Extremophiles (2009) 13:859–865
DOI 10.1007/s00792-009-0273-z

ORIGINAL PAPER

Membrane-bound acid pyrophosphatase from *Sulfolobus tokodaii*, a thermoacidophilic archaeon: heterologous expression of the gene and characterization of the product

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Received: 8 December 2008 / Accepted: 3 August 2009 / Published online: 21 August 2009

Abstract Membranes of *Sulfolobus tokodaii*, a thermoacidophilic archaeon that grows optimally at pH 2–3, 75–80°C, show the ability to hydrolyze PPi with an optimum pH of 2–3. This acid PPase is proposed to be a dolicholpyrophosphatase that participates in glycoprotein biosynthesis. In the present study, the archaeal membranes hydrolyzed isopentenylpyrophosphate and geranylpyrophosphate, compounds related to dolicholpyrophosphate, at pH 3. However, the dolicholpyrophosphate-binding antibiotic bacitracin failed to inhibit the acid PPase. To investigate further the function and structure of the acid PPase, the gene was cloned and heterologously expressed in *Escherichia coli.* The membranes from recombinant *E. coli* showed PPase activity with similar pH and temperature dependence, substrate specificity, and kinetic parameters to those reported for *Sulfolobus* membranes. The acid PPase was solubilized and purified to electrophoretic homogeneity from the recombinant *E. coli*. The purified enzyme showed similar K_m values for PPi, ATP, and ADP to the membrane-bound enzyme. Lipids from the *Sulfolobus* membranes enhanced the activity to about threefold. Studies involving deletion mutants indicated that basic amino acids in the N-terminal (Arg2 and Lys3), as well as the residues (4th–69th) possibly twice-spanning the membrane, are essential for integration of the enzyme into membranes.

Keywords Pyrophosphatase · Ectoenzyme · Dolichol · Archaea · Thermophile · *Sulfolobus*

Communicated by H. Santos.

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Introduction

Thermoacidophilic archaea belonging to the genus *Sulfolobus* live optimally at pH 2–4, and 75–85°C (Brock et al. 1972; Grogan 1989). The intracellular pH is around 6–6.5 (Lübben and Schäfer 1989). A large pH gradient is maintained across the cytoplasmic membrane, which is surrounded by a surface cell layer (S-layer) composed of glycoproteins (Inatomi et al. 1983).

The membrane fraction of *Sulfolobus tokodaii* [formerly *Sulfolobus* sp. strain 7 (Wakagi et al. 1998), or *Sulfolobus acidocaldarius* stain 7 (Wakagi and Oshima 1985, 1986; Wakagi et al. 1992)] shows the ability to hydrolyze PPi, ATP and ADP with an optimal pH of 2–3, suggesting this activity is located on the outer surface of the membrane (Wakagi and Oshima 1985, 1986). The activity is not that of a non-specific phosphatase, because AMP, *p*-nitrophenylphosphate (pNPP), and glucose-6-phosphate are not hydrolyzed. The membrane-bound acid PPase is different from both the cytosolic PPase (Wakagi et al. 1992, 1998) and acid phosphatase (Kurosawa et al. 2000) from *Sulfolobus*.

The growth of *Sulfolobus acidocaldarius* at pH 4.7 (not at pH 3) is suppressed by bacitracin (Meyer and Schäfer 1992), an antibiotic that inhibits bacterial isoprenyl pyrophosphatase (Stone and Strominger 1971; Storm and Strominger 1973). Bacterial isoprenyl pyrophosphatase is a component of the glycosylation system of membrane proteins involved in membrane biogenesis (Young et al.

2002). Among archaea, dolichol-linked oligosaccharides have been found in *Haloferax volicanii* (Kuntz et al. 1997). *Sulfolobus* acid PPase is related to the archaeal dolichol pyrophosphatase (Meyer and Schäfer 1992; Moll and Schäfer 2004). However, direct inhibition of *Sulfolobus* acid PPase activity in the presence of bacitracin has not been examined so far. Thus, the physiological role of *Sulfolobus* acid PPase is still not clear. To elucidate the function and structure of the enzyme, efforts to purify the acid PPase from *S. acidocaldarius* (Meyer and Schäfer 1992) and *S. tokodaii* (Amano et al. 1993) revealed that the enzyme is comprised of a single subunit of 17–25 kDa. Based on the partial amino acid sequence, the gene for *S. acidocaldarius* PPase (*sepp*, *sac*1025) has been cloned and sequenced (Moll and Schäfer 2004). The gene is flanked by three other genes possibly constituting an operon, while the gene organization in other *Sulfolobus* species is completely different. Genome analyses revealed that the homologous PPase genes in other species of *Sulfolobus* are *st*2226 in *S. tokodaii* and *sso*2563 in *S. solfataricus*. These genes encode 21–22 kDa proteins which are characterized by (1) five possible transmembrane regions, (2) tripartite phosphatase motif found in several membrane integral lipid phosphatases, (3) homology to *Escherichia coli* YbjG, *Bacillus subtilis* YwoA, and *Bacillus lichenformis* BcrC which show undecaprenylpyrophosphate phosphatase activity that is essential for peptideglycan biosynthesis (Bernard et al. 2005; Touze et al. 2008), with conservation of 11 residues mostly overlapping with the above phosphatase motif (Moll and Schäfer 2004). A topological model of ST2226 (Fig. 1) indicates that basic residues are rich in the N-terminal and inside-loop (residues 70–75) regions. Conserved residues mentioned above are in both outside (residues 99–143) and inside (residues 158–169) loop regions.

In this study, we constructed expression vectors for the ST2226 gene to clarify whether the gene product is functional in the recombinant form. Using the recombinant system, the roles of amino acids in the N-terminal region were investigated. The ability of S-membranes to hydrolyze isopentenylpyrophosphate (C_5PP) and geranylpyrophosphate (C_{10}PP), compounds related to C_{55}-isoprenylpyrophosphate, was also investigated.

Materials and methods

Archaeal and bacterial strains

Sulfolobus tokodaii cells were grown at pH 3, and 75°C, collected by centrifugation and used to prepare a membrane fraction (designated as S-membranes) as described previously (Wakagi and Oshima 1985; Amano et al. 1993).

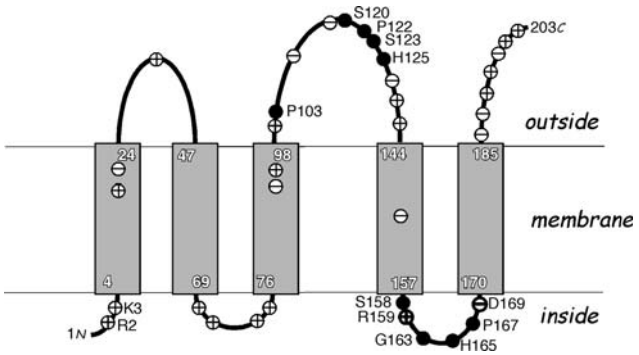

Fig. 1 Prediction of transmembrane topology of acid PPase (ST2226) from *S. tokodaii*. The model was constructed from the output of the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM) with a modification. *Shaded boxes* indicate transmembrane helices. A *circled plus* sign indicates Lys or Arg. A *circled minus* sign indicates Asp or Glu. *Filled circle* and *thick symbol* indicate 11 conserved residues described in the text

Sulfolobus lipids were prepared from *Sulfolobus acidocaldarius* as described elsewhere (Sugai et al. 1995).

Escherichia coli strain BL21(DE3) CodonPlus-RIL {F^-, *ompT hsdS*(r_B–m_B–) *dcm* + Tet^r *gal* λ(DE3) *endA* Hte [*argU ileY leuW* Cam^r]} and *E. coli* strain C43(DE3) (Miroux and Walker 1996) were used as host strains. The host strains were transformed with either pSTT or pSTH.

The reagents and procedures for DNA manipulation were as described previously. Bacitracin was purchased from Sigma.

Construction of expression plasmids

Genomic DNA was prepared and used as a template for PCR. The gene encoding ST2226 was amplified in a mixture comprising the template, oligo DNA primers, shown in Table 1, dNTPs, $MgCl_2$, a buffer and thermostable DNA polymerase (Easy-A high-fidelity PCR cloning enzyme, Stratagene). Two expression plasmids, pSTT and pSTH, which carry tandemly arranged genes encoding the ST2226 gene, HSV-tag and His-tag, were constructed from pET25b(+) (Stratagene) and the PCR products.

pSTT was designed to express only the ST2226 gene, while pSTH was designed to express ST2226, HSV-tag and His-tag at the C-terminal of the fused protein, so that the product could be easily purified by Ni^{2+}-affinity chromatography.

Construction of mutant plasmids

Plasmids, pSTHΔR and pSTHΔRK, were designed to express deletion mutants, STHΔR and STHΔRK, lacking Arg2 and Arg2-Lys3, respectively. Plasmids pSTH-70 and pSTH-76 lacking residues 4–69 and 4–75, respectively, were also designed to express deletion mutants STH-70 and

Table 1 Oligo DNA primers

prSTT/f:	5′-TATACATATGCGTAAATATTGGT-3′ *Nde*I R K Y W
prSTT/r:	5′-GATCTCGAGTCATGAAACTTTA-3′ *Xho*I stop S V K
prSTH/f:	5′-TATACATATGCGTAAATATTGGT-3′ *Nde*I R K Y W
prSTH/r:	5′-GATCTCGAGTGAAACTTTAAAT-3′ *Xho*I S V K F
prSTH-ΔRK/f:	5′-GACATACCATATGTATTGGTTCCTTTTA-3′ *Nde*I Y W F L L
prSTH-ΔR/f:	5′-GACATACCATATGAAATATTGGTTCCTTTTA-3′ *Nde*I K Y W F L L
prSTH-70/f:	5′-CATACCATATGCGCAAGTTTAAAAGGACTAGAAG-3′ *Nde*I **R2 K3 I70 K71**
prSTH-76/f:	5′- CATACCATATGCGCAAGATTGCTATAACACTTGCTGC-3′ *Nde*I **R2 K3 I76 A77**
prSTH-C/r:	5′-CCAATATTTACGCATATGTATATC-3′ W Y K R *Nde*I pET25b(+)

Restriction sites are underlined. The deduced amino acid sequence of acid PPase (ST2226) is 1-NH_2-M-R-K-Y-W-F-L-L-·····-L-F-K-V-S-COOH-203

STH-76 which presumably lack first two membrane spanning regions.

The oligo DNA primers used to construct deletion mutants STHΔR, STHΔRK, STH-70 and STH-76 are shown in Table 1. Using the primer set of prSTHΔR/f and prSTH-C/r, PCR was carried out to obtain pcrSTHΔR, and using the primer set of prSTHΔRK/f and prSTH-C/r, pcrSTHΔRK was obtained. The template was pSTH. The PCR products were digested with *Nde*I and self-ligated to obtain pSTHΔR and pSTHΔRK. pSTH-70 and pSTH-76 were obtained in the similar manner using the primers prSTH-70/f, prSTH-76/f and prSTH-C/r. These plasmids were used to transform *E. coli* BL21(DE3) Codonplus-RIL. A membrane fraction was prepared from these cells as described below and assayed for acid PPase.

Preparation of recombinant *E. coli* membranes

Recombinant *E. coli* strains were cultured in Luria–Bertani medium containing 0.1 mg/mL ampicillin (and 0.03 mg/mL chloramphenicol in the case of the Codonplus strain) at 37°C until $OD_{600} = 1$, and then 0.5 mM isopropyl-β-thiogalactoside (IPTG) was added and the culture was further continued for 3 h. The IPTG concentration and the time of culture after induction had been optimized in a preliminary experiment with 0, 0.5, 1 mM IPTG and 3, 6, 18 h culture, respectively.

Recombinant *E. coli* cells were collected, suspended in 10 mM Tris–HCl, pH 7.6, 1 mM phenylmethylsulfonylfluoride, in the ratio of 1:5 (cell wet weight:buffer weight), sonicated with a BIORUPTOR (COSMO BIO) at 220 W for 30 min at 0°C, and then centrifuged at 8,000×*g* for 5 min at 4°C. The resulting turbid supernatant was centrifuged at 15,000×*g* for 60 min at 4°C. The resulting pellet was washed twice with 10 mM Tris–HCl, pH 7.6. The fraction thus obtained was designated as R-membranes (recombinant *E. coli* membranes).

Solubilization and purification of the enzyme from R-membranes

To purify the enzyme from recombinant *E. coli* membranes, the enzyme was solubilized with various detergents such as Triton X-100, MEGA9, *n*-octyl-β-D-glucoside, and *n*-dodecyl-β-D-maltoside, which were added to the membrane suspension (about 1 mg of protein/mL) in the protein:detergent ratios (w/w) of 1:1, 1:3 and 1:5, gently mixed for 1 h at room temperature, and then centrifuged at 100,000×*g* for 1 h, at 25°C. The supernatants were saved, and assayed for protein and activity. For large-scale preparation, Triton X-100 was selected to solubilize the proteins from the *E. coli* membranes. The solubilized proteins were applied onto a HiTrap Chelating HP 5 mL column (Amersham Biosciences) charged with Ni^{2+}. The column had been pre-equilibrated with 10 mM Tris–HCl, pH 7.6, 0.5 M NaCl, 10 mM imidazole, and 1% Triton X-100 (Buffer A). Then the proteins were eluted with a linear gradient of imidazole from 10 to 250 mM (2 mL/

min × 30 min) in the presence of 1% Triton X-100. Each 6 mL fraction was collected, and then subjected to protein and activity assays, and SDS-PAGE.

SDS-PAGE was carried out in 12 or 16% (w/v) polyacrylamide gels. To detect recombinant proteins with a His-tag, proteins in an acrylamide gel were transferred to a PVDF membrane (MILLIPORE) and analyzed immunologically using an anti-His-tag antibody (produced by a mouse, purchased from Roche) as a primary antibody and an anti-mouse-serum-antibody labeled by alkaline phosphatase (WesternBreeze, purchased from Invitrogen) as a secondary antibody. Color development was performed with a BCIP/NBT system (Invitrogen). Proteins in the acrylamide gel were visualized by Coomasie brilliant blue or silver staining (Silver stain MS kit, Wako Pure Chemicals).

Activity and protein assays

Enzyme activity was determined at 70°C in 50 mM phthalate buffer, pH 3.0, 5 mM NaPPi and an appropriate amount of sample enzyme. The reaction was started by adding the enzyme and terminated by cooling on ice. To determine substrate specificity, PPi was replaced with ATP, ADP, AMP, pNPP, C_5PP (a kind gift from Dr. T. Kuzuyama, the University of Tokyo), or C_{10}PP (Sigma). To determine the pH optimum, phthalate buffer (pH 2–4) and Na-acetate buffer (pH 4–6) were used. To determine orthophosphate, the reaction mixture was mixed with BIOMOL GREEN Reagent [BIOMOL Research, improved from (Hess and Derr 1975)] and absorbance at 620 nm was measured. The enzyme concentration was adjusted so that the time course of the reaction was linear. To determine the kinetic parameters, three experiments were performed in parallel. One unit (U) was defined as the production of 1 μmol orthophosphate per min.

Protein was determined by the BCA method (Pierce) with bovine serum albumin as a standard.

Results and discussion

Substrate specificity of the enzyme in S-membranes

We have reported that S-membranes show hydrolytic activity with an optimal pH around 2–3 with PPi, ATP, and ADP. Since the role of this enzyme is suggested to be that of a dolichol pyrophosphatase in the protein glycosylation system (Meyer and Schäfer 1992), we evaluated the ability of S-membranes to hydrolyze C_5PP and C_{10}PP, compounds related to dolichol pyrophosphate. The Pi-releasing activities of PPi, C_5PP, and C_{10}PP (0.5 mM in 25 mM phthalate buffer, pH 3.0, 70°C) were 0.99, 0.41, and 0.26 U/mg of protein, respectively. pNPP was not hydrolyzed. C_5PP and C_{10}PP were hydrolyzed by the enzyme, suggesting it plays some role in the dolichol cycle.

We confirmed that equimolar Pi was released from 50 nmol C_{10}PP after enough time of incubation with the enzyme (data not shown). Therefore, the product derived from C_{10}PP should be geranylphosphate, which did not release Pi any more.

Effect of bacitracin on the enzyme activity in S-membranes

Bacitracin inhibits the growth of *S. acidocaldarius* at pH 4.7, but not at pH 3 (Meyer and Schäfer 1992). Bacitracin inhibits C_{55}-isoprenyl pyrophosphatase by forming a complex with the pyrophosphate moiety and Mg^{2+} at pHs higher than 5 (Stone and Strominger 1971; Storm and Strominger 1973). So we investigated the effect of bacitracin on the PPase activity of R-membranes.

Bacitracin (0.2, 1, 2 mM) was added to a reaction mixture comprising 0.5 mM PPi and 2 mM $MgCl_2$ in either 50 mM phthalate buffer, pH 3.0, or 50 mM Na-acetate buffer, pH 5.0, and either S-membranes or R-membranes from *E. coli* harboring pSTH (about 1 mg of protein/mL). The activity of S-membranes was 1.7 ± 0.2 U/mg of protein at pH 3.0, and 0.35 ± 0.1 U/mg of protein at pH 5.0, in the presence of 0–2 mM bacitracin. The activity of R-membranes was 3.4 ± 0.3 U/mg of protein at pH 3.0, and 0.6 ± 0.1 U/mg of protein at pH 5.0, in the presence of 0–2 mM bacitracin. No inhibition of PPase activity was observed for either S- or R-membranes at any pH.

The interaction of bacitracin with C_{55}-isoprenylpyrophosphate is greater than the interaction of bacitracin with C_5PP or PPi by 10^2, and the optimal bacitracin action is observed around neutral pH (Stone and Strominger 1971; Storm and Strominger 1973). Whether bacitracin interacts with *Sulfolobus* acid PPase and whether the PPase functionally acts as an archaeal dolichol pyrophosphatase remain unknown.

The pH dependence of the PPase activity in S-membranes and R-membranes (from *E. coli* harboring pSTH or pSTT) was similar to each other with optimal pH around 2.5–3.0 (in phthalate buffer whose pH was adjusted at room temperature). S-membranes show specific activity of 1.4 U/mg of protein at an optimal pH of 2.5, these values being close to those previously reported (Wakagi and Oshima 1985). R-membranes from both *E. coli* (pSTT) and *E. coli* (pSTH) show an optimal pH of 2.5–3 and maximal activity of 3.5–4 U/mg of protein. Both plasmids successfully expressed the acid PPase in *E. coli* membranes with higher specific activity than in *S. tokodaii* membranes. Judging from the specific activity, 2.1–2.9-fold enrichment

of the enzyme in the membranes was achieved. This indicates that the expression plasmid works effectively in *E. coli*, and the product is efficiently incorporated into the *E. coli* membranes.

For the following experiments, R-membranes from *E. coli* (pSTH) rather than *E. coli* (pSTT) were chosen, because His-tag is useful for purification.

The temperature dependence of the acid PPases in S- and R-membranes was investigated. Both PPases show the same optimum temperature (80°C) and similar temperature dependence, even though the lipid structures surrounding the enzymes are different. The lipids from *Sulfolobus* are composed of tetraether, in contrast to the ester type lipid bilayer of *E. coli* (Elferink et al. 1995).

Strains of *E. coli* other than C43(DE3) were also examined. R-membranes from *E. coli* BL21(DE3) Codonplus-RIL showed slightly higher specific activity of acid PPase than those from C43(DE3), so this stain was used in the experiments in Table 2. R-membranes from *E. coli* C43(DE3)pLys showed lower specific activity.

Substrate inhibition

One of the outstanding features of the acid PPase in *Sulfolobus* membranes is its independence of divalent cations such as Mg^{2+} (Meyer and Schäfer 1992; Amano et al. 1993), and another is substrate inhibition (Amano et al. 1993). The latter has not been described for *S. acidocaldarius* acid PPase, which exhibits "simple Michaelis–Menten kinetics" (Meyer and Schäfer 1992). In *S. tokodaii* membranes, the rates of hydrolysis of PPi, ATP and ADP were maximal with substrate concentrations around 1–2 mM, and above that, the rates decreased concomitantly as the concentrations increased (Amano et al. 1993). Such inhibition was also observed for R-membranes (Fig. 2). In general, substrate inhibition can be explained in several ways: most simply by the formation of an abortive complex from the enzyme and excess substrate (Segel 1975). The mechanism of the substrate inhibition for *Sulfolobus* acid PPase is unknown, but Fig. 2 suggests that the substrate inhibition is not affected by the state of the enzyme, i.e., whether it is bound to membranes or solubilized.

Solubilization and purification of acid PPase from R-membranes

We tested Triton X-100, MEGA9, *n*-octyl-*β*-D-glucoside, and *n*-dodecyl-*β*-D-maltoside as to solubilization of the acid PPase from R-membranes. Every detergent was effective. The optimum protein: detergent ratio (w/w) was 1:5. In the presence of a detergent, the specific activity increased by about 50%.

Using Triton X-100, the acid PPase was solubilized from R-membranes (specific activity of 4.2 U/mg of protein), applied onto a Hi-trap column, and eluted with a linear gradient of imidazole. Three fractions were collected with imidazole concentration from 10 to 24 mM (fraction 1), from 24 to 36 mM (fraction 2), and from 36 to 48 mM imidazole (fraction 3), with a specific activity of 2.7, 4.1, and 18 U/mg of protein, respectively. About 4.2-fold purification from the R-membranes was achieved. SDS-PAGE revealed that the fraction 3 gave almost a single band on silver staining, and ant-His-tag blotting (Fig. 3).

K_m and V_{max} determination of the purified and membrane-bound PPases

Kinetic parameters as to hydrolysis of three substrates, PPi, ATP and ADP, at low concentrations and pH 3, were determined using S-membranes, R-membranes, and the purified enzyme as summarized in Table 2. The purified enzyme from R-membranes showed similar K_m values to

Table 2 Kinetic parameters for S-membranes, R-membranes and purified enzyme

Source	Substrate	K_m (mM)	V_{max} (μmol/min mg of protein)
S-membranes from *S. tokodaii*	PPi	0.42 ± 0.012	0.96 ± 0.02
	ATP	0.52 ± 0.003	0.33 ± 0.001
	ADP	0.56 ± 0.002	0.41 ± 0.001
R-membranes from *E. coli* (pSTH)	PPi	0.15 ± 0.002	3.08 ± 0.01
	ATP	0.17 ± 0.007	2.49 ± 0.05
	ADP	0.42 ± 0.004	2.22 ± 0.01
Purified PPase	PPi	0.39 ± 0.006	30.3 ± 0.31
	ATP	0.58 ± 0.007	20.4 ± 0.16
	ADP	0.58 ± 0.013	19.3 ± 0.30

Kinetic parameters were obtained from four sets of experiments for three samples (S-membranes, R-membranes and purified PPase). Each set of experiment was composed of five different concentrations of substrate (either ATP, ADP or PPi) <1 mM to avoid substrate inhibition (Fig. 2). The K_m and V_{max} values were calculated from Eadie to Hofstee plots

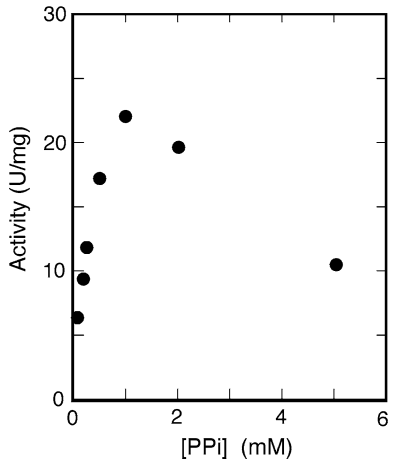

Fig. 2 Substrate inhibition of acid PPase of R-membranes. The R-membranes (1 mg of protein/mL) was incubated with each concentration of PPi in 50 mM phthalate buffer, pH 3.0 at 70°C for 5 min. Released Pi was determined

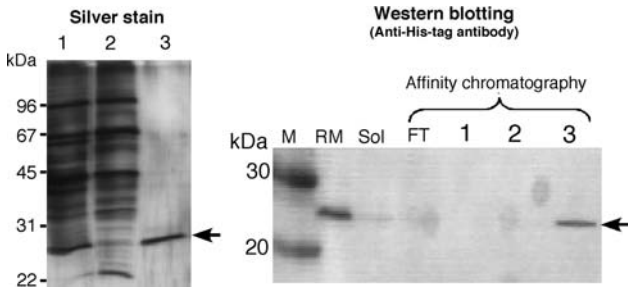

Fig. 3 Purification of acid PPase from R-membranes. *RM* R-membranes (30 μg protein), *Sol* solubilized fraction with 5% Triton X100 (5 μg protein), *FT* flowthrough from a Ni^{2+}-column (2 μg protein), *1*, *2*, and *3* three fractions (fraction 1, 2, and 3, respectively, with 0.5 μg protein each) eluted with increasing concentrations of imidazole (see text for detail)

those of S-membranes, but those of R-membranes were smaller, especially for PPi and ATP. The V_{max} value for PPi appears to be twofold higher than that for ATP, but catalytic turn-over seems almost the same, since two moles of Pi is released from one mole PPi.

The K_m values for PPi with acid PPase from *S. acidocaldarius* have been reported to be 0.6 mM (membranes) and 0.8 mM (purified enzyme) (Meyer and Schäfer 1992). These values are larger than those determined here, i.e., 0.42 ± 0.12 mM (S-membranes) and 0.39 ± 0.06 mM (purified PPase).

Effect of archaeal lipids on purified acid PPase

We have reported that acid PPase purified from S-membranes was stimulated on the addition of *Sulfolobus* membrane lipids (Amano et al. 1993). The activity of the purified PPase from the R-membranes was also enhanced upon the addition of *Sulfolobus* lipids in a similar manner (data not shown).

Acid PPase activity in the membranes of deletion mutants

The specific activities of the acid PPase in the R-membranes from the wild type *E. coli* (harboring pSTH), STHΔR, STHΔRK, STH-70, STH-76 and the control *E. coli* [harboring pET25b(+)] were 4.59 ± 0.42, 0.19 ± 0.012, 0.05 ± 0.004, 0.21 ± 0.027, 0.11 ± 0.025 and 0.07 ± 0.014 U/mg of protein, respectively. Deletions of Arg2, Arg2-Lys3, Tyr4-Ile69, and Tyr4-Arg75 resulted in 97, 100, 97 and 99% loss of activity, respectively. SDS-PAGE of these mutant membranes followed by Western blotting on to a PVDF filter and immunological analysis revealed that no His-tagged protein was present in the R-membranes of these mutants (data not shown). These results indicate that the positively charged basic amino acids in the N-terminal of ST2226, as well as the residues 4–69 (or 75) possibly twice-spanning membrane (Fig. 1), are also required for the protein to be integrated into the membrane.

The above results may be related to the general criterion that secretory signal peptides are characterized by a positively charged N-terminal region (von Heijne 1988).

Conclusion

Our present study revealed that; (1) S-membranes catalyzed hydrolysis of not only PPi (ATP, ADP) but also C_5PP and C_{10}PP (compounds related to dolicholPP); (2) C_5PP was hydrolyzed to C_5P (releasing Pi) but C_5P was not hydrolyzed any more; (3) the PPase activities in S- and R-membranes were not inhibited by bacitracin; (4) the ecto-PPase gene (*st*2226) was functionally expressed in *E. coli* membranes with optimal pH around 3 and optimal temperature around 80°C and substrate specificity toward PPi, ATP and ADP. Similar values were found in original S-membranes; (5) no additional gene such as *sabc* or *simp* co-transcribed with *sepp* in *S. acidocaldarius* (Moll and Schäfer 2004) was necessary for PPase activity; (6) positive charges at the N-terminal and the residues in the first two membrane-spanning regions were necessary for the enzyme to be incorporated correctly into the membrane.

Glycosylation of protein occurs across the membrane. Lipid carrier that charges glycan chain in the membrane is C_{55}-isoprenylpyrophosphate in bacteria and dolichol(pyro)phosphate in archaea and eukarya. The elaborate process for protein glycosylation in eukarya is not fully elucidated, but is thought to be originated from a simpler archaeal system. Bacitracin, an antibiotic acting on

bacterial C_{55}-isoprenylpyrophosphate phosphatase inhibits glycosylation of *Methanococcus* flagellins and *Sulfolobus* S-layer (Eichler and Adams 2004).

These suggest the relation of *Sulfolobus* membrane-bound acid pyrophosphatase to glycosylation of protein, although the enzyme was insensitive to bacitracin possibly due to low pH of the reaction.

Deletion of ST2226 or its homologue would bring informative results on the physiological function of the enzyme, since gene manipulation system of the genus *Sulfolobales* is rapidly in progress.

In *S. acidocaldarius*, heterologous expression of the acid PPase gene (*sepp*) has been reported. An 18-kDa product has been detected in an in vitro transcription/translation system with a reticulocyte lysate (Moll and Schäfer 2004). However, the yield seems quite low. Enzyme activity of the product has not been checked.

Our gene expression system produced *S. tokodaii* acid PPase integrated into the *E. coli* membranes, the content being higher than that in the original S-membranes. Homogeneous enzyme was obtained by solubilization from R-membranes followed by His-tag column chromatography. The expression system will be beneficial to the mutational studies to elucidate the role of each residues or any region of this novel enzyme, as well as crystallization studies, since it is highly stable against extreme condition such as high temperature.

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