A Chimeric Inorganic Pyrophosphatase Derived from *Escherichia coli* and *Thermus thermophilus* Has an Increased Thermostability†

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ABSTRACT: Factors contributing to the thermostability of inorganic pyrophosphatase (PPase) were investigated by examining chimeric PPases from *Escherichia coli* and *Thermus thermophilus* (*Tth*). Two chimeric PPase genes, T1−135E (residues 1−135 from the N terminus are comprised of *Tth* PPase and residues 136−173 are derived from the C terminus of *E. coli* PPase) and T1−149E [residues 1−149 from the N terminus are from *Tth* PPase and the rest (150−175) are from *E. coli* PPase], were constructed by random chimeragenesis. After the genes were overexpressed in the *E. coli* BL21(DE3) strain and the expression products were purified, we compared the characteristics of these chimeric PPases with those of the parental PPases. We found that the two chimeras had higher activity than either parent PPase at the optimum temperature. We also examined thermal stability in terms of CD spectra, fluorescence spectra, and thermal changes in enzyme activity. The results revealed that the thermal stability of T1−149E is similar to that of *Tth* PPase, but T1−135E is much more stable. This suggests that the four residues that are different between T1−135E and T1−149E may be critical for thermostability between the two chimeras. By comparing the three-dimensional structures of *Tth* and *E. coli* PPases, we deduced that the following two factors may contribute to differences in thermostability. (1) Two residues (Thr138 and Ala141 in the *Tth* PPase and His140 and Asp143 in the *E. coli* PPase) in the vicinity of the trimer−trimer interface were different. (2) The Ala144-Lys145 loop in the *Tth* PPase was deleted in the *E. coli* PPase and also in the T1−135E chimera. Therefore, we conclude that T1−135E was thermostabilized by these two factors, and also, the *Tth* PPase moiety may contribute to the structural integrity of the chimeric enzymes.

The factors dominating the stability of proteins have been extensively investigated. Hyperthermophile enzymes have been directly compared with mesophile enzymes, and the mechanism of thermostabilization has been deduced for some proteins (*1*, *2*). In addition, knowledge about three-dimensional structure of a protein combined with site-directed mutagenesis has been successfully used to investigate the stability of some proteins in detail (*3*). Stabilization of unstable proteins has also been accomplished, including that for an α-helix structure (*4*), α-helical dipoles (*5*), and a *β*-sheet (*6*) using this method. However, it has been rather difficult to generalize the rules for stability. Therefore, in this study, we utilized random chimeragenesis (*7*) as a tool for exploring the region(s) contributing to the thermostability of a thermophilic enzyme. This method takes advantage of homologous recombination in vivo and is a potent tool for selecting significant region(s) from numerous candidates. In this study, we applied this method to an inorganic pyrophosphatase (PPase,[1] EC 3.6.1.1), which hydrolyzes pyrophosphate to two orthophosphates in the presence of divalent cations such as Mg^{2+} (*8*). The PPases from various sources were categorized into two groups, on the basis of molecular mass and the oligomeric structure. One is the eukaryotic group, which contains PPases from *Saccharomyces cerevisiae* (Y-PPase) (*9*), *Kluyveromyces lactis* (*10*), *Schizosaccharomyces pombe* (*11*), *Arabidopsis thaliana* (*12*), and bovine retina (*13*). The quaternary structure of these PPases is composed of monomers or dimers, with about 290 amino acid residues each. The molecular masses of these PPases were ca. 30 kDa per subunit. The best studied PPase among them is Y-PPase, whose three-dimensional structure was reported at 2.2 and 2.0 Å (*14*), and a structure-based mechanism for catalysis has been proposed (*15*). The other group of enzymes is prokaryotic, which include PPases from *Escherichia coli* (*E. coli* PPase) (*16*), *Thermus thermophilus* (*Tth* PPase) (*17*), *Bacillus stearothermophilus* (*Bst* PPase) (*8*), and thermophilic bacterium PS-3 (*18*, *19*). These PPases consist of about 170 amino acid residues with molecular masses of about 19 kDa per subunit. The homology of the primary structure between prokaryotic PPases was about 40%, and their quaternary structures consist of oligomeric subunits, either trimers or hexamers. Overexpression systems for these four prokaryotic PPases has been established in *E.*

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[1] Abbreviations: PPase, inorganic pyrophosphatase; *Tth*, *Thermus thermophilus* HB8; Y, *Saccharomyces cerevisiae*; *Bst*, *Bacillus stearothermophilus*.

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coli cells. In addition, the three-dimensional structures of *E. coli* PPase at 1.9 Å (*20*) and *Tth* PPase at 2.0 Å (*21*) were analyzed. Both three-dimensional structures are very similar, but they differ in oligomeric interactions. It has been proposed that *Tth* PPase acquires stability by oligomerization of the subunits which induces numerous additional hydrogen bonds and ionic interactions (*22*).

Therefore, we selected *Tth* PPase and *E. coli* PPase as model enzymes for analysis of thermostability of thermophilic enzymes. In this work, we report on the exploration for factors contributing to the thermostability of *Tth* PPase by random chimeragenesis, the thermostability of the resultant chimeric PPases, and possible designs for constructing more thermostable enzymes.

EXPERIMENTAL PROCEDURES

Bacterial Strains. *E. coli* strains JM101 and JM109 were used for random chimeragenesis and transformation with the expression vector of chimeric PPase, respectively. The *E. coli* cells were grown in Luria-Bertani (LB) medium at 37 °C.

Chemicals. 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.

Wild-Type PPase Gene Constructs. The construction and overexpression of *E. coli* and *Tth* PPase genes in plasmids pSKE-1 and pETTP have been described previously (*17*). These enzymes were used as the parent enzymes for random chimeragenesis.

Chimeric PPase Gene Constructs. Random chimeragenesis was used to construct chimeric PPases (*7*). At first, the cloning vector for the *Tth* PPase gene, pUCTPPCR, was digested with *Xba*I and treated with Klenow fragment, followed by digestion with *Sal*I. The cloning vector for the *E. coli* PPase gene, pSKE-1, was digested with *Eco*RI and treated with Klenow fragment, followed by digestion with *Sal*I. The obtained *Tth* PPase gene fragment was ligated to the plasmid vector pSKE-1 by the methods given above. Then, we constructed a plasmid vector, pTE1, in which the *Tth* and *E. coli* PPase genes were ligated sequentially. Then, pTE1 was digested with *Sac*I and *Bam*HI, or *Sac*I and *Bgl*II. *E. coli* JM101 cells were transformed with the linearized fragments, and plasmids were prepared from the obtained transformants. After screening by digestion with restriction endonucleases, we obtained some chimeric PPase genes. The nucleotide sequences of these chimeric genes were confirmed by DNA sequencing. These chimeric genes were digested with *Sal*I and treated with Klenow fragment, followed by digestion with *Nco*I. The resultant DNA fragments were inserted into the *Bam*HI, which had already been treated with Klenow fragment, and *Nco*I sites of the expression vector pET15b.

Overexpression and Purification of Wild-Type and Chimeric PPases. The overexpression and purification procedures of the *E. coli* and *Tth* PPases (wild type) were carried out as reported previously (*17*). The two chimeric PPase genes constructed as described above were expressed in the *E. coli* BL21(DE3) strain. The purification of the chimeric PPases was performed as follows; *E. coli* BL21(DE3) cells transformed with the expression vector for chimeric PPases were cultured in 1 L of LB medium containing ampicillin at 37 °C for 20 h. Cells were harvested by centrifugation and lysed by sonication, and the soluble fraction was collected. This fraction was applied to a DEAE-Sephacel (Pharmacia) anion-exchange column followed by passage through a Sephacryl S-200HR (Pharmacia) gel filtration column. If necessary, anion-exchange column HPLC was performed until a single band of enzyme was shown on polyacrylamide gel electrophoresis.

Enzyme Assay. The activity of PPase was assayed at 37 °C (standard condition) essentially according to the method described previously (*23*), in which the liberation of inorganic phosphate was determined by the method of Peel and Loghman (*24*). Optimum temperature enzyme activity assays were performed between 40 and 90 °C for 10 min. Protein concentrations were determined by the method of Lowry et al. (*25*), using bovine serum albumin as the standard.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−polyacrylamide gel electrophoresis was performed by the method of Laemmli (*26*) on 15% polyacrylamide gels. The proteins were stained with Coomassie Brilliant Blue R-250.

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 length cuvette. CD data are expressed in terms of mean residue ellipticity, $[\theta]$, using the mean residue molecular mass from the primary structure.

Fluorescence Measurements. Fluorescence measurements were taken with an FP777 spectrofluorometer (JASCO) at room temperature using a 5 mm path length quartz cuvette. The concentration of protein was adjusted to 0.1 mg/mL in 20 mM Tris-HCl buffer (pH 7.8). Tryptophan excitation was at 295 nm, and tyrosine excitation was at 275 nm. Both emission spectra were set between 300 and 400 nm.

Evaluation of the Themostability for Wild-Type and Chimeric PPases. To evaluate the thermostability of the *E. coli*, *Tth*, and chimeric PPases, we used changes in CD spectra, fluorescence spectra, and enzyme activity after incubation at 40, 50, 60, 70, 80, or 90 °C for 1 h, in the absence or presence of 0.05 or 5% sodium dodecyl sulfate (SDS). However, enzymatic activity could not be measured in the presence of 5% SDS because of turbidity due to protein denaturation. The concentration of each sample was adjusted to 0.1 mg/mL. Although the *Tth* PPase (wild type) aggregated after heating at 90 °C for 1 h, each measurement was performed on the supernatant after centrifugation.

RESULTS

Construction of the Chimeric PPase Gene by Random Chimeragenesis. We constructed a plasmid vector, pTE1, containing both the *Tth* and *E. coli* PPase genes, digested it with *Sac*I−*Bam*HI or *Sac*I−*Bgl*II, and transformed the *E. coli* JM101 strain with the resultant fragment. Candidate plasmids were prepared from the resultant transformants and selected by digestion with restriction endonucleases and DNA sequencing. Four chimeric PPase gene plasmids were obtained. Each chimeric gene was inserted into the expression

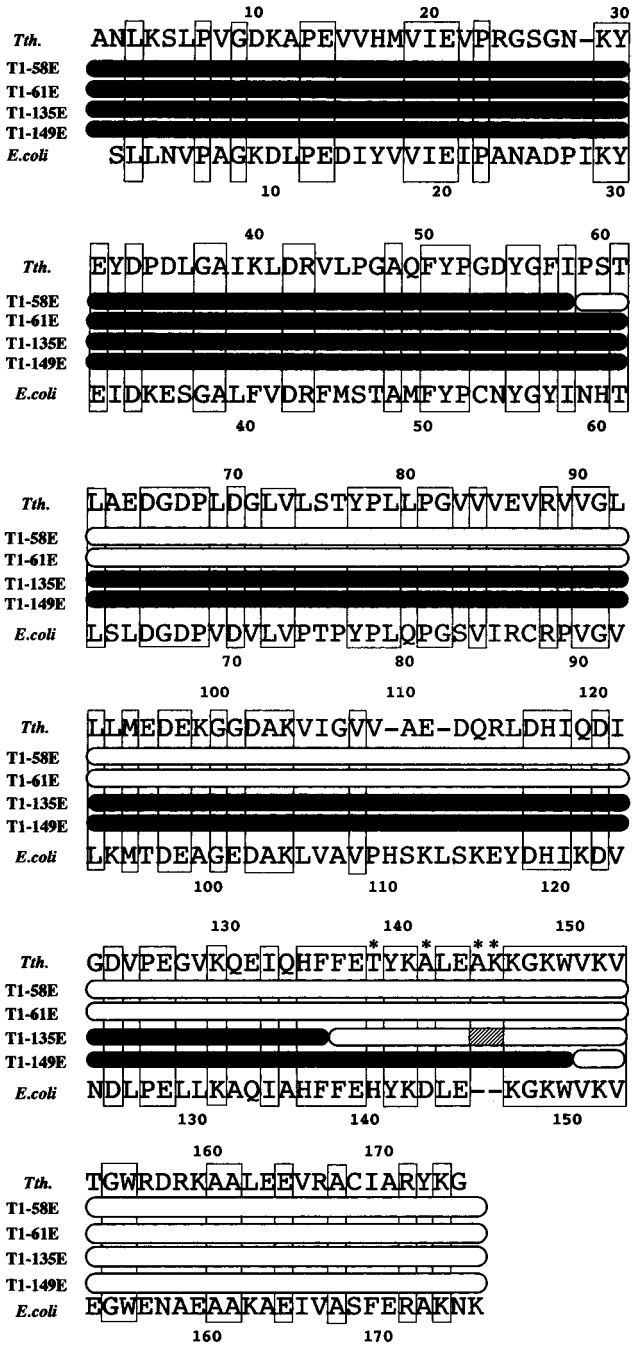

FIGURE 1: Amino acid sequences of *E. coli* and *Tth* PPases and four chimeric PPases (T1−58E, T1−61E, T1−135E, and T1−149E). Amino acid residues in *E. coli* and *Tth* PPases are denoted by one-letter symbols. The amino acid residues of the chimeric PPases are shown by schematic representation. The sequence corresponding to that of *Tth* PPase is represented by a black ellipse, and *E. coli* PPase is a white one. The boxed amino acids represent invariant residues among all PPases. The asterisks denote the four different residues between T1−135E and T1−149E. The two shaded residues are not present in T1−135E.

vector pET15b, resulting in chimeric PPases genes pT1−58E, pT1−61E, pT1−135E, and pT1−149E.

Preparation and Characterization of Chimeric PPases. We purified these four chimeric PPases according to the procedure described above. The recombinant region is shown in Figure 1. Four chimeric genes were expressed effectively in the *E. coli* BL21(DE3) strain. However, the purifications of T1−58E and T1−61E were unsuccessful. T1−135E and T1−149E were fully active and very stable, and we purified about 10 mg of each chimeric PPase from 1 L of LB medium to the homologous state electrophoretically.

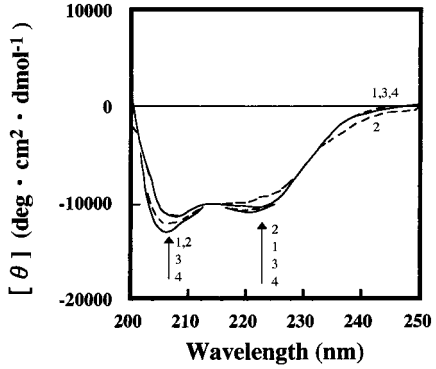

FIGURE 2: Far-UV CD spectra of *Tth*, *E. coli*, and chimeric PPases (T1−135E and T1−149E). The CD spectra were measured under the conditions described in Experimental Procedures. The numbers in the figure indicate (1) *Tth* PPase, (2) *E. coli* PPase, (3) T1−135E, and (4) T1−149E. Curves 1 and 4 are solid lines, and curves 2 and 3 are dashed lines.

We then characterized these chimeric PPases and the parental *Tth* and *E. coli* PPases. The specific activities at 37 °C of wild-type *Tth* and *E. coli* PPases were 498 and 1210 units/mg, respectively. The activities of the chimeric PPases T1−135E and T1−149E were 278 and 668 units/mg, respectively. Compared to that of the parental *Tth* PPase, the specific activity of T1−135E was reduced to 55.8%, while that of T1−149E was increased to 134%. We also examined the optimum temperature for the chimeric PPases. The optimum temperatures for the two chimeric PPases were identical at 75 °C, and the specific activities of T1−135E and T1−149E at this temperature were 7920 and 5530 units/mg, respectively. These chimeric PPases were more active than *Tth* PPase at their optimum temperatures (75 °C). The CD spectra in the far-UV region and fluorescence spectra of the parental *Tth*, *E. coli*, and two chimeric PPases were measured. We found that all four CD spectra (Figure 2) and fluorescence spectra (data not shown) were very similar to one another. Our results suggest that the secondary structure and the environment in the vicinity of the Trp residues may not be altered in the two chimeras. These results are summarized in Table 1.

Thermostability of Chimeric PPases. We investigated the thermostability of the two chimeric PPases and compared them with that of the parental PPases. The activity of these four enzymes was measured after heating at various temperatures. The wild-type *Tth* PPase was more thermostable than the wild-type *E. coli* enzyme, as reported previously (*17*). On the other hand, the two chimeric PPases exhibited different thermostabilities as shown in Figure 3a. T1−149E was still active after heating to 80 °C for 1 h, but mostly inactivated after heating to 90 °C. Meanwhile, T1−135E exhibited so-called "heat activation" after heating between 50 and 80 °C. Further, 40% of the activity of T1−135E remained even after heating to 90 °C for 1 h. These results show that the thermostability of T1−149E is similar to that of *Tth* PPase and T1−135E has a much higher thermostability.

Furthermore, we evaluated the thermostability of the four enzymes with far-UV CD and fluorescence spectra. The far-

Table 1: Characteristics of *E. coli*, *Tth*, and Chimeric PPases (T1−135E and T1−149E)

	enzyme activity			fluorescence spectra		far-UV CD spectra
	specific activity at 37 °C (units/mg)	specific activity at T_{opt}[a] (units/mg)	T_{opt} (°C)	max WL[b] (nm)	shift of WL[c] (nm)	$[\theta]_{222nm}$ (deg cm^2 dmol^{-1})
Tth PPase	498	3320	75	335.4	0	−11000
E. coli PPase	1210	2520	55	335.8	+0.4	−9720
T1−135E	278	7920	75	335.6	+0.2	−11200
T1−149E	668	5530	75	334.2	−1.2	−11000

[a] T_{opt} is the optimum temperature for enzyme activity. [b] Max WL is the maximum emission wavelength of tryptophan fluorescence spectra. [c] Shift of WL is the wavelength shift of the emission maximum of tryptophan fluorescence spectra. That of *Tth* PPase was taken as zero.

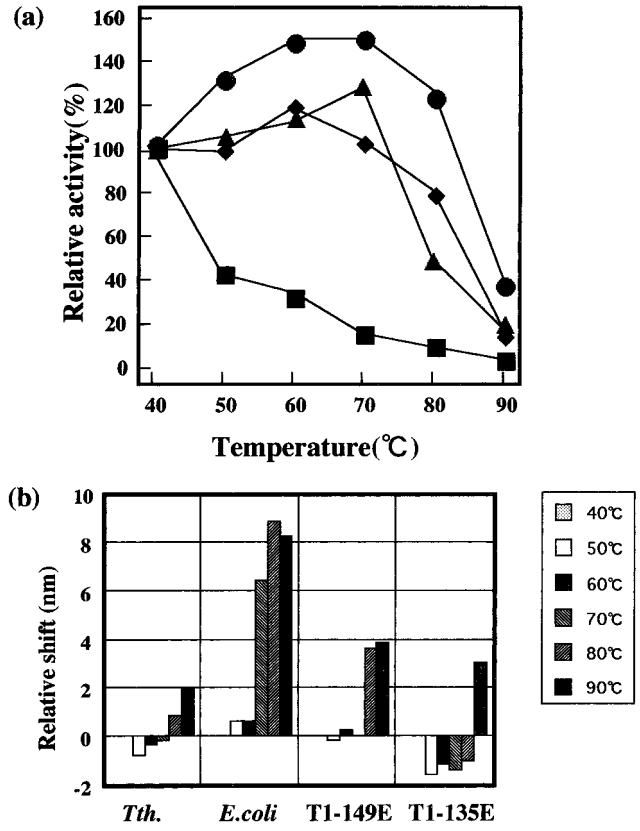

FIGURE 3: Thermostability of *Tth*, *E. coli*, and chimeric PPases (T1−135E and T1−149E). Enzymes (0.1 mg/mL, pH 7.8) were heated at the indicated temperatures for 1 h and cooled rapidly. The activity measurement was performed at 37 °C. (a) The remaining activity of all PPases after heat incubation. The activity after incubation at 40 °C was taken as 100%: (◆) *Tth* PPase, (■) *E. coli* PPase, (▲) T1−149E, and (●) T1−135E. (b) The relative shift in the emission maximum of the tryptophan fluorescence spectra. The excitation wavelength was 295 nm. The emission maximum of unheated proteins is listed in Table 1. This figure shows the shift of wavelength at the indicated temperatures relative to that of the unheated proteins.

UV CD spectra after heating below 80 °C were almost unaltered (data not shown); however, the emission maximum of the fluorescence spectra was different from that of the CD spectra after heating between 40 and 90 °C for 1 h (Figure 3b). For the *E. coli* PPase, the maximum of fluorescence shifted to >5 nm longer above 70 °C. In the *Tth* PPase, this red-shifted effect was observed slightly above 80 °C. The two chimeric PPases exhibited an opposite effect. The emission maximum of T1−149E exhibited a similar "red-shifted" effect, shifting by 3 nm above 80 °C. On the other hand, T1−135E exhibited a 2 nm blue shift below 80 °C. We suggest that the secondary structures of the four enzymes are not significantly altered after they were heated

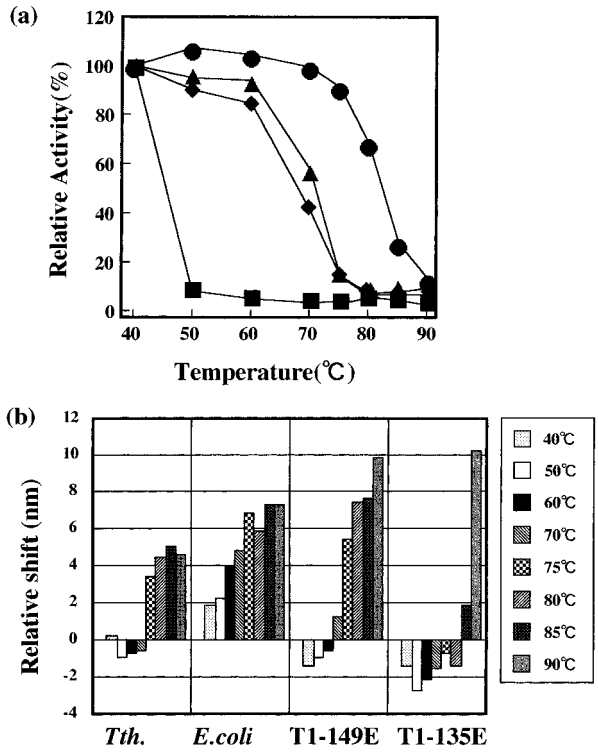

FIGURE 4: Thermostability of *Tth*, *E. coli*, and chimeric PPases (T1−135E and T1−149E) in the presence of 0.05% SDS. The experimental conditions were identical with those described in the legend of Figure 3 except for the presence of 0.05% SDS. (a) The remaining activity of all PPases after heat incubation for 1 h in the presence of 0.05% SDS. The activity after the incubation at 40 °C was taken as 100%: (◆) *Tth* PPase, (■) *E. coli* PPase, (▲) T1−149E, and (●) T1−135E. The activity measurements were performed under the conditions described in the legend of Figure 3. (b) The relative shift in the emission maximum of tryptophan fluorescence spectra in the presence of 0.05% SDS. The other symbol is the same as described in the legend Figure 3.

below 80 °C, but the environments around the Trp residues are different among these four enzymes. In particular, we propose that either or both Trp residues of T1−135E may become hydrophobic after heating below 80 °C, and this recombinant region may contribute to the thermostability of the molecule.

Thermostability of Chimeric PPases in the Presence of Sodium Dodecyl Sulfate (SDS). We examined the thermostability of activity for *E. coli*, *Tth*, and the two chimeric PPases in the presence of 0.05% SDS (Figure 4a). While a remarkable reduction in the thermostability for *E. coli* PPase was observed above 50 °C, only a few percent of *Tth* PPase and T1−149E activities were left after heating at 80 °C for 1 h. However, T1−135E was more heat stable than the other three PPases even in the presence of 0.05% SDS. Further-

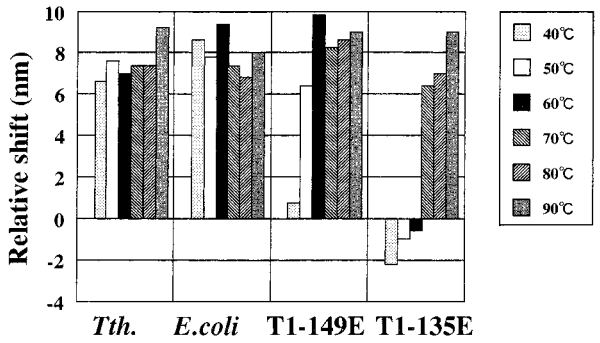

FIGURE 5: Relative shift in the emission maximum of tryptophan fluorescence spectra of *Tth*, *E. coli*, and chimeric PPases (T1−135E and T1−149E) in the presence of 5% SDS. The experimental conditions were identical with those described in the legend of Figure 3 except for the presence of 5% SDS.

more, we evaluated their thermostabilities with fluorescence spectra to detect subtle conformational changes. As shown in Figure 4b, the emission maximum of fluorescence spectra of *E. coli* PPase red-shifted by 2−7 nm after heating above 40 °C. On the other hand, the maxima of *Tth* PPase and T1−149E blue-shifted by 1−2 nm when they were heated to 50−70 °C. Since these proteins showed the opposite red shift when heated above 75 °C, we believe that their Trp residues might be exposed to solvent because of heat-induced denaturation. Meanwhile, T1−135E exhibited a small blue shift after heating between 40 and 80 °C, which possibly indicates stabilization, whereas the red shift effect occurred only above 85 °C.

We also measured the changes in fluorescence spectra of the four PPases after heating in the presence of 5% (w/v) SDS (Figure 5). The PPases other than T1−135E were destabilized under these conditions. The maxima of these three PPases red-shifted by over 6 nm when they were heated to 40−50 °C, whereas T1−135E exhibited a blue shift within this temperature range. We conclude that T1−135E has very stable enzyme activity and conformation, while T1−149E has the same degree of thermostability as *Tth* PPase. We also believe that the four different residues between T1−135E and T1−149E are crucial for thermostabilty (Figure 1).

DISCUSSION

The three-dimensional structures of *Tth* and *E. coli* PPases were resolved at 2.0 (*21*) and 1.9 Å resolution (*20*), respectively. Comparison of the three-dimensional structures suggests that *Tth* PPase acquires stability by oligomerization of its subunits and subsequent induction of additional hydrogen bonds and ionic interactions. It has also been suggested that *Tth* PPase is more stable than *E. coli* PPase because the C-terminal part of the *Tth* PPase is more resistant to thermal denaturation (*22*). Furthermore, we previously reported the establishment of expression systems for both PPase genes in *E. coli* cells, and characterization of the recombinant enzymes (*17*). On the basis of these insights, we explored the factors contributing to differences in thermostability between the *Tth* and *E. coli* PPases by utilizing random chimeragenesis.

As described above, we obtained four chimeric PPases. Among them, the two chimeric PPases, T1−135E and T1−149E, were fully active and stable. The recombinant region (residues 136−149 in the *Tth* PPase in Figure 1) was highly conserved in both chimeric enzymes. Two chimeras may be stable because the recombined region was in the junction between α-helix A and β-sheet 8 in the *Tth* PPase. These two chimeric PPases were more active than the parent enzymes at the optimum temperature (Table 1). Our results indicate that no critical conformational changes occurred in these chimeric PPases (Table 1 and Figure 2) and that the active sites of the two chimeras are similar to that of *Tth* PPase, on the basis of a similar thermal activation at high temperatures. Furthermore, we found that the thermostability of T1−149E is similar to that of *Tth* PPase, but T1−135E has a much higher thermostability in either the absence or presence of 0.05 (Figures 3 and 4a,b) or 5% SDS (Figure 5). We also found that T1−135E has a much more stable enzyme activity, conformation, and intramolecular interactions, while T1−149E had the same degree of stability as *Tth* PPase.

The difference in the primary structure between T1−135E and T1−149E was only four residues. His138 and Asp141 in T1−135E were substituted with Thr138 and Ala141 in T1−149E, and the other two residues were the deletion of Ala144 and Lys145 in T1−135E. The most remarkable feature of T1−135E was that its activity increased by up to 150% when it was heated to 60−70 °C (Figure 3a). In our previous paper, we found *Tth* PPase has a similar feature (*17*), but T1−135E is more activated than *Tth* PPase. T1−149E exhibited a similar degree of activation as *Tth* PPase. These results suggest that these four residues or the adjacent region may contribute to the heat activation effect. This suggestion is supported by spectroscopic data on conformational changes (Figure 3b). The blue-shifted effect exhibited by T1−135E reflects the environment around the Trp residues after heating, possibly indicating that the Trp147 and Trp153 residues in T1−135E are buried in a more hydrophobic environment after heating at 50−80 °C. The blue-shifted temperature range is similar to the range for heat activation. This suggests that heat activation is related to the environment of the Trp residues, and these four residues may contribute to this effect because these four residues are located in the vicinity of the two Trp residues and affect the active site cavity.

In this study, we analyzed the contribution of Ala144-Lys145 to differences in thermostability between *Tth* and *E. coli* PPases. Ala144 and Lys145 residues in T1−149E and *Tth* PPase are missing from T1−135E and *E. coli* PPase. These two residues reside in the 3_{10}-helix of the *Tth* PPase; in particular, Lys145 from one monomer [denoted as monomer A by Salminen et al. (*22*)] forms a hydrogen bond with Gln130 in another monomer (monomer D), which forms a hydrogen bond with Gln113 in another monomer (monomer C), which forms a hydrogen bond with Leu142 back in monomer A. The hydrogen bonding loop thus is A−D−C−A, joining Leu142 to Lys145 (*21*, *22*). Hence, this region is involved in the network of hydrophilic interactions in this intertrimer. Deletion of these residues in the T1−135E and *E. coli* PPase should induce loss of these interactions. The other residues that are different between T1−135E and T1−149E are His138 and Asp141 in T1−135E, which are substituted with Thr138 and Ala141 in T1−149E. These two residues correspond to Thr138 and Ala141 in *Tth* PPase, and His140 and Asp143 in *E. coli* PPase. Both the *Tth* PPase

and *E. coli* PPase three-dimensional structures (*20*−*22*) suggest that His140 and His136 form a network of intra- and intersubunit interactions in *E. coli* PPase, i.e., a His136′−Asp143−His140 network (prime indicates the residue in another monomer), and the network of interactions was completed by hydrophobic contacts between His140 and His140′ at 3.5 Å. Velichko et al. (*27*) and Baykov et al. (*28*) reported that the replacement of His136 and His140 by Gln resulted in a trimeric enzyme, and His136, His140, and Asp143 involved in α-helix A are important for *E. coli* PPase to remain in the hexameric state. On the other hand, Thr138 in a monomer of the *Tth* PPase forms an intertrimeric hydrophobic contact with Thr138′, and the intertrimeric hydrophilic contact with His134′ in another monomer (*21*, *22*). Additionally, we confirmed that His134 and Thr138 contribute to the hexameric stability in *Tth* PPase by site-directed mutagenesis (unpublished results). In the two chimeras, the positions in T1−135E corresponding to His134 and Thr138 are His138 and Asp141, which are identical with those of *E. coli* PPase. The corresponding residues in T1−149E are Thr138 and Ala141, which are identical with those of *Tth* PPase. Hence, we hypothesize that the quaternary structure and intertrimer interface of T1−135E may be very similar to those of the *E. coli* PPase. We subsequently examined these four PPases by gel filtration to determine whether they all exist as hexamers after heating. We found that T1−135E dissociated into monomers such as *E. coli* PPase after heating at 90 °C, whereas *Tth* PPase aggregated at 90 °C (unpublished data). This heat-induced aggregation was observed only for *Tth* PPase, but unfortunately, we could not determine whether the aggregation was derived from monomers or hexamers. Then, we deduced that these ionic residues (His138 and Asp141) and the deletion of Lys145 in T1−135E may contribute to the intersubunit interactions and thermostability. Although further investigations are needed in these viewpoints, we can conclude that the thermostabilization of T1−135E takes place via four residues between T1−135E and T1−149E, which are located in the vicinity of the trimer−trimer interface, and further that these residues contribute to the structural integrity of the *Tth* PPase moiety.

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REFERENCES

1. Cavagnero, S., Debe, D. A., Zhou, Z. H., Adams, M. W. W., and Chan, S. I. (1998) *Biochemistry 37*, 3369−3376.
2. Qu, C., Akanuma, S., Moriyama, H., Tanaka, N., and Oshima, T. (1997) *Protein Eng. 10*, 45−52.
3. Moriyama, H., Onodera, K., Sakurai, M., Tanaka, N., Kirino-Kagawa, H., Oshima, T., and Katsube, Y. (1995) *J. Biochem. 117*, 408−413.
4. Haack, T., Sanchez, Y. M., Gonzalez, M. J., and Giralt, E. (1997) *J. Pept. Sci. 3*, 299−313.
5. Motoshima, H., Mine, S., Masumoto, K., Abe, Y., Iwashita, H., Hashimoto, Y., Chijiiwa, Y., Ueda, T., and Imoto, T. (1997) *J. Biochem. 121*, 1076−1081.
6. Kvassman, J. O., Lawrence, D. A., and Shore, J. D. (1995) *J. Biol. Chem. 270*, 27942−27947.
7. Kim, J. Y., and Devreoyes, P. N. (1994) *J. Biol. Chem. 269*, 28724−28731.
8. Hachimori, A., Shiroya, Y., Hirato, A., Miyahara, T., and Samejima, T. (1979) *J. Biochem. 86*, 121−130.
9. Cohen, S. A., Sterner, R., Keim, P. S., and Heinrikson, R. L. (1978) *J. Biol. Chem. 253*, 889−897.
10. Stark, M. R., and Milner, J. S. (1989) *Yeast 5*, 35−50.
11. Kawasaki, I., Adachi, N., and Ikeda, H. (1990) *Nucleic Acids Res. 18*, 5888.
12. Kieber, J. J., and Signer, E. R. (1991) *Plant Mol. Biol. 16*, 345−348.
13. Yang, Z., and Wensel, T. G. (1992) *J. Biol. Chem. 267*, 24641−24647.
14. Harutyunyan, E. H., Kuranova, I. P., Vainshtein, B. K., Höhne, W. E., Lamzin, V. S., Dauter, Z., Teplyakov, A. V., and Wilson, K. S. (1996) *Eur. J. Biochem. 239*, 220−228.
15. Heikinheimo, P., Lehtonen, J., Baykov, A., Lahti, R., Cooperman, B. S., and Goldman, A. (1996) *Structure 4*, 1491−1508.
16. Lahti, R., Pitkäranta, T., Valve, E., Ilta, I., Kukko-Kalske, E., and Heinonen, J. (1988) *J. Bacteriol. 170*, 5901−5907.
17. Satoh, T., Samejima, T., Watanabe, M., Nogi, S., Takahasi, Y., Kaji, H., Teplyakov, A., Obmolova, G., Kuranova, I., and Ishii, K. (1998) *J. Biochem. 124*, 79−88.
18. Maruyama, S., Maeshima, M., Nishimura, M., Aoki, M., Ichiba, T., Sekiguchi, J., and Hachimori, A. (1996) *Biochem. Mol. Biol. Int. 40*, 679−688.
19. Ichiba, T., Takenaka, O., Samejima, T., and Hachimori, A. (1990) *J. Biochem. 108*, 572−578.
20. Harutyunyan, E. H., Oganessyan, V. Y., Oganessyan, N. N., Avaeva, S. M., Nazarova, T. I., Vorobyeva, N. N., Kurilova, S. A., Huber, R., and Mather, T. (1997) *Biochemistry 36*, 7754−7760.
21. Teplyakov, A., Obmolova, G., Wilson, K. S., Ishii, K., Kaji, H., Samejima, T., and Kuranova, I. (1994) *Protein Sci. 3*, 1098−1107.
22. Salminen, T., Teplyakov, A., Kankare, J., Cooperman, B. S., Lahti, R., and Goldman, A. (1996) *Protein Sci. 5*, 1014−1025.
23. Hachimori, A., Takeda, A., Kaibuchi, M., Ohkawara, N., and Samejima, T. (1975) *J. Biochem. 77*, 1177−1183.
24. Peel, J. L., and Loughman, B. C. (1957) *Biochem. J. 65*, 709−716.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Mol. Chem. 193*, 265−275.
26. Laemmli, U. K. (1970) *Nature 227*, 680−685.
27. Velichko, I. S., Mikalahti, K., Kasho, V. N., Durarenkov, V. Y., Hyytiä, T., Goldman, A., Cooperman, B. S., Lahti, R., and Baykov, A. A. (1998) *Biochemistry 37*, 734−740.
28. Baykov, A. A., Durarenkov, V. Y., Käpylä, J., Salminen, T., Hyytiä, T., Kasho, V. N., Husgafvel, S., Cooperman, B. S., Goldman, A., and Lahti, R. (1995) *J. Biol. Chem. 270*, 30804−30812.

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