論文要約（４（２）関係）

博士論文要約（Summary）

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Title	Functional and structural analysis of inorganic pyrophosphatase from Antarctic psychrotroph *Shewanella* sp. AS-11

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Introduction

Inorganic pyrophosphatase (PPase) is an essential enzyme in all living organisms, as it hydrolyzes inorganic pyrophosphate (PPi) to phosphate (P). There are 2 soluble PPase families, families I and II, and the PPases in these 2 families have completely different primary structures. Family I PPases are homodimer or homohexamers of one-domain subunits. Family II PPases are homodimers of two-domain subunits. Both families of PPases are only active in the presence of metal ion cofactors but different catalytic efficiencies and cofactor selectivity.

Shewanella sp. AS-11 is a bacterium isolated from the shellfish *Neobuccinum eatoni* and lives in the ice-covered seas of Antarctic, where the temperature is close to and often below 0°C. This bacterium grows most rapidly at 20°C, and grows well at 4°C, but cannot grow above 30°C; it is thus can be classified among the psychrotrophs bacteria. Enzymes derived from psychrophilic bacteria generally have higher activity at low temperatures, with lower thermostability, compared to their homologues from mesophilic bacteria. The characteristics of psychrophilic bacteria provide valuable alternatives to their mesophilic counterparts. The relatively high thermo-sensitivity of these enzymes allows rapid inactivation in complex mixtures by mild heat treatment, which can lead to preservation of product quality. Furthermore, the results of this study affirmed that inorganic pyrophosphatase from psychrotroph *Shewanella* sp. AS-11 (*Sh*-PPase) belongs to family II PPase. Family II PPases comprises N-terminal and C-terminal domains that joined by a hinge region. The open-close movement of their domains is through to be necessary for its catalytic activity. Therefore, it is interesting to study *Sh*-PPase as an ideal enzyme to elucidate the mechanism of cold-adaptation enzyme as well as relation between conformational changes and activity during catalysis. The aims of this study are to determine the functional characteristics of *Sh*-PPase, upon activation by divalent cations and to analyze its structural.

Experimental

The gene encoding the *Sh*-PPase was cloned and over expressed in *Escherichia coli* using pET-16b vector. Isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM) was expressed the enzyme when incubated at 20°C for 18–20 hr. The cell extracts of recombinant bacteria was purified by a

combination of ammonium sulphate fractionation and anion-exchange chromatography using Hi-Trap Q HP (GE Healthcare Bio-sciences, Sweden). For large amount of protein purification, Super-Q chromatography was used.

Metal-free (non–activated) *Sh*-PPase was prepared by ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) treatment of the enzyme followed by ultra filtration (Amicon ultra centrifugal filter devices) (30-kDa cutoff). The activated enzymes were prepared by incubation of 0.5 mg/mL metal-free enzyme with the optimal concentration of divalent cations that gave the highest activity, for 2 hr at 5°C. *Sh*-PPase containing Co^{2+}, Mn^{2+}, or Zn^{2+} ions in the high-affinity metal-binding site was prepared by a similar incubation, followed by a 20-fold dilution with buffer containing 40 µM activating metal ion, and then concentrated by using ultra filtration.

The activity of *Sh*-PPase was measured after reaction of 10 µL of enzyme and 110 µL of 1 mM substrate ($K_4P_2O_7$) in 100 mM Tris-HCl buffer, 50 mM KCL (pH 7.5), containing 5 mM $MgCl_2$ for 3 min at 25°C. The reaction was stopped by the addition of 30 µL of 50 mM H_2SO_4. The reaction mixture was colored by addition of 150 µL of 1% sodium ascorbate in 0.05 % K_2SO_4 and 1% ammonium molybdate in Milli-Q water. The amount of Pi liberated from the hydrolysis of PPi was measured at 750 nm using a microplate reader (Bio-Rad, model 680XR) and a standard Pi curve after 600s. The specific activities (U/mg) are reported as µmoles $Pi \cdot min^{-1} \cdot mg^{-1}$ of protein. One unit of pyrophosphatase activity was defined as the enzyme activity capable of transforming 1 µM of PPi into 2 µM of Pi per min under the above conditions.

Thermostabilities of non–activated and activated *Sh*-PPases were determined by measuring the residual activity after incubation of the enzyme at a concentration of 20 µg/mL at temperatures from 0 to 70°C for 15 min. Thermal inactivation was measured after incubation for various times at 50°C. The optimum temperature for activation of *Sh*-PPases was determined by measuring the activities of activated *Sh*-PPases at various temperatures (0–70°C).

The velocities of activated *Sh*-PPases were measured at 0°C and 25°C. K_m and k_{cat} values were determined from velocity data at various concentrations of substrate by non-linear regression on the Michaelis-Menten equation using the program GraphPad Prism (GraphPad Software Inc.).

The conformational changes of *Sh*-PPases in solution were analyzed by using fluorescence spectroscopy. Fluorescence emissions spectra were recorded on Hitachi 850 fluorescence spectrophotometer. Each spectrum followed from three scans average and the buffer spectrum was deduced from the sample spectrum taken in the same condition. The excitation wavelength was set at 295 nm in order to preferentially excite the two tryptophan residues per monomer for measurements of intrinsic fluorescence. In fluorescence quenching experiments, quencher stock (acrylamide) was added to protein samples and fluorescence spectra were recorded after each addition. The steady state fluorescence quenching data were analyzed by Stern–Volmer equations in order to obtain quantitative quenching parameters. The steady state fluorescence anisotropy was measured using the same instrument.

For ANS assays, the excitation wavelength was set at 380 nm while the emission was recorded between 400 and 600 nm. Concentration of ANS and enzyme were 50 µM and 0.1 mg/mL, respectively. All measurements were performed at 25°C.

Circular dichrosim (CD) spectra were recorded on a Jasco spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan). Samples were placed in a 0.1 mm path length rectangular quartz cell. The CD data are expressed in terms of mean residue ellipticity (θ).

The purified of protein non–activated and activated *Sh*-PPases crystals were grown by hanging-drop vapor diffusion method in 24-well Falcon plates. X-ray diffraction data were collected on beamline BL26B1 at Spring-8, Japan and Saga LS, Japan. Raw diffraction data were integrated, scaled and reduced using Crystal screen 2.0 program.

Results and discussions

The gene encoding the inorganic pyrophosphatase from psychrotroph *Shewanella* sp. AS-11 was successfully cloned and over expressed in *Escherichia coli* using pET-16b vector (DDBJ/EMBL/GenBank accession number: AB775531). The PPase activity was detected over 0.12 to 0.15 M KCl on the linear gradient. The sequential steps gave 10.9-fold augmentations in specific activity with a yield of 13.8%. The purified PPase migrated as a single band on SDS-PAGE.

The molecular masses of this enzyme were determined to be 34 kDa by SDS-PAGE, whereas 66, 62, and 64 kDa by gel filtration (superdex 75pg) after activation with Co^{2+}, Mn^{2+} and Zn^{2+} ions, respectively. Binding with divalent cations caused the dimerization of *Sh*-PPase, while non–activated *Sh*-PPase was monomer. The results indicated that *Sh*-PPase is a homodimer of 34-kDa subunits, belonging to family II PPase.

Sh-PPase was markedly activated by incubation with Co^{2+}, Zn^{2+}, and Mn^{2+}. However, no significant activation was shown with Cu^{2+}, Ba^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+}, or Sr^{2+}. Furthermore, no hydrolytic activity was observed in this research when enzyme was assayed in the absence of divalent cations. The optimal concentrations of Co^{2+}, Mn^{2+}, and Zn^{2+} ions for activation of *Sh*-PPase were 25, 15, and 0.5 mM, respectively. Co^{2+}, Mn^{2+}, and Zn^{2+} can activate *Sh*-PPase as a Lewis acid function of those metal ions, and thus they are likely to activate water more effectively for nucleophilic attack. PPi hydrolysis proceeds as a direct nucleophilic attack of a water molecule, which is activated by coordination with 2 metal ions on the phosphorus of PPi.

The non–activated, Co– and Mn–*Sh*-PPases were stable until 40°C, whereas Zn–*Sh*-PPase was stable until 50°C. Zn^{2+}–binding to the enzyme could protect it against thermal denaturation.

The order of thermostability of *Sh*-PPase, when incubated for 15 min at 50 °C, in the presence of various metal cations, was found to be Zn–*Sh*-PPase, non–activated *Sh*-PPase, Mn–*Sh*-PPase, and Co–*Sh*-PPase. Increasing the temperature at higher than 40°C was caused the inactivation of non-activated, Mn– and Co–*Sh*-PPases. Furthermore, Zn–*Sh*-PPase was more stable compare than non-activated *Sh*-PPase. On the other hand, Mn– and Co–*Sh*-PPases were more unstable compare than non-activated *Sh*-PPase. Thermal inactivation rates of *Sh*–PPases at 50°C indicated that binding with Co^{2+} or Mn^{2+} caused destabilization of this enzyme.

The optimal temperature for activity of Mg–*Sh*-PPase, Co–*Sh*-PPase and Zn–*Sh*-PPase was 40°C, 30°C, and 20°C, respectively. However, the optimal temperature for Mn–*Sh*-PPase was surprisingly low (5°C). The specific activities of *Sh*-PPase activated by Co^{2+}, Mn^{2+} and Zn^{2+} were 100-, 45- and 12-fold higher than that of Mg–*Sh*-PPase at 5°C. The results showed Co–*Sh*-PPase

also have high activity at low temperature. This result affirmed that *Sh*-PPase belongs to family II PPase, because families II PPases are more active with Mn^{2+} and Co^{2+} than with Mg^{2+}. The results also indicated that Co– and Mn–*Sh*-PPases with high activity at low temperature have the characteristics generally found with psychrophilic enzymes. Psychrophilic enzymes have a high specific activity at low and moderate temperatures and are inactivated easily or unstable by a moderate increase in temperature. At higher temperatures, denaturation of this enzyme occurs

The psychrophilic characteristics of Co– and Mn–*Sh*-PPases were confirmed by the kinetic results that the k_{cat} values of these activated enzymes at 0°C were not decreased from those at 25°C. On the other hand, k_{cat} values of Mg– and Zn–*Sh*-PPases at 0°C were decreased 5- and 7-fold compared with those at 25°C. It was related to the high activity of Co– and Mn–*Sh*-PPases at low and moderate temperatures. Co– and Mn–*Sh*-PPases had turnover number (k_{cat}) and physiological efficiency (k_{cat}/K_m) much higher than those of Mg–*Sh*-PPases both at 0 and 25°C. However, K_m values of all of these enzymes were not significantly different from each other.

From these results, it can be concluded that Co^{2+} or Mn^{2+} ions are required for *Sh*-PPase to gain cold-adapted characteristics. The high activity of cold-adapted enzymes at low temperatures may be attributed to the flexible structure around the active site, or may be related to the flexibility of the coordination geometry of the amino acids of the PPase and the metal ion. In the metal ion-binding site of the protein, the metal ion will be coordinated by different combinations of protein side chains caused by the variation in coordination geometries.

Activation with divalent cations caused some conformational changes of *Sh*-PPase. It was shown on the results of intrinsic tryptophan fluorescence spectroscopy of non–activated and activated *Sh*-PPases. Fluorescence spectra of non–activated, Co–, Mn–, Zn– and Mg–*Sh*-PPases exhibited an emission maximum at 348, 353, 352, 352, and 352 nm, respectively, when excited at 295 nm. The emission maximum of activated *Sh*-PPases shifted to longer wavelength (red shifted). The difference in fluorescence spectra of these enzymes indicated that the hydrophobicity of environment surrounding the tryptophan residues of *Sh*-PPases was decreased upon binding of divalent cation.

The fluorescence anisotropies of tryptophan of non–activated and activated *Sh*-PPases were similar, which indicates that the conformational fluctuation of the tryptophan residues did not change on activation with divalent cations. This shows the tryptophan residues were buried in the enzyme on activation with divalent cations, but the flexibility of the residues was not changed.

The activation with divalent cations decreased the quenching effect of acrylamide on tryptophan fluorescence of the enzyme, indicated that the accessibility of acrylamide to tryptophan residues was decreased. Furthermore, acrylamide quenching of non–activated and activated *Sh*-PPases showed linear Stern–Volmer plots. The linear Stern–Volmer plots indicated that both of Trp109 and Trp286 residues of non–activated and activated *Sh*-PPases are equally quenched by acrylamide. Accessibility of acrylamide to two tryptophan residues was not different. However, the emission maximum of tryptophan fluorescence was shifted upon activation with divalent cations to longer wavelength (red shifted). These results suggested that tryptophan residues become buried in the enzyme upon activation with divalent cation but interacting with a neighboring polar group.

The gel filtration of non–activated and activated *Sh*-PPases showed that dimerization of the enzyme occurred upon activation with divalent metal ions, which could possibly change the environment around the tryptophan residues.

The homology-modeled structure of *Sh*-PPase showed that the manganese ion-binding site was 24 Å from Cα of Trp109 and 23 Å from Cα of Trp286, but activation with manganese ions modified residues in the environment surrounding the tryptophan residues as results of intrinsic tryptophan fluorescence. This suggests that the conformation of the *Sh*-PPase molecule could be changed upon activation with divalent cations.

The results of extrinsic fluorescence spectroscopy using ANS probe also showed the conformational changes of *Sh*-PPase on activation with divalent cations. The non–activated *Sh*-PPase caused a marked blue shift (by 24 nm) and enhancement of ANS fluorescence from emission maximum wavelength of ANS. The blue shift of Mg–*Sh*-PPase was 10 nm from emission maximum wavelength of ANS. The blue shift of Co–, Mn–, and Zn–*Sh*-PPases were 5.0, 0.5, and 6.5 nm, respectively from emission maximum wavelength of ANS. The results shows activation with divalent cations caused the red shift in the emission maximum of ANS bound to the enzyme, proving that the environments of ANS-binding sites become less hydrophobic upon the activation. The finding also indicated that the degrees of red shift were different among the enzymes activated with different metal ions demonstrates that conformational changes were somewhat different depending on the bound metal species. These conformational changes may influence the temperature dependency and thermostability of activated enzymes.

The CD spectra of non–activated and activated *Sh*-PPases had two negative bands at 222 nm and 208 nm, and positive band at ~ 190 nm which were typical of α-helical structure. The results indicated that conformational changes of *Sh*-PPase upon activation with divalent metal ions in solution may be rather subtle, as detected by fluorescence spectra and did not change the secondary structure.

To further understand the mechanism of cold adaptation of *Sh*-PPase, the crystals of *Sh*-PPases were grown for X-ray crystallography. The crystals of non–activated and Mn–activated *Sh*-PPases were grown at neutral or acidic pH with polyethylene glycol as precipitant, but the quality of X-ray diffraction data of these crystals was not good enough to analyze the three dimensional structure.

From all of these data, it can be concluded that inorganic pyrophosphatase from *Shewanella* sp. AS-11 is a cold-adapted enzyme when activated with Co^{2+} or Mn^{2+}, which has higher activity at low temperatures and lower thermostability than Zn^{2+}–, and non–activated enzymes. Binding of divalent cations to the active site induces some conformational changes of *Sh*-PPase, which may influence temperature dependency and thermostability of the enzyme. The conformational change of *Sh*-PPase upon binding with Co^{2+} or Mn^{2+} could possibly lead to the flexible structure of this enzyme, which is required for high activity at low temperatures.