Effect 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

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Each of two histidine residues and one tryptophan residue in thermophilic bacterium PS-3 inorganic pyrophosphatase (PPase) was replaced by alanine. The activities of the H125A and W143A variants decreased to one-fifth, whereas the activity of H118A remained unaltered. CD spectra in the near-UV region indicated that the conformations of the first two variants changed with the substitution. In contrast with wild-type PPase, which is hexameric beyond an enzyme concentration of 0.1 μ M in the presence of Mg^{2+} , the H118A and H125A variants cannot be assembled from trimers into hexamers at less than an enzyme

concentration of 10 μ M even at a higher concentration of Mg^{2+} . In particular, H118A was irreversibly inactivated in a diluted state. In contrast, the enzyme concentration dependence of W143A PPase activity was almost the same as that of wild-type PPase. These results indicated that His-118 and His-125 are important for both trimer–trimer interaction and structural integrity, whereas Trp-143 is important structurally. The trimer–trimer interaction is absolutely necessary for the thermostability of the PS-3 enzyme.

INTRODUCTION

Alignment of the sequences of several soluble inorganic pyrophosphatases (PPases; EC 3.6.1.1) that have so far been studied indicated that the active-site residues are very well conserved, even though the overall level of sequence similarity is low [1]. All the polar residues located in the active-site cavity of the *Escherichia coli* and *Saccharomyces cerevisiae* PPases have been replaced [2–4]; some of the variant PPases have been characterized in detail [5–10]. Furthermore, the three-dimensional structures of the PPases that have so far been revealed were found to be similar in spite of few sequence similarities beyond the evolutionarily conserved residues of the active site and different oligomeric organizations [11–14].

We previously determined, through studies involving Edman degradation [15] and cloning and expression of the gene [16], the primary structure of PPase from thermophilic bacterium PS-3, which exhibits high sequence identity, including the well-conserved active-site residues, with the *E. coli* [17], *Thermus thermophilus* [13] and *Thermoplasma acidophilum* [18] enzymes. Earlier chemical modification studies on PS-3 PPase implied that His-118 and Trp-143 were involved in the active site or were located near the active site [19,20]. However, neither His-118 nor Trp-143 is one of the evolutionarily conserved residues of the active site, indicating that His-118 and Trp-143 have no direct role in catalysis and that the chemical modification is an indirect effect. Baykov et al. [21] reported that replacement of His-136 and His-140 with glutamine in *E. coli* PPase, the former corresponding to His-125 in the primary structure of PS-3 PPase, results in an enhancement of the catalytic activity in both the hydrolytic and synthetic directions owing to increases in the rate constants governing most of the catalytic steps. They also found that these variants dissociate readily from hexamers into trimers,

indicating that His-136 and His-140 are key residues at the trimer–trimer interface.

These observations prompted us to replace His-118 and His-125 with Ala in PS-3 PPase, to elucidate the roles of these residues in subunit–subunit interaction, because PS-3 PPase exists as a trimer in the absence of Mg^{2+} and assembles into a hexamer on addition of Mg^{2+} [22]. The replacement of Trp-143 with Ala in PS-3 PPase was also performed.

EXPERIMENTAL

Mutagenesis

Oligonucleotide-directed mutagenesis was performed by the PCR-mutagenesis method with MUT 1 (Takara Shuzo Co.) as the mutagenesis primer *in vitro*. The oligonucleotides used as primer 1 were as follows: 5'-TCAGTTTGGCCTGCGGG-3' (H118A), 5'-CAAAGAAGGCGCGATT-3' (H125A), and 5'-GCCCTCCGCTGTGCC-3' (W143A). The mutated wild-type codons encoding histidine and tryptophan are underlined. A plasmid, pTPP3, containing the intact PS-3 *ppa* gene [16], was used as the donor of the *ppa* gene manipulated in this work. pUC 118 was used as a vector for the mutagenesis. JM109 was used as a host for the mutagenesis. The mutations were checked by sequencing with the dideoxy chain-termination method [23].

Purification of PPases

E. coli JM109 cells expressing the wild-type and variant PPases were produced by inoculating 1 litre of Luria–Bertani medium containing 80 μ M isopropyl β -D-thiogalactoside and 50 μ g/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,

Abbreviations used: PPase, inorganic pyrophosphatase; PS-3, thermophilic bacterium PS-3.

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Figure 1 Structural model of the PPase from PS-3

The model of PS-3 PPase (B) was constructed by homology modelling with the program package QUANTA/CHARMm. The X-ray structure of *E. coli* PPase (A) served as a scaffold [12].

pH 7.8, containing 5 mM MgCl_2 (buffer A). Purifications of the wild-type and variant PPases were performed with the same procedure, as follows. $(\text{NH}_4)_2\text{SO}_4$ was added to a crude extract to 40% satn., and the sample was then applied to a column of Phenyl-Sepharose CL-4B (15 cm $\times$ 12 cm) previously equilibrated with buffer A containing 40%-satd. $(\text{NH}_4)_2\text{SO}_4$. The enzyme was eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (40–0% satn.) in buffer A. The active fractions were pooled and concentrated to 1 ml in a collodion bag. The concentrated sample was dialysed against buffer A containing 40%-satd. $(\text{NH}_4)_2\text{SO}_4$, then applied to a HPLC column of Phenyl-5PW (0.75 cm $\times$ 7.5 cm) previously equilibrated with buffer A containing 40%-satd. $(\text{NH}_4)_2\text{SO}_4$. The enzyme was eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (40–0% satn.) in buffer A at a flow rate of 0.75 ml/min, fractions of five drops being collected. The active fractions were collected and the purity was examined by native PAGE [24,25] and SDS/PAGE [26]. Under both conditions, all the wild-type and variant samples yielded a single band on staining with Coomassie Blue. The samples were concentrated in a collodion bag to a protein concentration of approx. 10 mg/ml and stored at -80°C .

Enzyme assay

PPase activity was determined by the procedure described previously [27]. One unit of specific activity is defined as 1 μmol of P_i liberated in 1 min by 1 mg of protein. The protein concentration in a solution was determined with a Pierce bicinchoninic acid protein assay kit, with BSA as standard.

Molecular mass estimation

The molecular mass of PPase was determined by gel filtration. The protein solution (100 μl) was applied to an HPLC column of TSK-gel G3000SW (0.75 cm $\times$ 60 cm), then eluted with a buffer comprising 20 mM Tris/HCl, pH 7.8, 2 mM MgCl_2 and 0.1 M KCl at a flow rate of 0.75 ml/min.

CD spectroscopy

CD spectra were obtained with a Jasco J-600 automatic recording dichrograph at room temperature. Cells of 1 and 5 mm path length were used for the measurements in the far- and near-UV

regions respectively. CD data were expressed in terms of mean residue ellipticity, $[\theta]$, with the mean residue molecular mass of 114.6 calculated from the amino acid sequence [16].

Chemicals

Restriction endonucleases, T4 DNA polymerase, *Taq* polymerase and a DNA ligation kit were purchased from Takara Shuzo Co.; antibiotics, egg-white lysozyme, ribonuclease A and low-gelling-temperature agarose (type S) were obtained from Nippon Gene Co. [α - ^{32}P]dATP (3000 Ci/mmol) and a DNA sequencing kit were obtained from Amersham. A Gene Clean II kit was obtained from Bio 101. Phenyl-Sepharose CL-4B and TSK-gel Phenyl-5PW were purchased from Pharmacia and Tosoh Co. respectively. All other chemicals used were of A.R. grade.

RESULTS

Three-dimensional structure model of PS-3 PPase

Figure 1 shows the three-dimensional structure of PS-3 PPase predicted by computer analysis of homology modelling with the QUANTA/CHARMm program (Molecular Simulation). The PS-3 model greatly resembles the three-dimensional structure of *E. coli* PPase, whose crystallographic data were used as a scaffold [12]. His-118 and His-125 of PS-3 PPase are located in the so-called helix A, and Trp-143 is just behind His-118.

Effect of replacement on the enzyme activity

The k_{cat} values of the wild-type and mutant PPases at pH 7.8 are shown in Table 1. The plasmid-driven wild-type PPase showed slightly higher enzyme activity than the authentic PPase from PS-3 cells [22]. Interestingly, the replacement of His-118 with Ala did not affect catalytic activity, whereas the mutant PPase with His-125 changed to Ala showed a decrease in catalytic activity to approx. one-fifth of that of the wild-type PPase. However, although H125A PPase was very stable in both the presence and the absence of Mg^{2+} , as was the wild-type PPase, H118A PPase was very unstable in a diluted state even in the presence of 50 mM Mg^{2+} ; it irreversibly lost its catalytic activity within 30 s at 4°C after dilution below a protein concentration of 5 μM , although complete inactivation was not observed. Therefore the

Table 1 Rate constants for the wild-type and variant PPases

The strains listed contain both the chromosomal and plasmid-encoded PPase. The plasmid-encoded PPase was amplified approx. 80-fold over the chromosomal PPase. The chromosomal-encoded PPase was taken into account as a blank value in each experiment. The number of individual rate measurements was 20. Values are means $\pm$ S.D.

Strain	k_{cat} (s^{-1})	$10^{-2}k_{\text{cat}}/K_m$ ($\text{s}^{-1} \cdot \text{mM}^{-1}$)
Wild-type	816.6 ± 26.6	13.6 ± 0.42
H118A	843.3 ± 27.4	14.1 ± 0.46
H125A	181.6 ± 5.8	3.0 ± 0.10
H118A/H125A	49.3 ± 2.3	0.8 ± 0.04
W143A	173.3 ± 5.2	2.9 ± 0.08

enzyme activity of H118A in Table 1 was measured within 15 s of dilution in 20 mM Tris/HCl, pH 7.8, containing 5 mM MgCl_2 and 1 mM sodium pyrophosphate, which made the enzyme quite stable, as with *E. coli* H140Q PPase [21]. The replacement of both His-118 and His-125 with alanine affected the enzyme activity more profoundly. The stability of H118A/125A PPase in a diluted state was almost the same as that of H118A PPase. The enzyme activity of W143A PPase also decreased to approx. one-fifth of that of the wild-type PPase, but its stability was not affected by the replacement.

The pH profile of the enzyme activity and the apparent K_m at pH 7.8 for magnesium pyrophosphate were not affected by the replacement.

Variation in activity with enzyme concentration

PS-3 PPase is a trimer in the absence of Mg^{2+} , and a hexamer in

the presence of Mg^{2+} [22]. Therefore the effect of replacement of His-118, His-125 and Trp-143 on the assembly to hexamers by Mg^{2+} was examined by measuring the recovery of specific activity at different protein concentrations. The stock solutions, containing 5 mM MgCl_2 , of the wild-type and variant PPases (approx. 0.5 mM, based on a molecular mass for the subunit of 20 kDa) were dialysed against 20 mM Tris/HCl, pH 7.8, containing 0.1 mM EDTA, after which treatment the enzymes dissociated into trimers. The PPases were then diluted to different protein concentrations with Tris/HCl, pH 7.8, containing either 2 or 50 mM MgCl_2 , and pre-equilibrated at 25 °C for 30 min for assembly into hexamers, except for H118A and H118A/H125A, whose activities were measured immediately after dilution with 20 mM Tris/HCl, pH 7.8, containing 1 mM PPI and either 2 or 50 mM MgCl_2 . As shown in Figure 2, the specific activities of the wild-type and W143A PPases increased abruptly with increasing enzyme concentration and reached maximal levels beyond 0.1 μM . No difference in the recovery of the specific activity of wild-type PPase was observed between 2 and 50 mM MgCl_2 in the medium during preincubation, whereas 50 mM MgCl_2 was more effective than 2 mM MgCl_2 for the W143A PPase. In contrast, the specific activities of H118A and H125A remained constant at a low level up to 1 μM , then increased gradually with increasing protein concentration to reach constant levels beyond 10–20 μM . The recovery of the specific activities of the H118A and H125A PPases again depended on the Mg^{2+} concentration: 50 mM MgCl_2 in the medium was more effective than 2 mM MgCl_2 . The behaviour of H118A/H125A was almost the same as that of H118A.

Molecular mass

The molecular masses of the wild-type and variant PPases were

Figure 2 Specific activities (units) of the wild-type and variant PPases preincubated at different enzyme and Mg^{2+} concentrations

(A) Wild-type PPase; (B) H118A PPase; (C) H125A PPase; (D) W143A PPase. Symbols: ○, 2 mM MgCl_2 ; ●, 50 mM MgCl_2 .

Figure 3 CD spectra of the wild-type and variant PPases in the near-UV

The solvent was 20 mM Tris/HCl, pH 7.8, containing 5 mM MgCl_2 . Solid line, wild-type PPase; dotted line, H118A PPase; dot-dashed line, H125A PPase; double-dot-dashed line, H118A/H125A PPase; broken line, W143A PPase.

measured in the presence of 2 mM MgCl_2 by gel-exclusion HPLC with TSK-gel G-3000SW. The stock solutions, containing 5 mM MgCl_2 , of the wild-type and variant PPases (approx. 0.5 mM) were dialysed against 20 mM Tris/HCl, pH 7.8, containing 0.1 mM EDTA; the samples were then preincubated for association into hexamers for 30 min in the presence of 2 mM MgCl_2 and 1 mM PPI at a protein concentration of 1 mg/ml (50 μM), except for H118A and H118A/H125A, which were applied to the column immediately after dilution. The wild-type and W143A PPases, whose specific activities after chromatography were 2500 and 410 units respectively, yielded a molecular mass of 110 kDa. In contrast, the molecular masses of H118A, H125A and H118A/H125A, whose specific activities after chromatography were 25, 80 and 15 units respectively, were all 52 kDa.

CD and fluorescence spectra

To determine the effect of the replacements on the protein structure, CD spectra were measured. The wild-type and variant PPases had almost the same CD spectra as that of the authentic enzyme from PS-3 cells in the far UV region [22], indicating that the polypeptide backbone remained unaltered by the replacement. Figure 3 shows the CD spectra of the wild-type and variant PPases in the presence of 5 mM MgCl_2 in the near UV region. The CD spectrum of the wild-type PPase was almost the same as that of the authentic enzyme from PS-3 cells [22]. The CD spectrum of H118A was also similar to that of the wild-type enzyme except for a decrease in the magnitude of the CD bands

Figure 4 Fluorescence spectra of wild-type and variant PPases

The concentrations of the enzymes were 20 μM in 20 mM Tris/HCl, pH 7.8, containing 5 mM MgCl_2 . Solid line, wild-type PPase; dotted line, H118A PPase; dot-dashed line, H125A PPase; double-dot-dashed line, H118A/H125A PPase; broken line, W143A PPase. All the above enzyme solutions were excited at 280 nm.

at 293 and 287 nm. However, the replacement of His-125 with Ala affected the state of aromatic amino acid residues: the magnitudes of the ellipticity around 270–280 nm and around 255–265 nm decreased and increased respectively. The changes in magnitude in ellipticity in these regions were more prominent for H118A/H125A and W143A. For W143A, furthermore, the positive CD band at 293 nm and the negative shoulder at 287 nm disappeared, indicating that these bands around 293 nm are due to the tryptophan residue, as PS-3 PPase contains only one tryptophan residue [15,16]. Because the CD band at 293 nm of H118A and H118A/H125A decreased, the replacement of His-118 with Ala affected the state of the tryptophan residue.

Intrinsic fluorescence due to the tryptophan residue also changed with the replacement on excitation at 280 nm, shifting the emission maximum from 322 to 330 nm, except for W143A, for which the emission maximum was at 305 nm, instead of 330 nm, owing to tyrosine residues. In H125A the intensity was almost the same as that of the wild-type PPase, but it was decreased in H118A/H125A and more profoundly in H118A (Figure 4). The results indicate that the replacement of His-118 with Ala markedly affected the environment of Trp-143, a result that agrees with that from CD spectroscopy.

Thermostability

To examine the effect of the replacements on the thermostability of PS-3 PPase, wild-type, H125A and W143A PPases were incubated at various temperatures at a protein concentration of either 2 or 20 μM in Tris/HCl buffer, pH 7.8, containing 2 or 50 mM MgCl_2 , and the enzyme activity remaining was measured at 37 °C at various times. The semilogarithmic plots of residual activity against time were almost linear.

Figure 5 shows the enzyme activity remaining after incubation at various temperatures for 1 h. As in the authentic PPase from PS-3 cells [22], the wild-type and W143A PPases at a protein concentration of 2 μM in the presence of 2 mM Mg^{2+} were

Figure 5 Effect of substitution on the thermostability of PS-3 PPase

The enzymes were incubated at various temperatures for 1 h in 20 mM Tris/HCl, pH 7.8, containing either 2 or 50 mM $MgCl_2$; the residual activities were then measured at 37 °C. Symbols: ○, wild-type PPase at a protein concentration of 2 μ M in the presence of 2 mM $MgCl_2$; ▲, W143A PPase at 2 μ M protein and 2 mM $MgCl_2$; △, H125A PPase at 20 μ M protein and 50 mM $MgCl_2$; ●, H125A at 2 μ M protein and 50 mM $MgCl_2$.

thermostable up to 70 °C. In contrast, H125A at a protein concentration of 2 μ M was thermolabile even in the presence of 50 mM $MgCl_2$, and it rapidly lost its enzyme activity irreversibly above 50 °C. However, it showed almost the same thermostability as wild-type PPase at a protein concentration of 20 μ M. The same behaviour was observed with H118A and H118A/H125A.

DISCUSSION

E. coli PPase is considered to be a dimer of trimers [12]. Baykov et al. [21] reported for *E. coli* PPase that His-136 and His-140 together with Asp-143, all of which are located at the C-terminus of helix A, form a network of intrachain hydrogen bonds with those of the anti-parallel α -helix in the facing monomer for association into a hexamer. A similar arrangement of two anti-parallel α -helices to form a trimer–trimer interface was also suggested for *T. thermophilus* PPase [21], in which the corresponding residues His-134 and Thr-138 [13] are thought to have an important role in the formation of intrachain hydrogen bonds.

As previously reported, the molecular mass of the authentic PPase from thermophilic bacterium PS-3 cells was estimated to be 54 kDa ($s_{20,w} = 3.52$ S) in the absence of Mg^{2+} , and 110 kDa ($s_{20,w} = 6.09$ S) in the presence of Mg^{2+} , indicating that PS-3 PPase assembles from trimers to hexamers in the presence of Mg^{2+} [22]. PS-3 PPase contains two histidine residues at positions 118 and 125, the latter corresponding to His-136 in *E. coli* PPase as judged by alignment of their primary structures [15]. However, these residues in the PS-3 enzyme are instead located at the N-terminus, not the C-terminus, of helix A, although the overall three-dimensional structural model of PS-3 PPase greatly resembles that of the *E. coli* enzyme (Figure 1). When the PS-3 enzyme was treated with diethyl pyrocarbonate, only His-118 was modified, with a concomitant loss of enzyme activity [19]. In the present study, however, the replacement of the histidine residue with alanine at position 118 in PS-3 PPase did not affect the enzyme activity at all when the enzyme was in the hexameric state, indicating that His-118 is not involved in the active site and

the chemical modification is an indirect effect. The replacement of His-125 with Ala, in contrast, decreased the enzyme activity to one-fifth of that of the wild-type PPase, but complete inactivation was not observed. This finding is in contrast with *E. coli* PPase; the replacement of His-136 and His-140 with glutamine results in an enhancement of catalytic activity in the hexameric state [21]. The decrease in enzyme activity of H125A might have been caused by a conformational change, as shown by the CD spectrum in the near-UV region (Figure 3); the magnitudes of the ellipticity around 270–280 and 250–260 nm were decreased and increased respectively. The CD band around 270–280 nm might be due to one or more tyrosine residues. Tyr-55 and Tyr-141 in *E. coli* PPase, which correspond to Tyr-46 and Tyr-130 respectively in PS-3 PPase, are reported to be important for structural integrity [3]. Thus the effect of replacement of His-125 with Ala in PS-3 PPase seems to be more drastic than that of replacement of His-136 and His-140 with glutamine in *E. coli* PPase. The replacement of the tryptophan residue with alanine also decreased the enzyme activity. The CD spectrum of W143A around 270–280 nm was very similar to that of H125A, although the positive band at 293 nm and the negative shoulder at 287 nm, due to tryptophan, disappeared, and the positive band around 250–260 nm increased greatly. The three-dimensional structural model indicated that Trp-143 is completely surrounded by hydrophobic residues, i.e. a hydrophobic cluster. Therefore the replacement of Trp-143 by alanine might change the conformation of this hydrophobic cluster and its surroundings, including tyrosine residue(s). The loss of enzyme activity on treatment with *N*-bromosuccinimide [20] might be for the same reason. In contrast, the CD spectrum of H118A was almost the same as that of the wild-type PPase, except for the decrease in the CD band at 293 nm. The effect of Trp-143 on the replacement of His-118 by alanine was also confirmed by the fluorescence spectrum (Figure 4). However, the change of the state of Trp-143 caused by this replacement might be too small to affect the conformation of the hydrophobic cluster and its surroundings. Therefore the replacement of His-118 by alanine did not affect the enzyme activity.

In contrast with the wild-type PPase, the recovery of specific activity of the PS-3 H118A and H125A variants was markedly affected by the concentrations of both the enzyme and Mg^{2+} , which was ascribed to the inability to assemble from trimers into hexamers at low protein and Mg^{2+} concentrations, as shown by molecular mass determination by HPLC exclusion chromatography. These findings clearly showed that His-118 and His-125 in PS-3 PPase are key residues at the dimer interface, as are His-136 and His-140 in the *E. coli* PPase. The effect of dilution was more profound on H118A than on H125A. The former was irreversibly inactivated at a low protein concentration and the latter was re-activated on reconcentration, suggesting that the properties of (or environments around) His-118 and His-125 in PS-3 PPase resemble those of His-140 and His-136 respectively in the *E. coli* enzyme [21]. Asp-143 in the *E. coli* PPase was also inferred to have an important role in trimer–trimer interaction. There are two aspartic residues at positions 114 and 132 at or near helix A in the PS-3 PPase [15]. At present it is unknown which of them is important for trimer–trimer assembly. If it is Asp-114, then the important residues for trimer–trimer assembly are arranged, interestingly, in the opposite direction to those in *E. coli* PPase. The roles of Asp-114 and Asp-132 are now being examined in our laboratory. It was clearly shown by the results on specific activity versus enzyme concentration that Trp-143 has no bearing on trimer–trimer interaction. These findings indicate clearly that His-118 and His-125 are important for both trimer–trimer interaction and structural integrity, whereas Trp-143 is important structurally.

The present study confirmed that the thermostability of the PS-3 enzyme depends fully on its hexameric state, as suggested previously [22]; the PS-3 enzyme is thermostable only in the presence of Mg^{2+} , with which the enzyme exists in a hexamer. The newly formed intrachain hydrogen bonds between trimers might be responsible for this thermostability.

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