

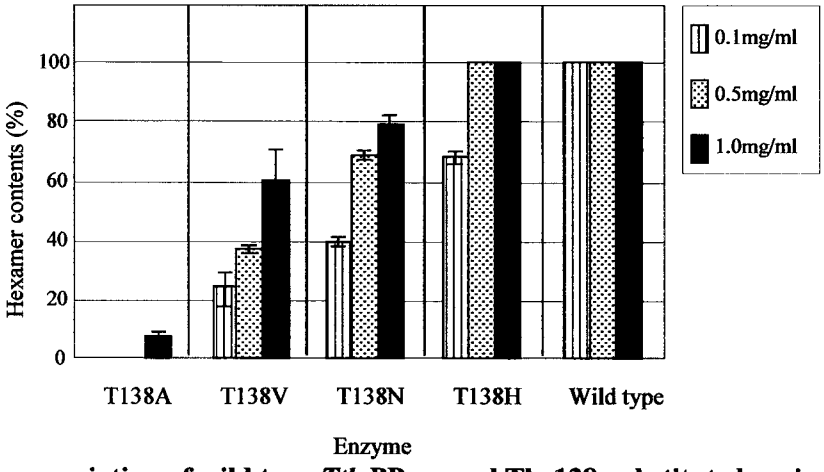

Fig.5 Hexamer reassociation of wild type *Tth* PPase and Thr138-substituted variants by concentration. The concentration of each sample was adjusted to 0.1mg/ml, followed by incubated at 4°C for 25days. Then, each sample was concentrated up to 0.5 and 1.0 mg/ml as described in Materials and Methods. 5μg of each sample at the indicated concentration in figure was analyzed by gel filtration chromatography under the same conditions as Fig.1 and Fig.4. Hexamer and trimer contents were estimated as described in the legend of Fig.4. The error bars indicate standard errors of the mean.

then, the peak of only hexamer was observed on gel filtration. On the other hand, since those of four variants were dependent on their concentration except for T138A, it might be possible for the reformation of hexamer by concentration. In particular, almost 100% of T138H variant formed hexamer when its concentration was increased up to 0.5mg/ml. The results indicate that hydrophilic effect (His and Asn) appeared to be more effective on the hexamer reformation than hydrophobic effect (such as Val and Ala) at this position, and then His might have high ability to form hexamer especially.

DISCUSSION

Inorganic pyrophosphatase from *Thermus thermophilus* (*Tth* PPase) forms hexamer, and it was suggested from X-ray studies that its intersubunit interactions stabilize the whole molecule [6]. In the three-dimensional structure of *Tth* PPase [6,15], it was reported that Nε2 atom of His134 interacts hydrophilically with O atom of Thr138' (in another subunit) at 3.21Å in the intertrimer interface of hexameric *Tth* PPase, while Cγ2 atom of Thr138 interacts hydrophobically with another Cγ2 atom of Thr138'. In addition, Cγ atom of His134 interacts hydrophobically with Cδ1 atom of Leu142 at 3.83 Å, and Cε atom of His134 with Cδ atom of Gln49 at 3.91 Å in this interface (Fig. 6). The trimer-trimer interface in hexameric *Tth* PPase is formed mainly by symmetry-related α-helices A which contains His 134 and Thr 138, and it had been considered that these interactions may relate to the tight packing of *Tth* PPase molecule and thermostability.

On the other hand, we suggested that the oligomeric thermostability of the recombinant *Tth* PPase exhibits two remarkable features differing from that of *E.coli* PPase in previous study [5,11]. That is, (1) Dissociation of hexamer into trimers was not observed in *Tth* PPase at high temperature (namely non-dissociation), and (2) thermal aggregation was observed after heating above 85°C. Furthermore, our previous study on the chimeric PPases exhibited that four amino acids (Thr138, Ala141, Ala144, and Lys145 in *Tth* PPase) may contribute to these difference in *Tth* and *E.coli* PPases [11], and subsequent study revealed that the deletion of Ala144 and Lys145 suppressed the thermal aggregation in *Tth* PPase at high temperatures [22].

On the basis of this information, we speculated that amino acids in intertrimeric interface of *Tth* PPase may determine the above thermal characteristics of *Tth* PPase, and then investigated the contribution of Thr138 residue in *Tth* PPase to the thermostability and quaternary structure by site-directed mutagenesis. Therefore, we constructed the T138H, N, V and A variants, and analyzed their characteristics and thermostability.

The specific activities of four variants were reduced to 68.4% (T138N), 58.0% (T138H), 29.7%

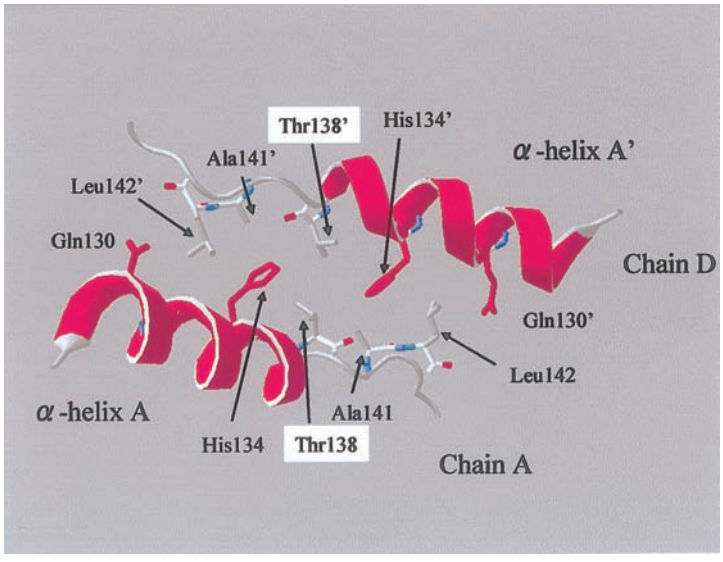

Fig.6 Intertrimer interface (α-helix A) between monomer A and D in *Tth* PPase.
The figure was drawn by a Molecular Graphics Program, Swiss-Pdb Viewer Ver.4.0 (Swiss Institute of Bioinformatics). The X-ray structural data for hexameric *Tth* PPase was kindly provided by Dr. Teplyakov, A., which corresponded to the PDB code 2PRD in monomer [4]. The stick representations indicate the main and side chains of intertrimer amino acid residues which are described in text. The α-helix A contains the residues from Glu126 to Thr138 in *Tth* PPase [4,13]. The residues without or with prime are located in monomer A and another monomer D, respectively.

(T138V) and 14.5 % (T138A) of wild type enzyme activity, respectively. From the spectroscopic data (Table 1) and gel filtration (Fig. 1), we suggest that this reduction of their enzyme activities may be due to the dissociation and additional conformational change of hexameric *Tth* PPase molecule by the Thr138 substitutions. And then, variants substituted with hydrophilic residues (T138N and H) might remain the much enzyme activity, conformation and quaternary structure relative to those with hydrophobic residues (T138V and A). Such a tendency was also observed in their thermostabilities of enzyme activity (Fig.2), fluorescence spectra (Fig.3) and quaternary structure (Fig.4). In particular, the thermostability of hexamer for T138H is the highest of all variants, whereas hexamer of T138A were disappeared after heating at 75°C completely. From these results, it was suggested that Thr138 in *Tth* PPase plays a key role in the formation of thermostable hexamer and intertrimer interface. Furthermore, the ability of hexamer reassociation was examined by concentrating enzyme (Fig.5). The results revealed that T138H contained the most hexamer content of all variants at a concentration of 1.0 mg/ml, whereas T138A was almost dissociated into trimers. Therefore, we assume that the bulkiness and hydrophilicity of side-chain at this position may relate with the oligomeric structural stability of molecule.

The average volume of Thr, His, Asn, Val and Ala are 122.1Å^3, 167.3 Å^3, 135.2 Å^3, 141.7 Å^3 and 91.5 Å^3, respectively [23]. On the other hand, hydrophobicity of Thr, His, Asn, Val and Ala are -0.7, -3.2, -3.5, 4.2 and 1.8, respectively [24]. Moreover, side-chain of Thr, His and Asn residue can form hydrogen bond, whereas those of the other two amino acids (Val and Ala) can not. On the other hand, Thr, Val and Ala have the possibility for forming hydrophobic interactions by side chain. Thus, it is considered that Thr might be more preferable than the other amino acids at this position because of its volume and amphiphilicity. From these reasons, we expected that both the hydrogen bond between His134' and Thr138 and the hydrophobic interaction between Thr138 and Thr138' stabilizes intertrimer interface of *Tth* PPase. However, it was proved from this study that the substitution of Thr138 with hydrophobic amino acid may perturb this interface interaction unfavorably, whereas one with polar amino acid like Asn or His could form hexamer to some extent. Consequently, we suggested that interactions between polar amino acids including Thr138 may contribute to hexamer formation and thermostability of *Tth* PPase, as compared with hydrophobic interactions.

Among Family I PPases, sequential homologous region on amino acid sequences is observed at Ile132-Leu142, which was mainly located in α-helix A. Thr138 residue in *Tth* PPase is not conservative among them, which corresponded to His140 of *E.coli* PPase and *Sulfolobus acidocardarius* PPase (*Sac* PPase) [4]. *Sac* PPase has higher thermostability than *Tth* and *E.coli* PPase [12], and its three-dimensional structure was already solved at 2.7Å resolution [14]. Although the three-dimensional structure of these PPases appeared to be very similar one another, it was deduced that ion triple His136'-Glu143-His140 might increase thermostability in hexameric *Sac* PPase. Equivalent ion triple is also observed in *E.coli* PPase (His136'-Asp143-His140), whereas the corresponding ion triple may not exist in *Tth* PPase (Glu143 or Asp143 corresponded to Ala141 in *Tth* PPase). In case of *E. coli* PPase, it was reported that the substitution of His with Gln (H140Q) showed no loss of the enzyme activity, although the dissociation from hexamer into trimers was observed [17, 18].

In our experiments on *Tth* PPase, when Thr138 was substituted to His (T138H), the thermostability of hexamer showed the highest of all Thr138-substituted variants. On the other hand, the substitution to Asn (T138N) exhibited the highest thermostability in enzyme activity, though the dissociation into trimers was observed after heating. In the case of T138N, it was considered that the introduced Asn might stabilize intratrimer and adjacent residues such as Tyr139 and Lys140 in the active site of enzyme, and then retain enzyme activity. The results of T138N seemed to resemble in the cases of above H136Q/H140Q in *E.coli* PPase, and it was considered that hexamer formation is not essential for the enzyme activity in *Tth* PPase, but trimers also can exhibit enzyme activity.

Consequently, we pointed out in this study the contributions of polar properties at 138 position of *Tth* PPase to hexamer formation and thermostability. Additionally, it was deduced that interaction between Thr138 and the other residues such as His134 and Ala141 also may be responsible for thermostability and intertrimer interface formation in *Tth* PPase, which stabilized in different ways from *E.coli* PPase and *Sac* PPase. In order to elucidate the relationship between these residues in intertrimer interface of *Tth* PPase, the site-directed mutagenesis studies on the Ala141-substituted variants is now undertaken.

From these investigations, we conclude that polar group in Thr138 of *Tth* PPase are more crucial than methyl group for thermostability and quaternary structure, and it may contribute to the formation of stable trimer-trimer interface.

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