J. Biochem. 129, 955–961 (2001)

Enhancement of the Thermostability of Thermophilic Bacterium PS-3 PPase on Substitution of Ser-89 with Carboxylic Amino Acids[1]

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Received January 9, 2001; accepted March 30, 2001

Serine 89 of the inorganic pyrophosphatase (PPase) subunit from thermophilic bacterium PS-3 (PS-3) was replaced with glycine, alanine, threonine, glutamic acid, or aspartic acid by the PCR-mutagenesis method with Mut-1 in order to determine the contribution of this serine residue to the thermostability and structural integrity of the enzyme molecule. S89G, S89A, and S89T showed reduced catalytic activity, whereas S89D and S89E showed increased enzyme activity. S89G, S89A, and S89T as well as the wild-type PPase were stable in the presence of 5 mM $MgCl_2$ at 70°C for 1 h, but were inactivated rapidly with time at 80°C. On the contrary, S89D and S89E were stable at 80°C, showing more than 95% of the original activity after 1 h incubation. The wild-type PPase, S89D and S89E were each a hexamer before and after incubation at 80°C for 1 h, while S89G and S89A comprised a mixture of a hexamer and a trimer both before and after incubation at 80°C for 1 h. On the other hand, S89T was a mixture of a hexamer, a trimer and a monomer, and it was partially precipitated during heat treatment at 80°C. The CD spectra of the recombinant enzymes in the far-ultraviolet region were the same as that of the wild-type PPase, whereas those of S89G, S89A, and S89T as well as the wild-type PPase were markedly different after heat treatment, although those of S89D and S89E did not change. The present study suggested that local small change(s) in the network of interactions among amino acid residues on replacement at position 89 led to the PS-3 PPase molecule being unable to form a hexamer from trimers or to dissociate into monomers in some cases without a significant change in the backbone conformation. It was also suggested that the partial disordering of the conformation of PS-3 PPase caused by heat depended on the degree of hydrophilicity in the vicinity of position 89.

Key words: inorganic pyrophosphatase, polymerase chain reaction, recombinant enzyme, thermophilic bacterium PS-3, thermostability.

Inorganic pyrophosphatase [EC 3.6.1.1] specifically catalyzes the hydrolysis of pyrophosphate to orthophosphate. This reaction provides a thermodynamic pull for many biosynthetic reactions (*1–3*) and is essential for life (*4–6*). PPases require bivalent metal ions for catalysis, with Mg^{2+} conferring the highest activity. Based on their primary structures, soluble PPases can be divided into two families which exhibit no sequence similarity to each other. Family I includes most of the currently known PPases, and Family II includes the PPases of *Bacillus subtilis* and *Methanococcus jannaschii* as well as the putative PPases of three other bacterial strains (*7–10*). Family I can be further divided into prokaryotic, plant and animal/fungal PPases. The best-studied PPases are those from *Escherichia coli* (*Eco*) and *Saccharomyces cerevisiae* in Family I, which have been extensively characterized by X-ray crystallography (*11–15*) and site-directed mutagenesis in combination with kinetic and thermodynamic measurements (*16–22*).

We previously determined, through studies involving Edman degradation (*23*), and cloning and expression of the gene (*24*), the primary structure of the PPase from thermophilic bacterium PS-3, which exhibits high sequence identity, including the evolutionarily well conserved active site residues, with the family I PPases (*7*). PS-3 PPase becomes a hexamer derived from trimers in the presence of Mg^{2+} (*25*). A hexamer expresses its full activity although a trimer may have very weak activity (*26*). Furthermore, PS-3 PPase becomes thermostable only in the presence of divalent cations (>1 mM), the stability being accompanied by a conformational change that was detected on the basis of the difference spectrum and circular dichroism (*25*).

We also found, through a study involving site-directed mutagenesis, that the newly formed interchain hydrogen bonds due to the trimer-trimer interaction (*26*) in the presence of Mg^{2+} (>1 mM) partially contribute to the thermo-

[1] This work was partially supported by a Grant-in-Aid, 10650782, for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: PPase, inorganic pyrophosphatase; PS-3, thermophilic bacterium PS-3; *Eco*, *Escherichia coli*; *Bst*, *Bacillus stearothermophilus*; *Tth*, *Thermus thermophilus*; CD, circular dichroism.

stability of PS-3 PPase. Teplyakov *et al.* (*27*) and Leppanen *et al.* (*28*) also found on X-ray crystallographic analysis that the tightly packed hexameric structure increasing the surface area of the trimer–trimer interface, which is characteristic of the *Thermus thermophilus* (*Tth*) and *Sulfolobus acidocaldarius* PPases, is important for the thermostability of these two highly thermostable PPases. Shinoda *et al.* (*29*) found that the hydrophobic interaction at the intratrimer interface also contributes to the thermostability of the PPase from *Bacillus stearothermophilus* (*Bst*).

Querol *et al.* (*30*) and Vogt *et al.* (*31*) have listed many other physical and chemical reasons, including better hydrogen bonding, better hydrophobic internal packing, enhanced secondary structure propensity, *etc.*, that researchers have reported in order to explain enhanced thermostabilization of proteins. Furthermore, Vogt *et al.* (*31*) pointed out that many of these structural properties can be attributed in whole or at least in significant part to increased hydrogen bonding to water molecules or amongst protein atoms. They found that an increased temperature of stabilization was related to an increase in the number of hydrogen bonds amongst protein atoms and an increase in fractional polar atoms exposed on the surface able to provide more hydrogen bonds with a solvent, on the analysis of 16 families of proteins with different thermal stabilities, the three-dimensional structures of sequence-homologous members of which are known.

On comparison of the primary structures of 37 PPases so far revealed (*7*), it was found that the amino acid residue corresponding to Ser-89 in PS-3 PPase is also serine in some other thermophilic PPases from *B. stearothermophilus*, *Thermococcus litoralis*, *Pyrococcus horikoshi,* and *Mycoplasma thermoautotorophicum*, whereas it is glutamic acid or aspartic acid in most of the PPases from bacteria, fungi, plants, and animals. Glu-98 in the *E. coli* PPase and the corresponding Ser-89 in the PS-3 PPase are located at the edge of the active site pocket on the surface of the protein molecule, as judged on X-ray crystallography (*11*) and computer analysis of the three-dimensional structures on homology modeling (*26*), respectively. Since the polarity of the side group of Ser is far smaller than that of those of Glu and Asp, the polarity and hydrophilicity in the vicinity of Ser-89 in the PS-3 PPase must be reduced compared with that in other mesophilic PPases, which contradicts the conclusion of Vogt *et al.*

In the present study, site-directed mutagenesis was conducted on PS-3 PPase to replace the serine residue at position 89 with glycine, alanine, threonine, glutamic acid, or aspartic acid to determine the contribution of the polar nature of the amino acid on the surface of the molecule to the thermostability.

MATERIALS AND METHODS

Materials—Restriction endonucleases, T4 DNA polymerase, Taq polymerase, and a DNA ligation kit were purchased from Takara Shuzo. Antibiotics, egg-white lyzozyme, ribonuclease A, and low-gelling-temperature agarose (type S) were obtained from Nippon Gene. [α-^{32}P]dCTP (3,000 Ci/mmol) and a DNA sequencing kit were obtained from Amersham. A Gene Clean II kit was obtained from Bio 101. Phenyl-Sepharose CL4B, and TSK-gel Phenyl-5PW and TSK-gel G3000 SW were purchased from Pharmacia and Tosoh, respectively. All other chemicals were of analytical grade.

Mutagenesis—Oligonucleotide-directed mutagenesis was performed by the PCR-mutagenesis method with MUT 1 (Takara Shuzo) as the mutagenesis primer *in vitro*. The oligonucleotides used as primers were as follows: 5′-TTC TTC ACC GCC GTC GAC -3′(S89G), 5′-TTC TTC ACC GGC GTC GCA-3′(S89A), 5′-TC TTC ACC GGT GTC GAC C-3′(S89T), 5′-TTC TTC ACC TTC GTC GAC C3′(S89E), and 5′-TC TTC ACC GTC GTC GAC-3′(S89D), the underlined nucleotides being the mutated codons. A plasmid, pTTP3, containing the intact thermophilic bacterium PS-3 [Thermophilic bacterium PS-3 was described in *J. Biol. Chem.* **250**, 7911–7916 (Yoshida, Y., et al).] *ppa* gene (*24*) was used as the template for the *ppa* gene manipulated in this work. pUC 118 was used as a vector, and JM109 as a host for mutagenesis. Sequencing by the dideoxy chain-termination method (*32*) was performed to check the mutations.

Purification of PPases—E. coli JM109 cells expressing the wild-type and variant PS-3 PPases were obtained by inoculating 1 liter of LB medium containing 80 μM isopropyl β-D-thiogalactoside and 50 μg/ml ampicillin with 2 ml of an overnight culture of the respective cells. Crude extracts were obtained from the respective cells by grinding with Al_2O_3 and extraction with 20 mM Tris-HCl buffer, pH 8.0, containing 5 mM $MgCl_2$. All the wild-type and variant PPases were purified to an electrophoretically homogeneous state according to the procedure reported previously (*26*). Samples were concentrated to approx. 10 mg/ml and then stored at −80°C.

Enzyme Assay—PPase activity was determined by the procedure described previously (*33*). The enzyme activity was expressed as k_{cat} (= V_{max}/[E]). The protein concentration in a solution was determined with a Pierce bicinchromic acid protein assay kit, with BSA as the standard.

Molecular Weight Estimation—The molecular mass of PPase was determined by gel filtration. The protein solution (0.1 mg of protein in 100 μl) was applied to a HPLC column of TSK-gel G3000SW (0.75 cm × 30 cm), and then eluted with a buffer comprising 50 mM Tris-HCl, pH 8.0, in the presence of 5 mM $MgCl_2$ at the flow rate of 0.75 ml/min. The molecular mass markers used were as follows; aldolase (158 kDa), bovine serum albumin (68 kDa), egg albumin (43 kDa), and trypsin inhibitor (23 kDa).

CD Spectroscopy—CD spectra were obtained with a Jasco J-600 automatic recording dichrograph at room temperature. A cell of 0.1 mm path length was used for the measurements in the far-UV region. CD data were expressed in terms of mean residue ellipticity, [θ], using the mean residue molecular mass of 114.6 calculated from the amino acid sequence (*24*).

RESULTS

Effects of Replacements on the Enzyme Activity—The k_{cat} values of the wild-type and mutant PPases at pH 8.0 are shown in Table I. The enzyme activities of S89G and S89A were only half that of the wild type PPase, and the K_M values of these variants were 10 times higher than that of the wild-type PPase, which resulted in considerable decreases in their catalytic efficiencies (k_{cat}/K_M). The effect of replacement with threonine, the same kind of amino acid as serine, was more marked, and both the k_{cat} and catalytic

efficiency decreased more considerably. On the contrary, the replacements with glutamic acid and aspartic acid enhanced the enzyme activity. The K_M value of S89E, however, was twice that of the wild-type PPase, which resulted in a slight decrease in the catalytic efficiency, whereas the K_M value of S89D was not affected by the replacement.

The enzyme activities of the wild-type and variant PPases were measured at various pHs, from 5.5 to 10.0, as shown in Fig. 1. The wild-type PPase showed the maximum activity between pH 7.5 and 9.0, and the activity decreased rapidly outside this pH range, as reported for the authentic PPase from PS-3 cells (*25*). Most of the variants showed a narrower pH-activity profile on the acidic side than that of the wild-type PPase. In particular, S89G showed a very sharp pH-activity profile; the maximum activity was observed at pH 8.0 and it decreased abruptly on both sides.

Effects of Replacements on the Thermostability—The wild-type PPase as well as the authentic enzyme from PS-3 cells (*25, 26*) was very stable up to 70°C in the presence of 5 mM $MgCl_2$ when incubated at the protein concentration of 0.1 mg/ml at pH 8.0, but its activity decreased rapidly with time during incubation beyond 80°C. To examine the effects of the replacements on the thermostability of the PS-3 PPase, the wild-type and variant PPases were incubated at 80°C at protein concentrations of 1 and 0.1 mg/ml in 50 mM Tris-HCl buffer, pH 8.0 (50 mM Tris-HCl, pH 8.73, at 25°C showed pH 8.0 at 80°C), containing 5 mM $MgCl_2$, and the enzyme activity remaining was measured at 37°C at various times. The results are shown in Fig. 2. The thermostabilities of the wild-type and variant PPases were dependent upon the protein concentration. At the protein concentration of 1 mg/ml, S89D and S89E were very thermostable at 80°C; no loss of the activity was observed after 1 h incubation. However, the wild-type PPase exhibited 70%, and S89A, S89G and S89T 30% of the original activity after 1 h incubation. These differences in thermostability were more obvious at lower protein concentration (0.1 mg/ml). The wild type PPase showed only 19% of the original activity even in the presence of 5 mM $MgCl_2$ after 1 h incubation at 80°C. S89G, S89A, and S89T behaved like the wild-type PPase as to temperature. On the other hand, S89E and S89D were very stable and they still showed more than 95% of the original activity after 1 h incubation at 80°C in the presence of 5 mM $MgCl_2$. Their activities decreased rapidly with time during incubation at a temperature over 85°C.

Effects of Replacements on the Subunit Assembly—We previously found, in a study on its sedimentation coefficient, that PS-3 PPase exists as a trimer in the absence of Mg^{2+}, and as a hexamer in the presence of Mg^{2+} (*25*). Thus, the effects of replacement of the serine with glycine, alanine, threonine, glutamic acid, and aspartic acid on the

TABLE I. **Rate constants for the wild-type and variant PPases.**

Strain	k_{cat} (S^{-1})	K_M (mM)	k_{cat}/K_M ($S^{-1}\cdot mM^{-1}$)
Wild-type	817.0	0.47	1,738.3
S89G	535.0	6.70	79.8
S89A	496.3	3.30	150.4
S89T	250.0	5.00	50.0
S89E	968.7	0.74	1,309.1
S89D	1,037.7	0.47	2,207.8

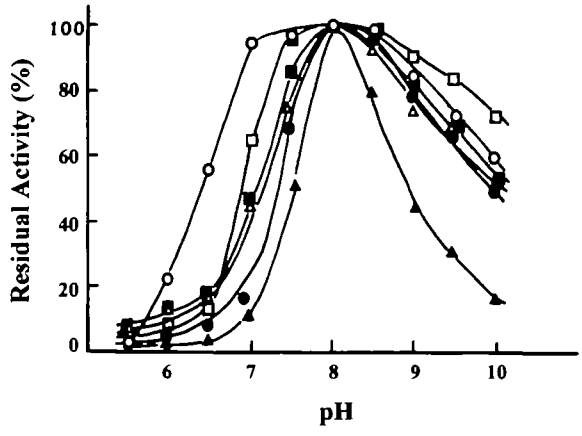

Fig. 1. **Effect of pH on the catalytic activities of the wild-type and variant PPases from thermophilic bacterium PS-3.** The activity of each enzyme at pH 8.0, at which all the PPases showed the maximum activity, was taken as 100%. The buffers used were 2-(*n*-morpholino)-ethane sulfonic acid (pH 5.5–7.0), Tris-HCl (pH 7.2–8.8), and glycine-NaOH (pH 9.0–10.5). ○, wild-type PPase; □, S89A; ■, S89T; ▲, S89G; ●, S89D; △, S89E.

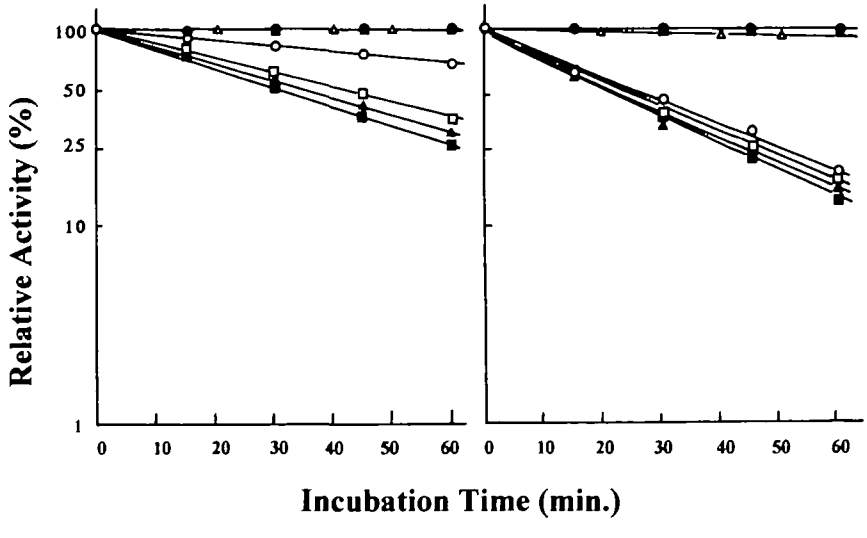

Fig. 2. **Thermal inactivation of the wild-type and variant PPases from PS-3.** The enzymes (A:1 mg/ml, B:0.1 mg/ml) in 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$ were incubated at 80°C. Aliquots were withdrawn at the indicated times and assayed at 37°C. The enzyme activity without heating was taken as 100%. The symbols used are the same as in Fig. 1.

subunit assembly before and after incubation at 80°C for 1 h at a protein concentration of 1 mg/ml in the presence of 5 mM $MgCl_2$ were examined by measuring the molecular mass by HPLC-gel chromatography. The results are shown in Fig. 3. The wild-type PPase in the presence of 5 mM $MgCl_2$ was a hexamer as reported, and incubation at 80°C for 1 h did not affect the elution pattern of the wild-type PPase, except that a tiny amount of monomer was observed. S89D and S89E both before and after 1 h incubation at 80°C were also eluted at the same retention time as a hexamer, like the wild-type PPase. On the contrary, S89G and S89A before incubation gave two protein peaks on elution, one for a hexamer and the other for a trimer, and a small amount of monomer was detected for both variants after 1 h incubation at 80°C. S89T gave three peaks on elution, corresponding to a hexamer, a trimer, and a monomer, respectively. During heat treatment at 80°C for 1 h, a small amount of precipitate was observed for S89T. Thus, the heat-treated S89T was applied to the HPLC-column after centrifugation, and it was found that the monomer peak was markedly decreased and the trimer peak slightly decreased, although the hexamer peak did not change, indicating that the monomer was easily precipitated during heat treatment.

CD Spectra—In order to determine the effects of replacements on the protein conformation, CD spectra of the wild-type and variant PPases were obtained at room temperature in the far-ultraviolet region before and after incubation at 80°C for 1 h at the protein concentration of 1 mg/ml in Tris-Hcl buffer, pH 8.0 (calibrated at 80°C), containing 5 mM $MgCl_2$. The wild-type and variant PPases all gave similar CD spectra in the far-ultraviolet region before heat treatment (Fig. 4A). As shown in Fig. 4B, S89E and S89D after heat treatment also gave identical spectra with those before heat treatment. The CD spectrum of the wild type PPase after heat treatment was slightly decreased. On the other hand, the CD spectra of S89G, S89A, and S89T, whose activities were markedly decreased, after 1 h incuba-

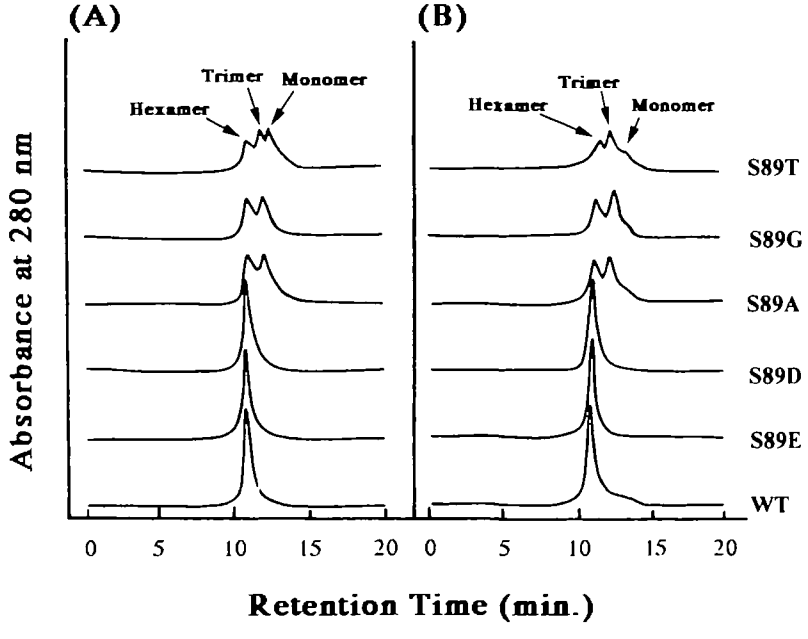

Fig. 3. **HPLC-gel chromatography elution profiles of the wild-type PS-3 and variant PPases before (A) and after (B) 1 h incubation at 80°C in 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$ at the protein concentration of 1 mg/mL**

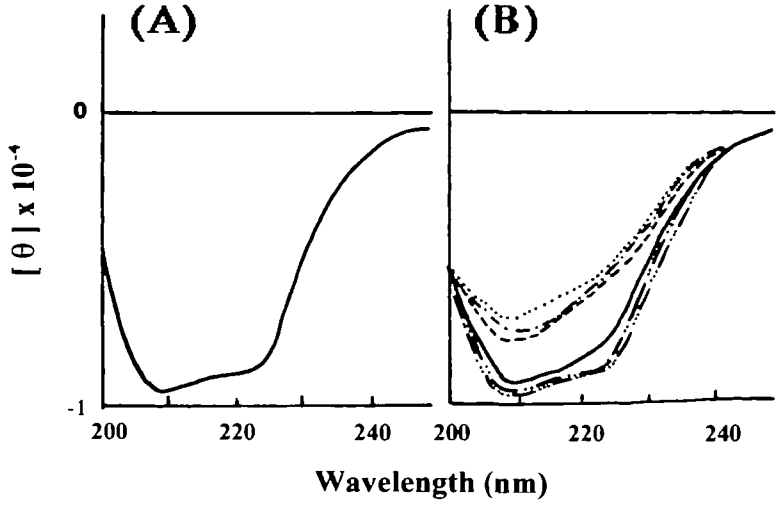

Fig. 4. **CD spectra of the PS-3 wild-type and variant PPases before (A) and after (B) 1 h incubation at 80°C in the presence of 5 mM $MgCl_2$.** The protein concentration was 1 mg/ml, and the solvent used was 50 mM Tris-HCl containing 5 mM $MgCl_2$. All the variants showed the same CD spectra as that of the wild-type PPase before the incubation. ——, wild-type PPase; ·····, S89A; – – –, S89T; ——, S89G; — —, S89D; —·—, S89E.

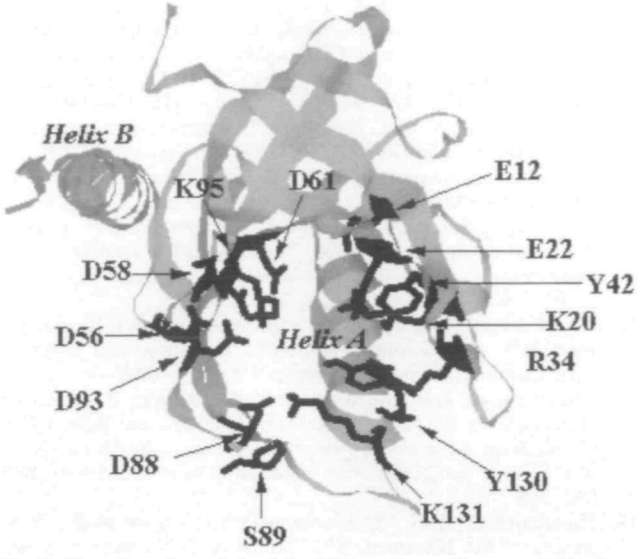

Fig. 5. **Structural model of the PPase from thermophilic bacterium PS-3.** A model of PS-3 PPase was constructed by homology modeling with the program package QUANTA/CHARMm. The X-ray structure of the Tth PPase served as a Scaffold (*27*). Ser-89 and the 13 putative active site residues are denoted in bold type. Helix A is part of the trimer–trimer interface.

tion at 80°C had changed considerably. These observations indicated that the replacement of Ser-89, and the resulting partial inability to form a hexamer and dissociation into monomers from a trimer did not affect their secondary structures. However, the heat treatment at 80°C destroyed the secondary structure of the trimer and the monomer, although the backbone structure of the hexamer was not affected profoundly.

DISCUSSION

Figure 5 shows the three-dimensional structure of PS-3 PPase, predicted by computer analysis on homology modeling with the QUANTA/CHARMm program (Molecular Simulation) using the crystallographic data for *Tth* PPase as a scaffold (*27*). The locations of Ser-89 and 13 putative active site residues are also illustrated. Ser-89 is located at the edge of the active site pocket on the surface of the molecule. Shinoda *et al.* reported that Arg-27–Ser-38, Thr-66–Asp-77, and Pro-100–Arg-105 in *Bst* PPase might be intratrimer regions, based on comparison of its primary structure with those of *Tth* and *Eco* PPases (*29*). The primary structure of PS-3 PPase is completely the same as that of *Bst* PPase except that the former has two more amino acids at the C-terminus (*34*). We confirmed the importance of Pro-72 in this region for the formation of a trimer from monomers (unpublished data). We also previously reported that Helix A, as shown in Fig. 5, is one of the trimer-trimer interface regions in PS-3 PPase (*26*). In *Eco* PPase, in addition to Helix A, Asn-24 and Asp-26, which correspond to Gly-14 and Gln-16 in PS-3 PPase, also participate in the trimer-trimer interface through hydrogen bonds with water molecules or Mg^{2+} (*12*, *35*). Such observations may rule out the direct participation of Ser-89 in PS-3 PPase in either the trimer-trimer or intratrimer interface.

Thus, the replacement of Ser-89 with other amino acid residues, which is located next to one of the putative active site residues, Asp 88, would be expected to affect Asp 88, and consequently cause distortion of the arrangement of the active site pocket leading to a decrease in the enzyme activity. The present study, however, indicated that the hydrophilic and polar nature of the Ser-89 side group may play a more important role in maintenance of the structural integrity of the protein molecule, as judged from the results of measurement of the subunit assembly by HPLC.

The replacement of Ser-89 with more hydrophobic and less polar amino acids, glycine and alanine, partially suppressed the formation of a hexamer from trimers in the presence of Mg^{2+}, indicating that the OH group of Ser-89 may play a very important role in the assembly of trimers. The lack of a OH group in S89G and S89A might cause the abolition of the interaction(s) with other amino acid residue(s), which might influence the arrangement of adjacent Helix A.

The replacement of Ser-89 with Thr, whose hydrophilicity and polarity are almost the same as those of Ser, had a more significant influence on the subunit assembly; a large amount of monomer was observed in addition to a hexamer and a trimer. The steric interference caused by the methyl group of a threonine side chain might more seriously affect the interactions among amino acid residues around position 89, which influences in turn the arrangement of not only adjacent Helix A but also some amino acid residue(s) at the intratrimer interface(s).

On the contrary, the replacement of Ser-89 with Glu and Asp had no influence on the subunit assembly in the presence of Mg^{2+}; those subunits become a hexamer with increasing concentration of Mg^{2+} up to 1 mM like the authentic PPase from cells (*25*), judging from the results of CD measurement at 293 nm and HPLC gel filtration. Since Ser-89 does not participate in the trimer-trimer interface, it is hard to consider that the replacement of serine with glutamic acid or aspartic acid strengthened the trimer-trimer interaction *via* metal ions. They may more tightly interact with the surrounding amino acid residues, to maintain the correct enzyme structure, than serine does, since the hydrophilicities and polarities of these two carboxylic amino acid residues are much higher than those of serine.

Judging from the results as to subunit assembly, the enzyme activities of the variants seemed to be almost proportional to the amount of hexamers they could form in the presence of Mg^{2+}. This well agreed with our previous finding that PS-3 PPase expresses its full activity in the hexamer form (*25*), although a trimer may have very weak activity, as confirmed for Eco PPase (*35*).

The thermostabilities of the wild-type and variant PPases were dependent upon the protein concentration. When the thermostability was examined by measuring the remaining activity after heat treatment at 80°C at the protein concentration of 1 mg/ml in the presence of 5 mM $MgCl_2$, S89D and S89E were found to be thermostable; no loss of the activity was observed after 1 h incubation at 80°C. However, the wild-type PPase was exhibited 70%, and S89A or S89G 30% of the original activity after 1 h incubation at 80°C. These differences in thermostability were more obvious at lower protein concentration (0.1 mg/ml). S89D and S89E were still thermostable, whereas the rest of the variants as well as the wild-type PPase were

rapidly inactivated with time at 80°C. We previously reported the contribution of the trimer-trimer interaction to the thermostability of PS-3 PPase (*26*). However, this may not have been the case in the present study, because HPLC-gel chromatography of the wild-type and all variant PPases indicated that the inability to form a hexamer from trimers or the dissociation of a hexamer into trimers and monomers scarcely occurred during heat treatment. Since the hexamer mainly expressed the full enzyme activity, as stated above, the reductions in the enzyme activities of the wild-type and some variant PPases during heat treatment at 80°C might be ascribed to the nature of the amino acid at position 89, as reported by Vogt *et al.* (*30*); a polar atom with high hydrophilicity on the surface seems to be necessary for increased thermostabilization of a protein. A hydropathy plot for the wild-type and variant PPases with the GENETYX program indicated that the hydrophilicity in the vicinity of position 89 is in the order of S89D = S89E ≫ Wild-Type PPase = S89T > S89G > S89A.

The present study suggested that Ser-89 in PS-3 PPase is located at a critical position for maintaining the structural integrity of the protein molecule, and also suggested that the correct overall structure of PS-3 PPase, which is maintained by many small interactions among amino acid residues, is easily disrupted even by local small change(s) in the network without a significant change in the backbone conformation.

While most of the PPases so far known have a aspartic acid or glutamic acid at the position corresponding to position 89 of PS-3 PPase, it is unknown at present why PPases from PS-3 as well as three other thermophilic bacteria have a serine residue here that decreases the thermostability.

REFERENCES

1. Kornberg, A. (1962) On the metabolic significance of phosphorolytic and pyrophosphorolytic reactions in *Horizons in Biochemistry* (Kasha, H. and Pullman, P., eds.) pp. 251–264, Academic Press, New York
2. Lahti, R. (1983) Microbial inorganic pyrophosphatase. *Microbiol. Rev.* **47**, 169–179
3. Baykov, A.A., Cooperman, B.S., Goldman, A., and Lahti, R. (1999) Cytoplasmic inorganic pyrophosphatase. *Prog. Mol. Subcell. Biol.* **23**, 127–150
4. Chen, J., Brevet, A., Fromant, M., Leveque, F., Schmitter, J.-M., Blanquet, S., and Plateau, P. (1990) Pyrophosphatase is essential for growth of *Escherichia coli*. *J. Bacteriol.* **172**, 5686–5689
5. Lundin, M., Baltscheffsky, H., and Ronne, H. (1991) Yeast PPA2 gene encodes a mitochondrial inorganic pyrophosphatase that is essential for mitochondrial function. *J. Biol. Chem.* **266**, 12168–12172
6. Sonnewald, U. (1992) Expression of *E. coli* inorganic pyrophosphatase in transgenic plants alters photo-assimilate partitioning. *Plant J.* **2**, 571–581
7. Sivula, T., Salminen, A., Parfenyev, A.N., Pohjanjoki, P., Goldman, A., Cooperman, B.S., Baykov, A.A., and Lahti, R. (1999) Evolutionary aspect of inorganic pyrophosphatase. *FEBS Lett.* **454**, 75–80
8. Shintani, T., Uchiumi, T., Yonezawa, T., Salminen, A., Baykov, A.A., Lahti, R., and Hachimori, A. (1998) Cloning and expression of a unique inorganic pyrophosphatase from *Bacillus subtilis*: evidence for a new family of enzymes. *FEBS Lett.* **439**, 263–266
9. Young, T.W., Kuhn, N.J., Wadeson, A., Ward, S., Burges, D. and Cooke, G.D. (1998) *Bacillus subtilis* ORF yybQ encodes a manganese-dependent inorganic pyrophosphatase with distinctive properties: the first of a new class of soluble pyrophosphatase? *Microbiology* **144**, 2563–2571
10. Kuhn, N.J., Wadeson, A., Ward, S., and Young, T.W. (2000) *Methanococcus jannaschii* ORF mj0608 codes for a class C inorganic pyrophosphatase protected by Co^{2+} or Mn^{2+} ions against fluoride inhibition. *Arch. Biochem. Biophys.* **379**, 292–298
11. Kankare, J., Neal, G.S., Salminen, T., Glumoff, T., Cooperman, B.S., Lahti, R., and Goldman, A. (1994) The structure of *E. coli* soluble inorganic pyrophosphatase at 2.7 Å resolution. *Protein Eng.* **7**, 823–830
12. Kankare, J., Salminen, T., Lahti, R., Cooperman, B.S., Baykov, A.A., and Goldman, A. (1996) Crystallographic identification of metalbinding sites in *Escherichia coli* inorganic pyrophosphatase. *Biochemistry* **35**, 4670–4677
13. Heikinheimo, P., Lehtonen, J., Baykov, A.A., Lahti, R., Cooperman, B.S., and Goldman, A. (1996) The structural basis for pyrophosphatase catalysis. *Structure* **4**, 1491–1508
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) X-ray structure of yeast inorganic pyrophosphatase complexed with manganese and phosphate. *Eur. J. Biochem.* **239**, 220–228
15. Harutyunyan, E.H., Oganessyan, V.Yu., Oganessyan, N.N., Avaeva, S.M., Nazarova, T.I., Vorobyeva, N.N., Kurilova, S.A., Huber, R., and Mather, T. (1997) Crystal structure of holo inorganic pyrophosphatase from *Escherichia coli* at 1.9 Å resolution. Mechanism of hydrolysis. *Biochemistry* **36**, 7754–7760
16. Salminen, T., Käpylä, J., Heikinheimo, P., Kankare, J., Goldman, A., Heinonen, J., Baykov, A.A., Cooperman, B.S., and Lahti, R. (1995) Structure and function analysis of *Escherichia coli* inorganic pyrophosphatase: is a hydroxide ion the key to catalysis? *Biochemistry* **34**, 782–791
17. Pohjanjoki, P., Lahti, R., Goldman, A. and Cooperman, B.S. (1998) Evolutionary conservation of enzymatic catalysis: quantitative comparison of the effects of mutation of aligned residues in *Saccharomyces cerevisiae* and *Escherichia coli* inorganic pyrophosphatases on enzymatic activity. *Biochemistry* **37**, 1754–1761
18. Käpylä, J., Hyytiä, T., Lahti, R., Goldman, A., Baykov, A.A., and Cooperman, B.S. (1995) Effect of D97E substitution on the kinetic and thermodynamic properties of *Escherichia coli* inorganic pyrophosphatase. *Biochemistry* **34**, 792–800
19. Volk, S.E., Dudarenkov, V.Y., Käpylä, J., Kasho V.N., Voloshina, O.A., Salminen, T., Goldman, A., Lahti, R., Baykov, A.A., and Cooperman, B.S. (1996) Effect of E20D substitution in the active site of *Escherichia coli* inorganic pyrophosphatase on its quaternary structure and catalytic properties. *Biochemistry* **35**, 4662–4669
20. Avaeva, S., Rodina, E., Kurilova, S.A., Nazarova, T.I., and Vorobyeva, N.N. (1996) Effect of D42N substitution in *Escherichia coli* inorganic pyrophosphatase on catalytic activity and Mg^{2+} binding. *FEBS Lett.* **392**, 91–94
21. Avaeva, S., Ignatov, P., Kurilova, S.A., Nazarova, T.I., Rodina, E., Vorobyeva, N., Oganessyan, V. and Harutyunyan, E. (1996) *Escherichia coli* inorganic pyrophosphatase: site-directed mutagenesis of the metal binding sites. *FEBS Lett.* **399**, 99–102
22. Fabrichniy, L., Kasho, V., Hyytiä, T., Salminen, T., Halonen, P., Dudarenkov, V., Heikinheimo, P., Chernyak, V., Goldman, A., Lahti, R., Cooperman, B.S., and Baykov, A.A. (1997) Structural and functional consequences of substitutions at the tyrosine 55-lysine 104 hydrogen bond in *Escherichia coli* inorganic pyrophosphatase. *Biochemistry* **36**, 7746–7753
23. Ichiba, T., Takenaka, O., Samejima, T., and Hachimori, A. (1990) Primary structure of the inorganic pyrophosphatase from thermophilic bacterium PS-3. *J. Biochem.* **108**, 572–578
24. Maruyama, S., Maeshima, M., Nishimura, M., Aoki, M., Ichiba, T., Sekiguchi, J., and Hachimori, A. (1996) Cloning and expression of the inorganic pyrophosphatase gene from thermophilic bacterium PS-3. *Biochem. Mol. Biol. Int.* **406**, 79–88
25. Hachimori, A., Shiroya, Y., Hirato, A., Miyahara, T. and Samejima, T. (1979) Effects of divalent cations on thermophilic inorganic pyrophosphatase. *J. Biochem.* **86**, 121–130
26. Aoki, M., Uchiumi, T., Tsuji, E., and Hachimori, A.(1998)

Effects of replacement of His-118, His-125 and Trp-143 by alanine on the catalytic activity and subunit assembly of inorganic pyrophosphatase from thermophilic bacterium PS-3. *Biochem. J.* **331**, 143–148
27. Teplyakov, A., Obmolova, G., Wilson, K.S., Ishii, K., Kaji, H., Samejima, T., and Kuranova, I. (1994) Crystal structure of inorganic pyrophosphatase from *Thermus thermophilus*. *Protein Sci.* **3**, 1098–1107
28. Leppanen, V.M., Nummelin, H., Hansen, T., Lahti, R., Schäfer, G., and Goldman, A. (1999) *Sulfolobus acidcaldarius* inorganic pyrophosphatase: structure, thermostability, and effect of metal ion in an archael pyrophosphatase. *Protein Sci.* **8**, 1218–1231
29. Shinoda, H., Hattori, M., Shimizu, A., Samejima, T., and Satoh, T. (1999) Hydrophobic interactions of Val-75 are critical for oligomeric thermostability of inorganic pyrophosphatase from *Bacillus stearothermophilus*. *J. Biochem.* **125**, 58–63
30. Querol, E., Perez-Pons, J.A., and Mozo-Villarias, A. (1996) Analysis of protein conformational characteristics related to thermostability. *Protein Eng.* **9**, 265–271
31. Vogt, G., Woell, S., and Argos, P. (1997) Protein thermal stability, hydrogen bonds, and ion pairs. *J. Mol. Biol.* **269**, 631–643
32. Sanger, F., Nicklen, S., and Coulson, A.R. (1981) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467
33. Hachimori, A., Takeda, A., Kaibuchi, M., Ohkawara, N., and Samejima, T. (1975) Purification and characterization of inorganic pyrophosphatase from *Bacillus stearothermophilus*. *J. Biochem.* **77**, 1177–1183
34. Satoh, T., Shinoda, H., Ishii, K., Koyama, M., Sakurai, N., Kaji, H., Hachimori, A., Irie, M., and Samejima, T. (1999) Primary structure, expression, and site-directed mutagenesis of inorganic pyrophosphatase from *Bacillus stearothermophilus*. *J. Biochem.* **125**, 48–57
35. Baykov, A.A., Dudarenkov, V.Y., Käpylä, J., Salminen, T., Hyytiä, T., Kasho, V.N., Husgafvel, S., Cooperman, B.S., Goldman, A., and Lahti, R. (1995) Dissociation of hexameric *Escherichia coli* inorganic pyrophosphatase into trimers on His-146 → Gln or His-140 → Gln substitution and its effect on enzyme catalytic properties. *J. Biol. Chem.* **270**, 30804–30812