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Deletion of Ala144-Lys145 in *Thermus thermophilus* Inorganic Pyrophosphatase Suppresses Thermal Aggregation

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The regions contributing to the thermostability of inorganic pyrophosphatase (PPase, EC 3.6.1.1) from *Thermus thermophilus* (*Tth*) were deduced in our previous study by random chimeragenesis, one of them being estimated to be Ala144-Lys145 [Satoh, T., Takahashi, Y., Oshida, N., Shimizu, A., Shinoda, H., Watanabe, M., and Samejima, T. (1999) *Biochemistry* 38, 1531-1536]. Therefore, we investigated the contributions of these two residues in *Tth* by preparing a deletion mutant (del.144-145 mutant) of *Tth* PPase. We examined its thermostability in terms of the CD and fluorescence spectra, and the thermal change in the enzymatic activity. The thermostability of the enzymatic activity of the del.144-145 mutant was similar to that of the wild type *Tth* PPase, whereas this mutant was more stable against heating. Furthermore, we compared the thermal aggregation of the wild type with that of the del.144-145 mutant. We found that the thermal aggregation of the mutant was reduced relative to that of the wild type. Moreover, the molecular weight of the mutant after heating at 90°C was higher than that of the unheated one, whereas the wild type aggregated under the same conditions. Therefore, we can conclude that although the Ala144-Lys145 residues in *Tth* PPase may partly cause thermal aggregation, the deletion of these residues may stabilize the *Tth* PPase molecule structurally against heating and suppress thermal aggregation.

Key words: aggregation, deletion, inorganic pyrophosphatase, thermostability, *Thermus thermophilus*.

Inorganic pyrophosphatase (PPase, EC 3.6.1.1) catalyzes the hydrolysis of pyrophosphate to orthophosphate in the presence of divalent cations (*1*). PPase has been isolated from various sources such as procaryotes, eucaryotes, and archaebacteria. Regarding procaryotic PPases, the genes of *Escherichia coli* PPase (*2*), *Thermus thermophilus* PPase (*Tth* PPase) (*3*), thermophilic bacterium PS-3 PPase (*4*), and *Bacillus stearothermophilus* PPase (*5*) were effectively expressed in *E. coli* cells, respectively. Furthermore, the former two PPases form a hexameric structure, and their three-dimensional structures have been elucidated by X-ray crystallography at 1.9 and 2.0 Å resolution, respectively (*6, 7*). Their three-dimensional structures are very similar, but they differ in the oligomeric interactions. In this respect, it was deduced that *Tth* PPase acquires stability through the oligomerization, and thereby many additional hydrogen bonds and ionic interactions are induced (*8*). On the contrary, the latter two PPases form a trimer or hexamer, and their three-dimensional structures are unknown. From these viewpoints, the former two PPases have been used as models as to thermostabilization of thermophilic enzymes. Several key residues contributing to the oligomeric structure were revealed by studies involving site-directed mutagenesis. In *E. coli* PPase, His136 and His140 are crucial for maintaining the trimer-trimer interface (*9, 10*), and substitutions of Glu20 with Asp (*11*), Tyr55 with Phe, and Lys104 with Arg (*12, 13*) induced dissociation from the hexamer to the trimer. Meanwhile, trimeric *Bst* PPase dissociated into monomers on substitution of Val75 with hydrophilic residues (*14*). However, since these PPases had oligomeric structures, such as the hexamer and trimer, the factors contributing to their thermostability may be more intricate than globular monomeric proteins (*15*), and it must be difficult to apply this information to the design of *de novo* thermostable enzymes. We approached this problem by random chimeragenesis in the previous study (*16*). We constructed two chimeric PPases between the *E. coli* and *Tth* PPases, T1-135E (residues 1-135 from *Tth* PPase and residues 136-173 from the C-terminal region of *E. coli* PPase) and T1-149E [residues 1-149 from *Tth* PPase and the rest (150-175) from *E. coli* PPase], and then pointed out the

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Abbreviations: PPase, inorganic pyrophosphatase; *Tth*, *Thermus thermophilus*; del.144-145, Ala144 and Lys145 deletion mutant of *Thermus thermophilus* inorganic pyrophosphatase; T1-135E, thermostable chimeric inorganic pyrophosphatase derived from *Thermus thermophilus* and *Escherichia coli*.

factors contributing to the thermostability, discussing the possibility of constructing more thermostable enzymes. As a result, we suggested that four residues are candidate factors for the thermostabilization of thermostable T1-135E, *i.e.* Thr138, Ala141, Ala144, and Lys145 in *Tth* PPase. On the contrary, although *Tth* PPase was thermostable, thermal aggregation was observed on heating at 90°C (*3*). We deduced that this thermal aggregation was the origin of the thermal inactivation and instability of *Tth* PPase at high temperature. Thermal aggregation has been observed for some proteins (*17, 18*), and generally occurs with unstable proteins. Although these examples were different from in the case of *Tth* PPase, we considered that *Tth* PPase may become more thermostable if thermal aggregation could be suppressed. Therefore, we attempted to stabilize the *Tth* PPase molecule by deletion of its Ala144-Lys145 residues (del.144-145 mutant), which comprised the candidate factor for thermostabilization, as described previously (*16*). In this paper, we report the differences in the thermal characteristics of wild type *Tth* PPase, the del.144-145 mutant, and thermostable chimeric PPase T1-135E. In particular, we observed the suppression of thermal aggregation by this deletion, and inferred the relation of this aggregation with a change in the quaternary structure on heating.

MATERIALS AND METHODS

Chemicals—The restriction endonucleases were purchased from Takara Shuzo, Toyobo, Biolabs, and Nippon Gene. DEAE-Sephacel and Sephacryl S-200 HR were purchased from Pharmacia. The reagents for enzyme assays (see below) were obtained from Wako Pure Chemicals.

Overexpression and Purification of Wild Type Tth PPase, the del.144-145 Mutant, and Chimeric PPase T1-135E—The overexpression and purification of wild type *Tth* PPase and chimeric PPase T1-135E were performed as reported previously (*3, 16*). The construction of the del. 144-145 mutant was performed by site-directed mutagenesis using the polymerase chain reaction (PCR). The sense-primer for the del.144-145 mutant was 5′-CCTCGAGAAGGGGAAGTGGGTCAAGG-3′ (26mer, corresponding to Ala141-Glu143 and Lys146-Val 152). The antisense-primer for del.144-145 mutant was 5′-CGCGGAGACGTCAGAGCTTGT-3′ (22mer, which was complementary to a sequence in the pUC118 vector). The amplified fragment was digested with *Xho*I and *Bam*HI, and then inserted into pUCTPPCR after digestion with the same endonucleases. Then, it was sequenced, digested with *Nco*I and *Bam*HI, and then inserted into the same site of the pET15b vector, the resultant mutant being named pETDEL144-145. The purification of the del.144-145 mutant was performed as described previously (*3*).

Enzyme Assay—The activity of PPase was assayed at 37°C essentially according to the method described previously (*19*), in which the liberation of inorganic phosphate was determined by the method of Peel and Loughman (*20*). Protein concentrations were determined by the method of Lowry *et al.* (*21*), using bovine serum albumin as a standard.

Circular Dichroism (CD) Spectra Measurements—CD spectra were recorded with a J-600 automatic recording dichrograph (JASCO) at room temperature with protein concentrations of 0.1-0.2 mg/ml. The far-UV CD spectra were measured between 200 and 250 nm in a 1 mm optical path cuvette. CD data are expressed in terms of mean residue ellipticity, $[\theta]$, using the mean residue molecular weight determined from the primary structure.

Fluorescence Measurements—Fluorescence measurements were made with an FP777 spectrofluorometer (JASCO) at room temperature, using a 5 mm path length quartz cuvette. The protein concentration was always adjusted to 0.1 mg/ml in 20 mM Tris-HCl buffer (pH 7.8). Tryptophan excitation was performed at 295 nm. The emission spectra were set between 300 and 400 nm.

Thermostability—As a means of evaluating the thermostability of wild type *Tth* PPase, chimeric PPase T1-135E and the del.144-145 mutant, we adopted the changes in the CD spectra, fluorescence spectra and enzyme activity on incubation at 40, 50, 60, 70, 80, and 90°C for 1 h. The concentration of each sample was adjusted to 0.1 mg/ml. Although the wild type *Tth* PPase aggregated after heating at 90°C for 1 h, each measurement was performed using the supernatant after centrifugation.

Thermal Aggregation Monitoring—The thermal aggregation of wild type *Tth* PPase, chimeric PPase T1-135E, and the del.144-145 mutant on heating at 90°C for 10, 20, 30, and 40 min was monitored as to the absorbance at 308, 360, and 600 nm as the criterion of turbidity according to Lee *et al.* (*18*). The absorbance of each unheated protein at the same wavelength was defined as the heating time of 0 min.

Analysis of the Quaternary Structure before and after Heating—The molecular weight of each of wild type *Tth* PPase, chimeric PPase T1-135E, and the del.144-145 mutant was determined on a HiLoad 26/60 Superdex 75 pg column (Pharmacia) with bed dimensions of 2.6×60 cm. The protein samples for molecular weight markers were used to obtain the calibration curve as reported previously (*14*). The column was run at room temperature with 50 mM Tris-HCl buffer (pH 7.8) as the eluent (flow rate, 3.0 ml/min). The protein concentration of applied samples was adjusted to 0.10 mg/ml. Samples were unheated and heated at 90°C for 1 h, followed by filtration with Samprep LCR25-LG (pore size, 0.2 mm; Millipore) and injection onto the column. The ratio of each peak was determined by estimating each peak area on gel filtration with a Shimadzu Chromatopac C-R6A.

RESULTS

In the previous study we constructed two chimeras, T1-135E and T1-149E, the former being more thermostable than wild type *Tth* PPase, whereas the thermostability of the latter was similar to that of wild type *Tth* PPase (*16*). Their primary structures differed in only four residues. Therefore, we focused on the deletion of Ala144-Lys145 in T1-135E, and attempted to stabilize the *Tth* PPase by deleting these residues (del.144-145 mutant). Schematic representation of wild type *Tth* PPase, T1-135E, and the del.144-145 mutant is shown in Fig. 1.

Firstly, we purified the del.144-145 mutant of *Tth* PPase, wild type *Tth* PPase and chimeric PPase T1-135E to homogeneous states electrophoretically. As a result, we obtained about 10 mg of each PPase from 1 liter culture of

LB medium. Then, we investigated the characteristics of these three PPases. The specific activities of wild type *Tth* PPase, chimeric PPase T1-135E, and the del.144-145 mutant were measured, and the results are shown in Table 1. As compared to that of the wild type *Tth* PPase, the specific activity of the del.144-145 mutant was reduced to 59.6%. We reported a reduction of specific activity of T1-135E in the previous paper (*16*), and deduced that the deletion of two residues (Ala144-Lys145 in *Tth* PPase) affected the enzyme activity. From the present results, we also confirmed that the deletion of this sequence affected the enzyme activity. Therefore, we examined whether a structural change of the del.144-145 mutant occurred or not. CD spectra in the far-UV region (data not shown) and fluorescence spectra (Table I) were measured. Consequently, we observed no drastic conformational change in the del. 144-145 mutant, whereas the environment in the vicinity of Trp residues became slightly more hydrophobic than in the wild type. Thus, we suggested that the discrepancy in the enzyme activity may be related slightly with the conformational change around the Trp residues.

As described above, no drastic conformational change of these PPases occurred in the native state. Therefore, we investigated the thermostability of wild type *Tth* PPase, chimeric PPase T1-135E, and the del.144-145 mutant. Firstly, the thermostability of their enzyme activities was examined, as shown in Fig. 2. A slight heat activation effect was observed for the del.144-145 mutant, whereas a significant effect was seen for chimeric PPase T1-135E. Furthermore, the wild type *Tth* PPase and del.144-145

TABLE I. **The characteristics of wild type *Tth* PPase, the del. 144-145 mutant, and chimeric PPase T1-135E.**

	Enzymatic activity		Fluorescence spectra	
	Specific activity (units/mg)	Relative activity[a] (%)	Max. WL.[b] (nm)	Shift of WL[c] (nm)
Wild type	498	100	335.4	0
del.144-145	297	59.6	332.6	−2.8
T1-135E	278	55.8	335.6	+0.2

[a]The specific activity of wild type *Tth* PPase was taken as 100%. [b]Max. WL indicates the emission maximum wavelength of tryptophan fluorescence spectra. [c]Shift of WL indicates the wavelength shift of the emission maximum of tryptophan fluorescence spectra. The emission maximum of wild type *Tth* PPase was taken as zero.

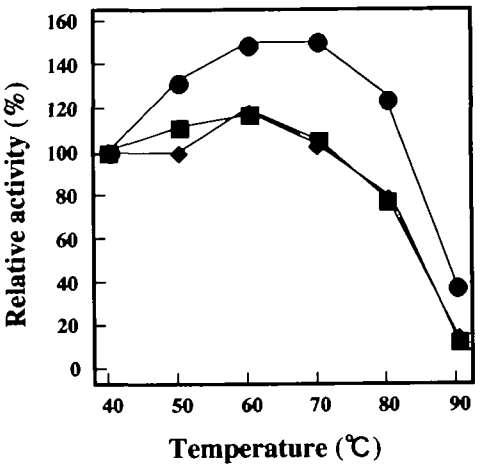

Fig. 2. **The thermostability of wild type *Tth* PPase, del.144-145, and chimeric PPase T1-135E.** Each enzyme (0.1 mg/ml, pH 7.8) was heated at the indicated temperatures for 1 h. The activity after incubation at 40°C was taken as 100%. Symbols: ◆, wild type *Tth* PPase; ■, del.144-145; ●, chimeric PPase T1-135E.

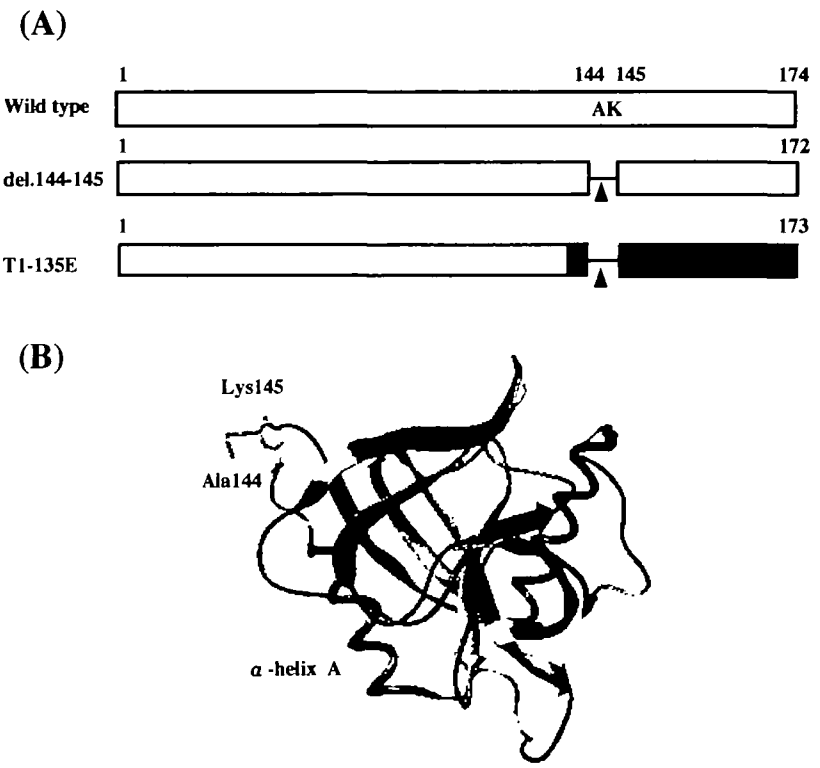

Fig. 1. **Schematic representation of the primary structures of wild type *Tth* PPase, del. 144-145, and chimeric PPase T1-135E (A), and a ribbon representation of the three-dimensional structure of *Tth* PPase (B).** (A) Schematic representation of the three PPases examined in this study. The open box indicates the sequence of *Tth* PPase, whereas the shaded box is that of *E. coli* PPase. The arrowhead indicates the deleted of the Ala144-Lys145 sequence. (B) The position of Ala144-Lys145 in *Tth* PPase. The X-ray data for *Tth* PPase were cited from the Protein Data Bank (PDB code 2prd). α-Helix A is the intratrimer interface of hexameric *Tth* PPase.

mutant were almost completely inactivated on heating at 90°C for 1 h, whereas about 40% of the activity of T1-135E remained even after heating at 90°C. These results suggested that the deletion of Ala144-Lys145 in the *Tth* PPase did not induce such significant thermostabilization as for T1-135E.

Secondly, from the viewpoint of the secondary structure, we evaluated the thermostability of these three PPases by means of far-UV CD spectra (data not shown). Their far-UV CD spectra were measured after heating at 40–90°C for 1 h, and those after heating below 80°C were almost unchanged. However, we observed a significant change after heating at 90°C. The wild type *Tth* PPase exhibited a drastic reduction of CD intensity because of thermal aggregation, while T1-135E and the del.144-145 mutant showed less reduction than the wild type. Such a tendency was also observed in the thermostability of fluorescence spectra, as shown in Fig. 3. For the wild type, the maximum of fluorescence spectra shifted to a longer wavelength as the temperature increased. On the other hand, that of the del. 144-145 mutant showed a slight blue-shift below 80°C, and abruptly red-shifted by over 5 nm on heating at 90°C. That of T1-135E also exhibited a blue-shift effect on heating below 80°C. We suggested that their secondary structures and the environment around Trp residues were unaltered after heating below 80°C, and the difference in their thermostabilities may be caused by the conformation after heating at 90°C.

Furthermore, we examined the thermal aggregation, which was observed for the wild type *Tth* PPase on heating at 90°C. As shown in Fig. 4, the turbidity after heating at 90°C was measured at 600 nm. For the wild type, the thermal aggregation rapidly occurred with heating and was saturated after 10 min. On the other hand, that of the del. 144-145 mutant was suppressed to about 10% of the wild type level. Moreover, chimeric PPase T1-135E did not show thermal aggregation at all. These results suggested that deletion of the Ala144-Lys145 sequence may suppress

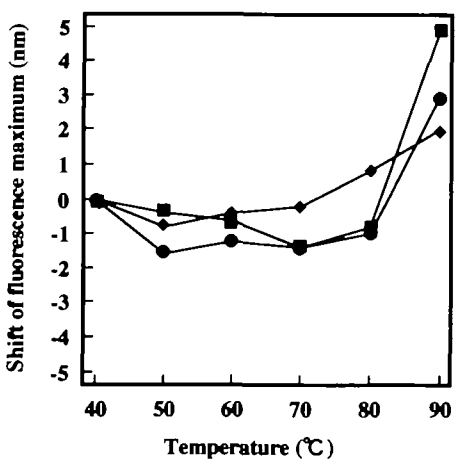

Fig. 3. **The relative shifts of the emission maxima of tryptophan fluorescence spectra of wild type *Tth* PPase, del.144-145, and chimeric PPase T1-135E.** The excitation wavelength was 295 nm. The emission maximum wavelengths for the unheated proteins are listed in Table I. The figure shows the shift of the wavelength at each indicated temperature relative to that on heating at 40°C. Symbols: ◆, wild type *Tth* PPase; ■, del.144-145; ●, chimeric PPase T1-135E.

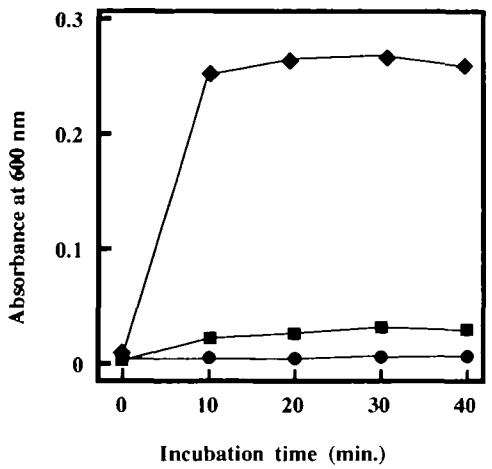

Fig. 4. **Thermal aggregation of wild type *Tth* PPase, del. 144-145, and chimeric PPase T1-135E.** The concentration of each enzyme was as follows; wild type, 0.15 mg/ml; del.144-145, 0.25 mg/ml; T1-135E, 0.2 mg/ml, respectively. Enzyme solutions were heated at 90°C for the indicated times, and then cooled down to room temperature. The absorbance of the three PPases was measured at 600 nm. The unheated protein was taken as zero. Symbols: ◆, wild type *Tth* PPase; ■, del.144-145; ●, chimeric PPase T1-135E.

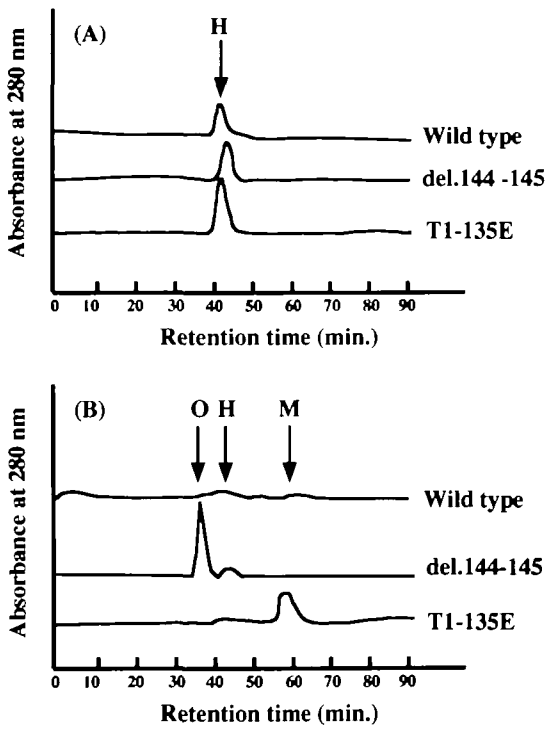

Fig. 5. **Elution profiles of wild type *Tth* PPase, del.144-145, and T1-135E on gel filtration before (A) and after heating at 90 °C (B).** The gel filtration chromatography was performed under the conditions described under "MATERIALS AND METHODS." (A) Profile before heating. The letter H indicates the position of the hexameric state deduced from the calibration curve for molecular weight. (B) Profile after heating at 90°C. The letter O shows the position of the oligomeric state, while M denotes that of the monomeric state. The calculated molecular weight corresponding to each peak was given in the text.

thermal aggregation.

In order to investigate their quaternary structures after heating at 90°C, gel filtration analysis of these PPases was performed. The results are shown in Fig. 5, A and B. At first, the native PPases (before heating) were analyzed on a Superdex 75 pg column (Fig. 5A). The calculated molecular weights of three PPases were 120–150 kDa, suggesting hexamer formation. However, those of the three PPases after heating at 90°C were different (Fig. 5B). The wild type *Tth* PPase exhibited no peak in its profile because of the thermal aggregation, while the del.144-145 mutant showed higher oligomerization than the native state. Furthermore, hexameric T1-135E was dissociated into monomer on heating. On the basis of these results, we can suggest that the deletion of Ala144-Lys145 in *Tth* PPase suppressed the thermal aggregation, and that the apparent thermostabilization of T1-135E may be due to the suppression of this thermal aggregation, and the dissociation into monomers.

DISCUSSION

In the previous paper, we reported that chimeric T1-135E exhibited the increased thermostability (*16*). Then, we suggested that the following two factors may contribute to the difference in thermostability between *Tth* and *E. coli* PPase; (i) the two residues in the vicinity of the trimer-trimer interface were different, *i.e.* His140 and Asp143 in *E. coli* PPase were substituted by Thr138 and Ala141 in *Tth* PPase. (ii) The Ala144-Lys145 loop in *Tth* PPase was deleted in the *E. coli* PPase. In this study, we investigated the contribution of the latter factor to the *Tth* PPase. The del.144-145 mutant of *Tth* PPase was constructed, and its characteristics in the native state were analyzed (Table I). As a result, the specific activity of the del.144-145 mutant was found to be reduced to 59.6% of that of the wild type. Such a reduction in specific activity was also observed for T1-135E, and this deleted region may affect the active site of *Tth* PPase indirectly. Tyr139, Lys140 *etc.* involved in the active site (*7*) are located in the proximity of this deleted sequence. Thus we deduced that the deletion of Ala144-Lys145 affected the orientation of these residues in the active site of *Tth* PPase. However, other spectroscopic characteristics, such as CD and fluorescence spectra, were almost identical to those of the wild type. Then we confirmed that a drastic conformational disorder did not occur with this deletion. Next, the thermostabilities of wild type *Tth* PPase, the del.144-145 mutant, and T1-135E were examined. The thermostability of the del.144-145 mutant was almost the same as that of the wild type in terms of enzymatic activity (Fig. 2). On the other hand, the CD (data not shown) and fluorescence spectra (Fig. 3) of the del. 144-145 mutant and T1-135E after heating at 90°C were different from those of the wild type. An extremely reduced CD profile after heating at 90°C was observed for the wild type because of thermal aggregation, while a slight reduction was observed for the del.144-145 mutant and T1-135E, respectively. Thus, we suggested that the del.144-145 mutant was more thermostable than the wild type structurally, and that the differences in thermostability may be due to the conformational change on heating at 90°C. Therefore, we focused on two thermal characteristics of these three PPases after heating at 90°C. One was thermal aggregation (Fig. 4), and the other was the dissociation of the hexamer into monomers (Fig. 5). The former phenomenon was observed for *Tth* PPase after heating at 90°C, but not for *E. coli* PPase. The latter was observed for *E. coli* PPase, but not for *Tth* PPase. These thermal characteristics may be defined as the *Tth* and *E. coli* PPase types. Which type does the del.144-145 mutant or T1-135E belong to? Turbidity measurement of these PPases after heating at 90°C revealed that the del.144-145 mutant may belong to the *Tth* PPase type, but T1-135E to the *E. coli* PPase type (Fig. 4). In spite of the common deletion of Ala144-Lys145 in the del.144-145 mutant and T1-135E, they exhibited different behavior as to thermal aggregation. Therefore, we investigated the dissociation of these PPases on heating at 90°C (Fig. 5). *Tth* PPase exhibits no significant peak because of the thermal aggregation, while the del.144-145 mutant was highly oligomerized. The molecular weight was estimated to be 388 kDa, which represents an about 20mer of the monomer. We suggested that this oligomerization may represent the pre-aggregated state, and the del.144-145 mutant suppressed thermal aggregation by terminating further higher oligomerization. On the contrary, the estimated molecular weight of the native T1-135E was 156 kDa, which was dissociated into 28.8 kDa components. Although these molecular weights were not identical with the theoretical ones, we estimated that the hexameric T1-135E was dissociated into monomers on heating at 90°C, because the molecular weight of the dissociated component was 1/5.4 of that of the native one. From these results, we can conclude that the thermostability and subunit interface of the del.144-145 mutant may belong to the *Tth* type, whereas those of T1-135E being to the *E. coli* type.

From the three-dimensional structure (*7*) and limited proteolysis results (*3*) for *Tth* PPase, it was suggested that this Ala144-Lys145 region was located in the loop region in the vicinity of α-helix A, and also in a rather flexible and hydrophilic region on the surface of the molecule. We can suggest on the basis of this study that this region may be the destabilizing factor of *Tth* PPase. However, *Tth* PPase may have adapted these residues for intrinsic thermostabilization evolutionarily. We consider that the role of these residues was as a regulating factor preserving the parallel packing of the intratrimer interface of *Tth* PPase. It has been reported that α-helices A of both *Tth* and *E. coli* PPases formed the trimer-trimer interface of the hexamer (*6, 7*). However, the packing and interactions of α-helices were very different between them; α-helices A of the intratrimer interface in *Tth* PPase were antiparallel (*7*), whereas those of *E. coli* PPase were antiparallel and slid (*9*). On the basis of these information, we found previously that the Lys residues in *Tth* (*3*) and *E. coli* PPases (unpublished results) exposed to solvents were readily digested with *Acromobacter* protease I because of this difference in the intratrimer interfaces between *Tth* and *E. coli* PPases. We are now analyzing the intratrimer interfaces of the del. 144-145 mutant and T1-135E by limited proteolysis, expecting to confirm this speculation, and the relation between their intratrimer interfaces and thermostability.

In general, there have only been several successful examples of stabilizing proteins by means of deletion mutagenesis (*22, 23*). In this study, we suggested that the deletion of Ala144-Lys145 in *Tth* PPase suppressed thermal aggregation, and that this deletion may contribute to

the thermostabilization of T1-135E. We have already deduced another factor thermostabilizing T1-135E, that is, His140 and Asp143 in *E. coli* PPase were substituted by Thr138 and Ala141 in *Tth* PPase (*16*). We presumed that since these intratrimeric interactions in *Tth* PPase are strong, *Tth* PPase can not dissociate into monomers, that is, the conformational rearrangement for avoiding a reduction in solubility like thermal aggregation may be perturbed. If thermal aggregation of *Tth* PPase could be completely suppressed by modification of these residues on the subunit interface, we may possibly construct more thermostable PPases. However, we need to perform further investigations from the viewpoint of these subunit interactions. Now detailed studies involving site-directed mutagenesis and limited proteolysis of these residues on the subunit interface are underway.

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