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Effect of Cys168 substitutions on the Thermostability and the Thermal Aggregation of *Thermus thermophilus* Inorganic Pyrophosphatase

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ABSTRACT

Thermus thermophilus Inorganic pyrophosphatase (*Tth* PPase) is comprised of homohexamer, and exhibits high thermostability. However, the thermal aggregation containing the cross-linked dimer was observed after heating above 85°C. Therefore, we focused on the sole cysteine (Cys168) in C-terminal region, and evaluated the effects of substitutions at this position on thermostability and thermal aggregation of *Tth* PPase.

Firstly, we prepared the four Cys168-substituted variants (C168A, L, I, and F) by site-directed mutagenesis. Although all variants formed hexamer in native state, C168A variant exhibited the highest thermostabilities for the enzyme activity and quaternary structure in wild type and all variants, while the other variants decreased them drastically as the side chain at the 168 position was much more bulky and hydrophobic in *Tth* PPase. Moreover, suppression of thermal aggregation for C168A variant was observed in the ANS fluorescence experiments. Therefore, we suggest that the small volume and less hydrophobicity of side chain at 168 position may contribute to the conformational thermostability, and substitution with Ala is the most suitable for thermostabilization and suppression of thermal aggregation of *Tth* PPase.

Keywords: Inorganic pyrophosphatase; Thermal aggregation; Bulkiness; Thermostability; Thermus thermophilus

INTRODUCTION

Inorganic pyrophosphatase (PPase, EC 3.6.1.1) catalyzes the hydrolysis of pyrophosphate to orthophosphate in the presence of divalent cations such as Mg^{2+}, and plays an important role in providing a thermodynamic driving force for many biosynthetic reactions in cell [1]. So many PPases had been reported from various source, and categorized into two families, namely Family I and II based on characteristics, primary and oligomeric structures[2]. PPases from prokaryotes are mainly belonging to subfamily Ia in the Family I PPases, which comprised of 162-220 amino acids per monomer, and their quaternary structures are trimer, tetramer or hexamer. These prokaryote subfamily PPases varied in the thermostabilities and quaternary structure, and thus far, thermostabilities of PPases from various archaeal and thermophilic bacterium had been reported such as *Sulfolobus acidocardarius* (*Sac* PPase) [3], *Thermoplasma acidophilum* [4], *Pyrococcus horikoshii* (*Pho*)[5], *Aquifex pyrophilus*[6], *Thermus thermophilus* (*Tth* PPase) [7], Thermophilic bacterium PS-3 [8], and *Geobacillus stearothermophilus* (*Bst* PPase) [9], which are higher than those of mesophilic ones like *Escherichia coli* PPase (*E.coli* PPase). In this subfamily PPases, three-dimensional structures also had been solved in *Pho*, *Sac*, *Tth* and *E.coli* PPases [10-14], and then these main-chain fold in subunit were similar one another, which forms the common β-barrel structure. Moreover, the factors contributing to the difference in their thermostabilities

Table 1 Comparison of C-terminal amino acid sequences among prokaryotic and archaeal PPases.

Enzyme source	C-terminal amino acid sequences (148 ... 174)	Amino acids
T.thermophilus	KWVKVTGWRDRKAALEEVRACIARYKG	174
G.stearothermophilus	KRTEIGTWEGPEAAAKLIDECIARYNEQK	164
Thermophilic Bacterium PS3	KRTEIGTWEGPEAAAKLIDECIARYNEQK	164
T.acidophilum	KETKVLGWEGKEAALKEIEVSIKMYEEKYGKKN	179
P.horikoshii	KEIIVEGWEGAEAAKREILRAIEMYKEKFGKKE	178
S.acidocaldarius	KYVKISGWGSATEAKNRIQLAIKRVSGGQ	173
E.coli	KWVKVEGWENAEAAKAEIVASFERAKNK	175
	* * * ↑	

C-terminal amino acid sequences (148-174) of *Tth* PPase are shown, compared with corresponding residues in Family I PPases. Arrow indicates Cys168 in *Tth* PPase, and the corresponding residues in Family I PPases. Identical amino acids among PPases are indicated by asterisks. The numbers of amino acids in *Tth* PPase are also shown.

were suggested by comparing these three-dimensional structures: *i.e.* (1) Intermonomer hydrophilic contacts including intertrimeric interactions between α-helix A, (2) Ionic interations between C-terminal residues and the rest of the molecule or within C-terminal α-helix B. (3) The increase of the number of Arg residues [11-13].

Based on these investigations, we have explored on the thermostabilizing factors in *Tth* PPase thus far, which consists of 174 amino acids per monomer and forms thermostable homohexamer [7]. As described above, it had been considered that *Tth* PPase may be thermostabilized by the increases of above three factors relative to *E.coli* PPase. In previous study on chimeric PPases between *Tth* and *E.coli* PPases, we suggested that the four amino acids in the vicinity of intertrimer interface (α-helix A) of *Tth* PPase, *viz.*Thr138, Ala141, Ala144, and Lys145, may contribute to differences in thermostability between them [15]. Furthermore, subsequent site-directed mutagenesis study revealed that deletion of Ala144-Lys145 suppressed thermal aggregation which caused the thermal inactivation of *Tth* PPase [16], whereas polarity of Thr138 may play a key role in its hexamer formation (Kouzai, M. *et al.*, in press.). In spite of these thermostabilizing factors, the inactive thermal aggregation occurs in *Tth* PPase after heating above 85°C (Fig.1 (A)). It was suggested that this thermal aggregation might be caused by oligomerization of partly denatured molecule [16], which contains the cross-linked dimeric component (Fig.1 (B)).

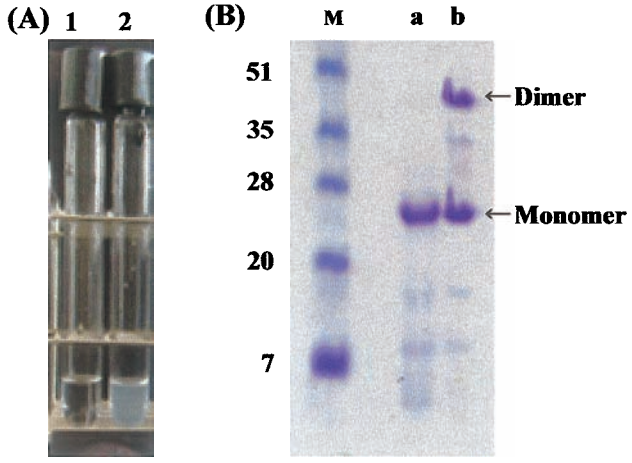

Fig.1 Thermal aggregation and its SDS-PAGE analysis of wild type *Tth* PPase.
(A) Thermal aggregation of wild type *Tth* PPase. Enzymes before (tube 1) and after heating at 90 ℃ for 1h (tube 2) were shown. (B) SDS-PAGE analysis of aggregates of wild type *Tth* PPase. Aggregates after heating at 90 ℃ for 1h were collected by centrifugation and analyzed by SDS-PAGE (15% gel). Wild type *Tth* PPase was analyzed in reducing condition (140mM 2-mercaptoethanol, lane 1) and in non-reducing conditions (lane 2). Lane M is the molecular weight marker (7, 20, 28, 35, 51 kDa).

In this work, we turn our attention to the contribution of the sole cysteine, Cys168 in the α-helix B, to the thermostability and the thermal aggregation of *Tth* PPase. This Cys168 is not strictly conservative among prokaryotic and archaeal PPases (Table 1). As described above, it was deduced that one of the thermostabilizing factors is ionic interactions in the α-helix B of *Tth* PPase such as

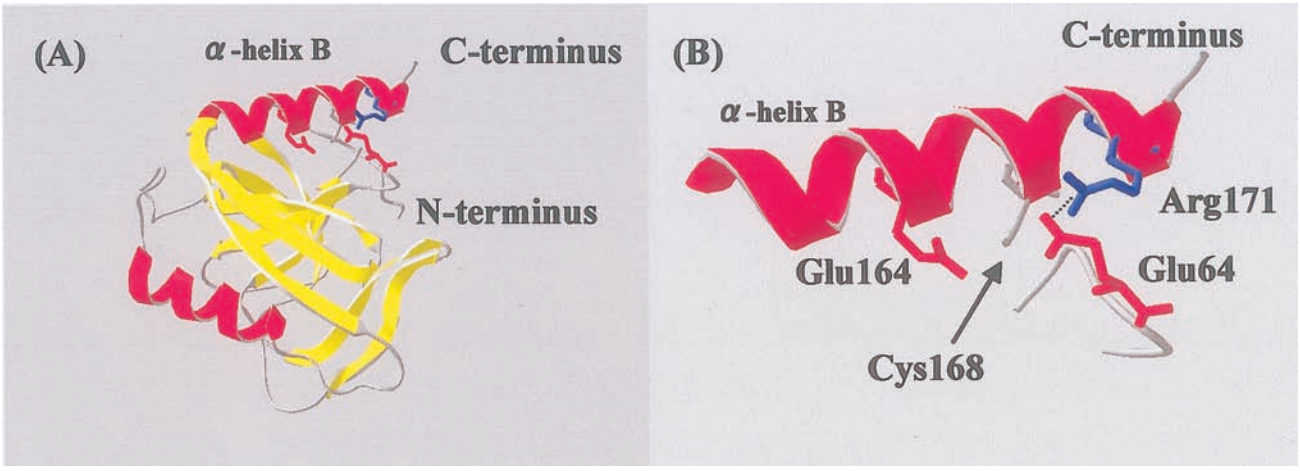

Fig. 2 Three-dimensional structure of *Tth* PPase and amino acid residues in the vicinity of Cys168. (A) The three-dimensional structure of monomeric *Tth* PPase. The figure was drawn by a Molecular Graphics Program, Swiss-Pdb Viewer Ver.4.0 (Swiss Institute of Bioinformatics). The X-ray structural data for *Tth* PPase was obtained from the Protein Data Bank (PDB code 2PRD) [12]. (B) Ionic interactions in the vicinity of Cys168. The part of α-helix B (163-174) and the loop for the active site residue (59-67) of the *Tth* PPase are shown in the panel (B). The stick representations indicate the main and side chains of Glu64, Glu164, Cys168, and Arg171 residues, where ionic interaction is formed between Glu64 and Arg171.

Arg171—Glu64, Arg158—Asp120, and Glu163—Arg166—Glu14 [11,13]. For the formation of these correct ionic interactions in *Tth* PPase, we expected that the microenvironments in the vicinity of these Arg residues also must be important. Here Cys168 is one of candidate for maintaining the suitable microenvironment, because it was deduced to be located in the same side as Arg171 and Glu164 of α-helix B, and oriented to internal moiety of *Tth* PPase molecule (Fig.2). Meanwhile, it was deduced that the thermal aggregation and oligomerization might be caused by the cross-linking of Cys168 residues between subunits above 85°C (Fig.1 (B)). Therefore, we investigated the relationship between the microenvironment at this 168 position in *Tth* PPase and the thermostability involving the formation of thermal aggregation by four Cys168-substituted variants (C168A, L, I, and F).

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. Molecular weight marker for SDS-PAGE was purchased from Bio-Rad Laboratories. 8-Anilino-1-Naphthalene Sulfonic acid (ANS) was obtained from MP Biomedicals, Inc. *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 [7].

Site-Directed Mutagenesis by Polymerase Chain Reaction

Site-directed mutagenesis was performed by means of polymerase chain reaction. The genes of Cys168-substituted variants were constructed as described below. The sense-primer for C168A, L, I and F was 5'- GACCTACAAGGCCCTCGAGGC CAAGAA-3' (27mer, corresponding to Glu137—Lys146). The antisense-primer for C168A was 5'-AGAGGATCCTTAGCCCTTGTAGCGCGCGATGG CGGCC -3' (37mer, corresponding to Arg166—Gly174, and stop codon), whereas antisense-primers for C168L, I, and F were 5'- TAGAGGATCCT TAGCCCTTGTAGCGCGCGATGANGGCC-3' (38mer, corresponding to Arg166—Gly174, and stop codon). The amplified fragment was digested with *Xho* I and *Bam* HI, 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 pETTPC168A, L, I and F, 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 [7]. 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 (PAGE) 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 [17], the liberation of inorganic phosphate being determined by the method of Peel and Loughman [18]. Protein concentrations were determined by the method of Bradford [19], 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.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was performed by the method of Lammli [20], on 15% polyacrylamide gel. The proteins were stained with Coomassie Brilliant Blue R-250. For the analysis of thermal aggregates, each enzyme solution was heated at 90℃ for 1h, and the resultant aggregates were collected by centrifugation, followed by analyzed on 15% SDS-PAGE with or without 2-mercaptoethanol (reducing or non-reducing condition) in sample buffer.

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, $[\theta]$, 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). The excitation wavelength for Tryptophan fluorescence was performed at 295 nm, and the emission spectra were set between 320 and 400 nm. Samples after heating were centrifuged, and each supernatant was measured in thermostability experiments. In case of the ANS fluorescence experiments [21], each enzyme solution was heated at 85℃ for 1h, and final 40μM ANS was added, followed by measured the fluorescence spectra. The excitation wavelength for ANS fluorescence was performed at 390 nm, and the emission spectra were set between 400 and 700 nm.

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, 65 (only C168L, I, and F), 70, 75, 80, 85 (except for C168L, I, and F) and 90 °C for 1 h, followed by cooling, filtration with Millex filter (pore size 0.45 mm, Millipore) and injected to the column. The ratio of subunit composition (hexamer, trimer, monomer, and so on) was determined by estimating each peak area of the gel filtration with a Shimadzu Chromatopac C-R6A.

RESULTS

As described above, some ionic interactions are involved in the α-helix B of *Tth* PPase [11-13], and it was predicted that Cys168 may affect on thermostability, structural integrity and formation of thermal aggregation in the *Tth* PPase molecule. Therefore, in order to examine the role of microenvironment at 168 position in thermostability and conformational stability of *Tth* PPase, Cys168 was substituted to hydrophobic amino acids (Ala, Leu, Ile and Phe). According to the protocol as described in MATERIALS AND METHODS, we prepared four Cys-substituted variants, namely C168A, L, I and F, respectively.

Firstly, we measured the specific activities of Cys168-substituted variants (C168A, L, I and F) after the dilution to a concentration of 0.1 mg/ml (Table 2). The specific activities of C168A, L, and I showed about 104.5%, 98.5% and 89.7% of the activity of the wild type *Tth* PPase, respectively, whereas that of C168F were decreased to 58.3%. These results indicate that the variants except for C168F might retain enzyme activity, whereas C168F showed the decreased enzyme activity. Furthermore, we examined whether conformational changes of Cys-substituted variants occurred or not. CD spectra in the far-UV region (Fig.3 and Table 2) and Tryptophan fluorescence spectra were measured (Table 2). As results, though drastic conformational changes were not observed in their CD spectra and fluorescence spectra relative to those of wild type, the

Table 2 The characteristics of wild type *Tth* PPase and Cys168-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.cm^2/dmol)	α-helix content [d] (%)
Wild type	890	100	335.0	2770	-8480	20.3
C168A	930	104.5	335.0	2480	-8270	19.6
C168L	877	98.5	335.0	2440	-7930	18.4
C168I	798	89.7	335.0	2610	-8030	18.8
C168F	519	58.3	335.5	2120	-8250	19.5

[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 [24]; α - helix content (%) = $-([\theta]_{222nm} + 2{,}340) \times 100 / 30{,}300$.

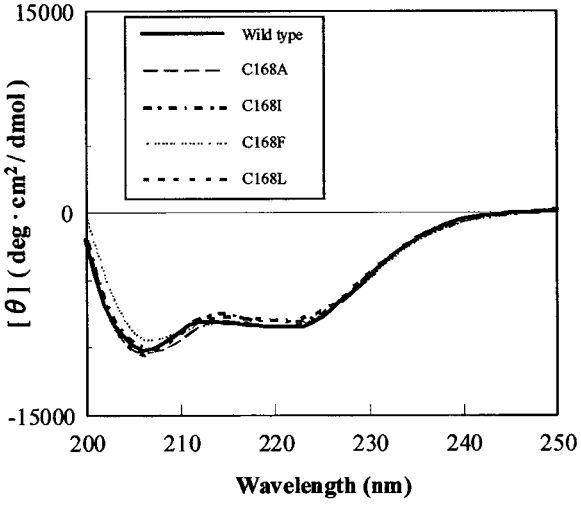

Fig.3 Far-UV CD spectra of wild type *Tth* PPase and Cys168-substituted variants.
The CD spectra were measured under the conditions described in MATERIALS AND METHODS. The each spectrum for wild type *Tth* PPase and its variants was drawn by the indicated line in Figure.

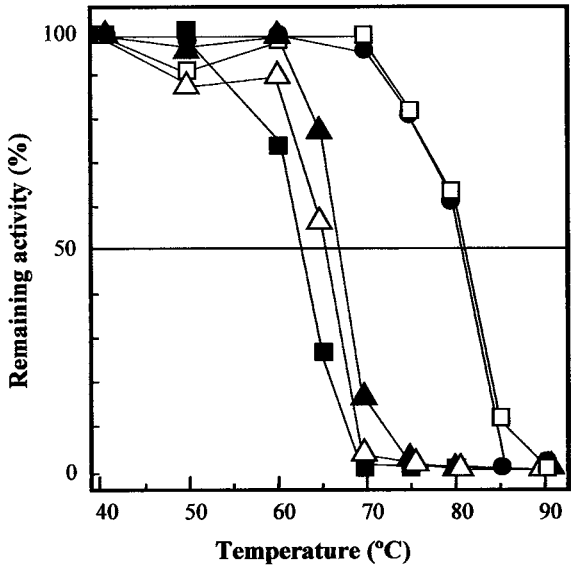

Fig.4 Thermostability of enzyme activity for wild type *Tth* PPase and Cys168-substituted variants.
The enzyme (0.1mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at the indicated temperatures for 1 h. Then the enzyme activity was measured at 37℃ after rapid cooling, and the activity at 40℃ was taken as 100%. Symbols: ●, wild type; □, C168A, ▲, C168L; ■, C168I; Δ, C168F.

environment in the vicinity of Trp seemed to be slightly perturbed in C168F variant. Subsequently, we analyzed quaternary structure of Cys-substituted variants, C168A, L, I, and F, at the concentration of 0.1mg/ml. Under this condition, all enzymes including wild type formed only hexamer (Data not shown). From these investigations, it was suggested that substitutions of Cys168 to hydrophobic amino acids might not affect on the conformation and oligomeric structure drastically in the native state, though the enzyme activity of C168F was decreased to some extent.

Secondly, we examined the thermostabilities of their enzyme activities, and Trp-excited fluorescence spectra, and quaternary structure in order to confirm

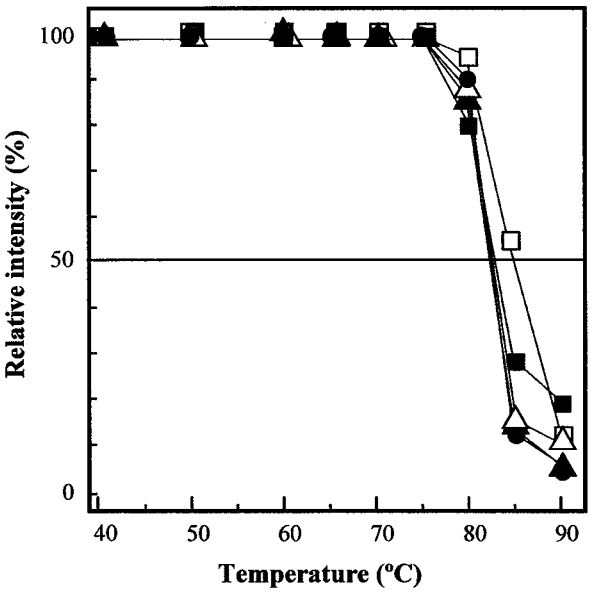

Fig.5 Thermostability of Tryptophan fluorescence spectra for wild type *Tth* PPase and Cys168-substituted variants

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 °C, 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. Symbols: ●, wild type; □, C168A, ▲, C168L; ■, C168I; Δ, C168F.

whether the conformational integrity of whole molecule may be susceptible to the substitution of Cys168, or not. In the thermostability of enzymatic activity (Fig.4), those of C168L, I, and F variants reduced significantly, and they were inactivated even at 75°C completely, while that of C168A variant was almost the same as wild type. Then, the results revealed that larger volume of side chain at 168 position of *Tth* PPase have the active site of enzyme unstable, which is located in the opposite side of molecule.

As to thermostabilities of Tryptophan fluorescence spectra (Fig.5), wild type exhibited remarkable perturbation due to the thermal aggregation after heating above 85 °C. Meanwhile, thermostabilities of C168A and I might increase at 85 °C relative to wild type, whereas those of C168L and F showed the same as wild type. Then, it was suggested that the substitution of Cys168 to Ala might stabilize the environment around Trp residues (Trp149 and 155) indirectly, and then thermal aggregation was suppressed.

Furthermore, we investigated the oligomeric thermostability of Cys-substituted variants (Fig.6). As described above, all these variants formed hexamer, and their dissociation into trimers or monomers does not occur in the native state. As shown in Fig.6, hexamer of C168A contained approximately 33% of hexamer after heating even at 85°C, whereas that of wild type enzyme almost disappeared. On the contrary, C168L, I, and F showed drastic destabilization of hexamer, and then their hexamer were dissociated into monomer rather than trimer even at 65°C. In addition, these monomer components were increased as temperature was raised up to 75°C, followed by decreased above 80°C due to thermal aggregation. These results suggested that hydrophobic and bulky amino acids at 168 position might have reduced oligomeric thermostability, especially intermonomeric interactions of *Tth* PPase.

Finally, we examined the thermal aggregation of wild type *Tth* PPase and Cys168-substituted variants by hydrophobic fluorescence probe, 8-Anilino-1-Naphthalene Sulfonic acid (ANS). As described above, thermal aggregates of wild type *Tth* PPase contain the cross-linked dimeric component on non-reducing SDS-PAGE (Fig.1). And then, we also examined thermal aggregates of Cys168-substituted variants by the same methods. As a result, the cross-linked dimeric component was not observed in case of Cys168-substituted variants (Data not shown). Hence, it was suggested that Cys168 is one of the causes of the formation of thermal aggregation in the *Tth* PPase. On the other hand, to detect the state of aggregates in wild type and Cys168-substituted variants, fluorescence spectroscopic experiments were performed by using hydrophobic fluorescence probe ANS (Fig.7). Each enzyme solution was heated at 85°C for 1h, and then added final 40μM ANS, followed by measured the ANS-derived fluorescence spectra. Although the maximum fluorescence wavelength of ANS was 515nm in buffer, its blue-shift to shorter wavelength (approximately 470nm) was observed in the heated wild type *Tth* PPase and its variants, because it is considered that ANS binds to the exposed hydrophobic region of the protein after heating. Furthermore, the maximum ANS fluorescence intensities of variants were decreased as the volume of amino acid at 168 position is smaller. Therefore, we concluded that thermal aggregation of *Tth* PPase after heating was caused by the exposure of hydrophobic region of molecule, the cross-linking of Cys168 among subunits, and oligomerization.

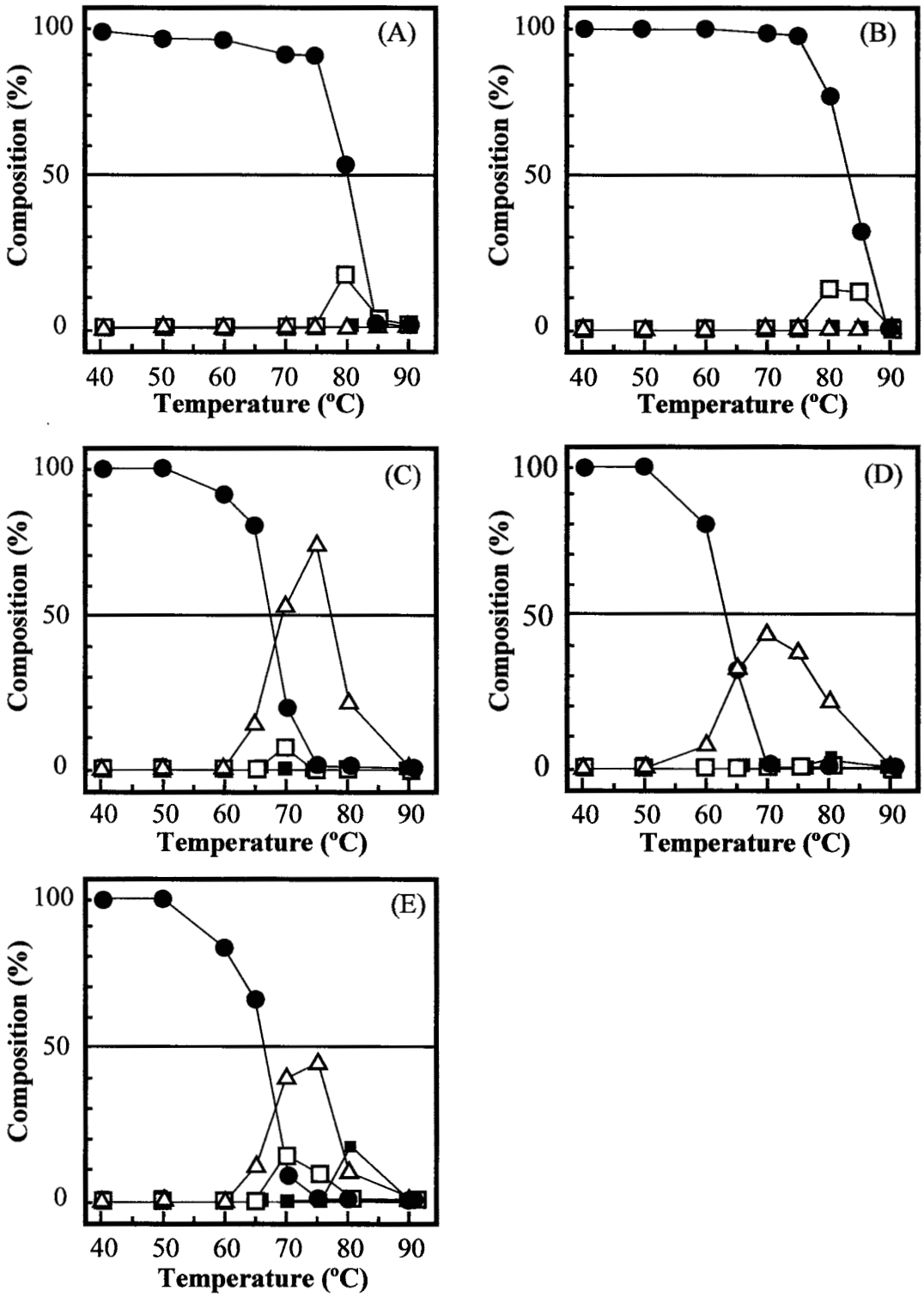

Fig. 6 Oligomeric thermostability for wild type *Tth* PPase and Cys168-substituted variants.
Panels: (A) Wild type, (B) C168A, (C) C168L, (D) C168I, (E) C168F. The enzyme (0.1mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at the indicated temperatures for 1 h. After rapid cooling, the gel filtration chromatography was performed as described in MATERIALS AND METHODS. Composition was estimated from each peak area of hexamer, trimer, monomer, and oligomer (component having much higher molecular weight than hexamer) in the HPLC elution profiles. 100% was taken as the sum of all peak area after heating at 40°C. Symbols: ●, hexamer; □, trimer; Δ, monomer; ■, oligomer.

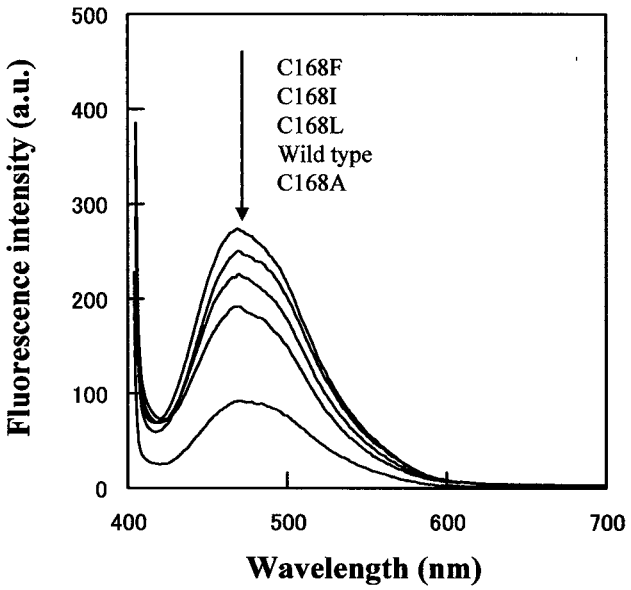

Fig.7 ANS binding to thermal aggregates of wild type *Tth* PPase and Cys168-substituted variants monitored by fluorescence.

Emission spectra of final 40 μ M ANS mixed with enzymes (0.05mg/ml) after heating at 85 °C. The excitation wavelength was 390 nm. All spectra were recorded at room temperature in 20 mM Tris-HCl buffer, pH 7.8.

From these investigations, we revealed that the volume of side chain at the 168 position may contribute to the conformational thermostability and the formation of thermal aggregation in the *Tth* PPase, and then small and less hydrophobic amino acids like Cys and Ala must be favorable for stabilizing peripheral environments including ionic interactions, in particular, Ala for suppressing the thermal aggregation.

DISCUSSION

Inorganic pyrophosphatase from *Thermus thermophilus* (*Tth* PPase) forms hexamer, and showed higher thermostability than *E.coli* PPase. Based on the studies of the three-dimensional structure of *Tth* PPase [11-13], it was deduced that the C-terminal part of the *Tth* PPase is more resistant to thermal denaturation, because a few ionic interactions are different between *Tth* PPase and *E.coli* PPase [13]. Although both three-dimensional structure are similar each other, which are comprised of β-barrel structure and two α- helices, namely α-helix A and α-helix B, the difference in ionic interactions within a monomer are mainly located in C-terminal α-helix B. In *Tth* PPase, ionic interactions such as Glu14-Arg166-Glu163, Glu64-Arg171, and Asp120-Arg158 are formed between C-terminus and the rest of the protein within a monomer. As to *E.coli* PPase, only Glu13-Lys162-Glu159 interactions were observed in α-helix B. Furthermore, thermostable *Sulfolobus acidocardarius* PPase (*Sac* PPase) has an equivalent ionic interaction, Glu64-Arg171-Glu63-Arg164, and then its C-terminus appeared to be more stabilized by these interactions [11]. However, in spite of investigations on these three-dimensional structures, it had not been reported on the contribution of the other amino acids in α-helix B to thermostability and structural integrity of *Tth* PPase functionally. Therefore, we focused on the sole cysteine, Cys168, in *Tth* PPase, because it is located in the same side as above Arg171 and Glu164 (Corresponding to Arg164 in *Sac* PPase) of α-helix B, and oriented to internal moiety of molecule (Fig.2) [11]. In addition, we expected that Cys168 may be responsible for the thermal aggregation of *Tth* PPase, because we have observed the cross-linked dimer in the precipitation of thermal aggregation when *Tth* PPase was heated at 90°C followed by analyzed on the SDS-PAGE in the absence of the reducing reagent (Fig.1). In view of above information, we investigated the possibility that Cys168 may affect on structural integrity and thermostability of *Tth* PPase molecule by substitution to hydrophobic amino acids (A, L, I and F).

Firstly, we analyzed some characteristics of Cys168-substituted variants in the native state (Table 2 and Fig.3). The specific activities of four variants were almost the same as that of wild type enzyme except for C168F (58.3%). Meanwhile, the drastic conformational changes were not detected in the CD spectra and Tryptophan fluorescence spectra of these variants. Additionally, each quaternary structure might be insensitive to the substitution of Cys168, which was comprised of only hexamer. Accordingly, the reduction of enzyme activity for C168F seemed to be due to slight torsion in the vicinity of the substituted Phe residue.

In contrast to these results, the thermostabilities of enzyme activity, conformation and quaternary structure for C168L, I, and F variants were decreased remarkably, as shown in Figs. 4, 5, and 6. Especially, C168I variant appeared to be the most unstable among them, and then almost 40% of its monomer was observed after heating even at 65°C. On the other hand, C168A variant showed the highest thermostabilities of all enzymes including wild type enzyme. Significant peaks for its hexamer and trimer were detected on gel filtration chromatography after heating even at 85°C, whereas most of wild type molecule aggregated under this condition (Fig.6). This aggregates of wild type *Tth* PPase contained dimeric component (Fig.1), whereas it was not observed in the aggregates of Cys168-subsutituted variants. These results were supported by the ANS

fluorescence experiments of thermal aggregation (Fig.7). It was suggested that thermal aggregation was suppressed in C168A variant, though all enzymes resulted in the thermal aggregation. Therefore, we concluded that thermal aggregation of *Tth* PPase after heating was caused by the exposure of hydrophobic region of molecule, the cross-linking of Cys168 among subunits, and oligomerization, and C168A variant must suppress the thermal aggregation and stabilized whole *Tth* PPase molecule, because the substituted Ala residue might adjust the adjacent environment such as ionic interactions for thermostability.

As described above, C-terminal region of *Tth* PPase was deduced to be responsible for high thermostability and stabilization of stable conformation [11-13]. As expected from the structural investigations, our results suggested the environment at 168 position in *Tth* PPase would affect much on the thermostabilities for the enzyme activity and quaternary structure. Then, we deduced that these remarkable differences in thermostabilities might be caused by volume and hydrophobicity at this 168 position in *Tth* PPase. In three-dimensional structure of *Tth* PPase, it was not reported that Cys168 interacts with other amino acids including ones in α-helix B. Then, we expected that the small cavity might be located in the vicinity of this residue (Fig.2). Given that the average volume of Ala, Cys, Leu, Ile, and Phe are 91.5 $Å^3$, 105.6 $Å^3$, 167.9 $Å^3$, 168.8 $Å^3$, and 203.4 $Å^3$, while the hydrophobicity of them are 1.8, 2.5, 3.8, 4.5, and 2.8, respectively [22,23], thermostabilities of Cys-substituted variants might be related closely to the volume and hydrophobicity of side chain at 168 position in *Tth* PPase. As the hydrophobicity of side chain increased, whole *Tth* PPase molecule would be less thermostable. Consequently, the small volume and appropriate hydrophobicity such as Cys and Ala probably might be suitable for this deduced cavity and adjacent ionic interactions, whereas larger side chain than them seemed to be attributable to unfavorable conformational perturbation by steric hindrance or irrelevant interactions. In particular, the substitution of Cys168 with Ala is the most preferable for thermostabilization and suppression of thermal aggregation of *Tth* PPase. On the basis of these investigations, we approached more artificial thermostabilization of *Tth* PPase by the combination of the thermostabilizing factors including C168A, and further studies are now undertaken.

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