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Functional Enhancement by Single-residue Substitution of *Streptomyces coelicolor* A3(2) H^+-translocating Pyrophosphatase

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H^+-translocating pyrophosphatase converts energy from hydrolysis of pyrophosphate to active H^+ transport across biomembranes. Mutational analysis of *Streptomyces coelicolor* A3(2) enzyme revealed that amino acid substitution of Phe-388 and Ala-514 altered the enzyme activity. Both residues are located at the interface between the transmembrane domains and cytosolic loops, in which the catalytic domain exists. Systematic amino acid substitution was carried out using the *Escherichia coli* heterologous expression system. Two of the 38 mutant enzymes, F388Y and A514S, showed a high ratio of H^+-pump to substrate hydrolysis without decrease in the substrate hydrolysis activity, indicating high energy-coupling efficiency.

Key words: energy coupling, H^+-pyrophosphatase, proton pump, site-directed mutagenesis, structure–function relationship.

Abbreviations: H^+-PPase, H^+-pyrophosphatase; ScPP, *Streptomyces coelicolor* A3(2) H^+-PPase; PPi, pyrophosphate.

Proton-translocating inorganic pyrophosphatase (H^+-PPase) is a proton pump that transports H^+ across membranes using energy generated by substrate hydrolysis. Because this enzyme consists of a single polypeptide and uses a simple substrate pyrophosphate (PPi) (*1*), the enzyme might be a useful molecule to study the energy-coupling mechanism of the proton pump. Many functional residues and motifs of H^+-PPases have been identified by site-directed mutagenesis for the enzymes of *Arabidopsis thaliana* (*2*–*4*), *Vigna radiata* (*5*–*7*), *Rhodospirillum rubrum* (*8*, *9*), *Carboxydothermus hydrogenoformans* (*10*) and *Streptomyces coelicolor* A3(2) (*11*–*15*).

In this study, we focus on *S. coelicolor* A3(2) H^+-PPase (ScPP), which is comprised of 794 amino acid residues with 17 transmembrane domains, and can be expressed in *Escherichia coli* (*11*, *16*). To analyze the structure–function relationship of H^+-PPase, we prepared more than 3000 ScPP mutants and determined their activity. Through comprehensive analysis, we have evaluated the functional contribution of individual residues to the substrate hydrolysis, proton translocation and energy coupling (*14*, *15*). Most ScPP mutants lost or decreased PPase and H^+-pump activities. Interestingly, a few mutant enzymes efficiently pumped protons without suppression of PPase activity. For example, a mutant of A514G had the same PPase activity and two-fold H^+-pump activity compared with the wild-type (WT) ScPP (*15*). Further analysis provided a similar mutant enzyme, whose residue was exchanged at Phe-388. Here we focused on these two residues Phe-388 and Ala-514, and prepared a series of ScPP mutants to determine the effect on enzyme function.

We substituted Phe-388 and Ala-514 of ScPP with all of the other residues. Mutant derivatives of ScPP were generated from a synthetic DNA of ScPP with a QuikChange site-directed mutagenesis kit (Stratagene) as described previously (*5*, *11*). The nucleotide sequences of the mutants were confirmed by DNA sequencing. Expression of ScPP and mutant derivatives in *E. coli* and preparation of membrane from the cells were carried out as described previously (*14*, *16*). *Escherichia coli* cells contain a soluble-type PPase (*1*). To eliminate the activity of the soluble PPase, we measured the activity in the presence of sodium molybdate and sodium fluoride, inhibitors of soluble-type PPases and phosphatases (*16*). As a result, the membranes prepared from *E. coli* cells having a vacant vector did not give PPase activity (*16*). The protein content was quantified using the Bradford method (*17*). We confirmed the accumulation of ScPP proteins by immunoblotting with an antibody against the C-terminal region of ScPP (positions 769–786, KRRGIAMGDEDDADPEPK), which was newly prepared in this study. Most mutants were accumulated as an 80 kDa protein in *E. coli* membranes at a level similar to that of the WT enzymes, although several Ala-514 variants were accumulated at relatively high levels (Fig. 1A). The results of F388L and F388S are not shown here, because these two mutants were examined previously (*15*).

PPi hydrolysis was measured at 30°C as described previously (*18*, *19*), in 5 mM Bicine-NaOH (pH 8.0), 100 mM KCl, 1 mM $MgCl_2$, 0.15 M sucrose, 0.4 mM Na_4PPi, 1 mM sodium molybdate and 0.5 mM NaF. The actual substrate for H^+-PPase is the Mg_2PPi complex (*1*, *19*) and the concentration of Mg_2PPi under the assay condition was

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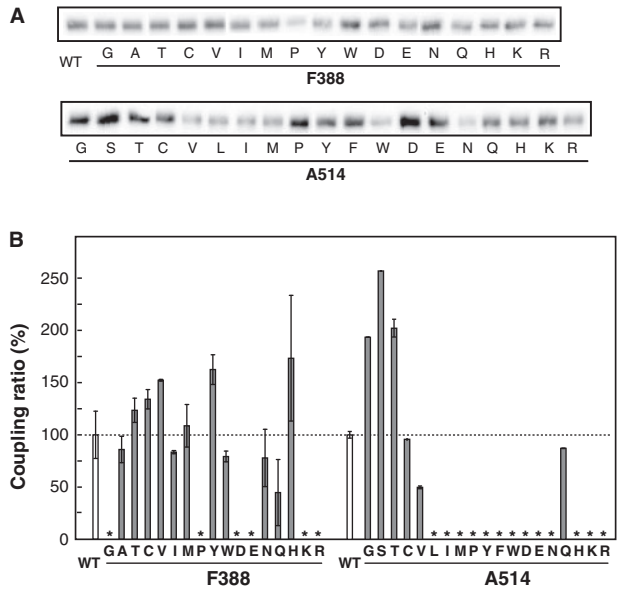

Fig. 1. **Site-directed mutants at Phe-388 and Ala-514.** The Phe-388 and Ala-514 of ScPP were substituted with other amino acids and the resulting 38 mutant enzymes were expressed in *E. coli*. (A) Membrane vesicles were prepared from *E. coli* cells expressing WT ScPP and site-directed mutants as indicated. Membranes (5 μg) were subjected to immunoblotting with anti-ScPP antibodies. (B) PPase and H^+-pump activities were measured and the coupling efficiencies were calculated as the ratios of H^+-pump activities to PPase activities, and are expressed relative to that of WT ScPP. PPase activity, H^+-pump activity and coupling ratio of WT ScPP were 87 nmol PPi min^{-1} mg^{-1}, 71.8%ΔF min^{-1} mg^{-1}, and 0.80%ΔF $nmol^{-1}$ PPi, respectively. Asterisks show the mutant variants with no H^+-pump activity.

calculated to be 71 μM, which is sufficient for expression of the full activity of ScPP (*16*). PPi-dependent H^+-transport activity was measured as the initial rate of fluorescence quenching of acridine orange (1 μM) in the same buffer at 25°C with a Shimadzu RF-5300PC fluorescence spectrophotometer (*18*, *20*). Then we calculated the coupling ratio, *i.e.* the relative ratio of H^+-pump activity to PPase activity.

PPase activities of the 15 ScPP mutants (F388G, F388P, F388D, F388K, F388R, A514P, A514Y, A514F, A514W, A514D, A514E, A514N, A514H, A514K and A514R) were <20% of WT ScPP (Supplementary Fig. S1A). These 15 mutants and another 4 mutants (F388E, A514L, A514I and A514M) exhibited no H^+-pump activity. The relative values of the remaining 17 mutants are shown in Fig. 1B. The protein level of ScPP varied with the variant (Fig. 1A). However, the coupling ratio might not depend on the protein level. Several mutants (F388A, F388T, F388C, F388I, F388M, F388N, A514C and A514Q) kept the same energy-coupling efficiency and two mutants (F388Q and A514V) showed about 50% lower efficiency than the WT ScPP. The coupling efficiency of two mutants F388L and F388S was ~40% as reported previously (*15*). Six mutants (F388V, F388Y, F388H, A514G, A514S and A514T) had increased coupling ratios. We selected F388Y and A514S, which had the highest ratio among the Phe-388 and Ala-514 variants, respectively, for further analysis. The value of F388H was high, but varied with the preparation. This may be due to the instability of the mutant protein in the *E. coli* membrane.

In addition to F388Y and A514S mutants, we prepared an additional ScPP mutant with both substitutions of F388Y and A514S (referred to as double mutant hereafter) to examine synergistic effect of the two substitutions. We newly prepared membrane fractions from *E. coli* cells expressing WT ScPP and these three ScPP mutants. In this experiment, we quantified the ScPP protein immunochemically with an antibody to the substrate-binding region of H^+-PPase that is highly conserved among H^+-PPases in various organisms (*21*). H^+-PPase was purified from mung bean hypocotyls as described previously (*18*) and used in immunoblotting as a standard protein (Fig. 2A). From the obtained standard curve, the protein amounts of ScPP and its variants were calibrated to be 43–53 ng in 5 μg of *E. coli* membranes (Fig. 2B).

To evaluate the energy-coupling efficiency, we assayed the PPase and H^+-pump activities under the same conditions, namely, protein concentration, buffer pH, temperature and the medium components including KCl, $MgCl_2$, sucrose and Na_4PPi. It should be noted that the initial reaction velocity is essential for comparison with the PPase activity because H^+-pump activity is apparently decreased when a steep pH gradient is formed in membrane vesicles. This is due to leak of protons from the membrane vesicles. In the present study, the initial velocities were determined and shown as the velocity per min. In a short assay period (20 s), the product inhibition by orthophosphate did not occur. H^+-PPase was not inhibited by orthophosphate at 0.1 mM, but affected at 0.4 mM (data not shown).

Molybdate, which was added to inhibit the soluble-type PPase, may bind orthophosphate in the reaction medium and to affect the enzyme activity. Molybdate has no effect on the PPase (*18*) and H^+-pump activities under the present assay conditions (data not shown). Thus, there was no product inhibition or side effect of molybdate under these assay conditions. We concluded that the PPase and H^+-pump activities were adequately determined under the same conditions.

Under the same conditions we observed relatively high PPase and H^+-pump activities reproducibly. F388Y, A514S and double mutants had 20%, 60% and 70% higher PPase activity, respectively, than the WT ScPP (Fig. 2C). The H^+-pump activity was 2.5-, 4-, and 4.2-fold that of the WT ScPP, respectively (Fig. 2D and E). They had coupling ratio of more than 200% of the WT ScPP (Fig. 2F). We examined whether or not the introduction of ScPP changed the membrane integrity and the activity of an endogenous proton pump of *E. coli* membranes. There was no change in the activity of ATP-dependent H^+ pump, which is an *E. coli* F-type ATPase, between the WT and mutant-harboring *E. coli* membranes (Fig. S2). This suggests the absence of marked differences in membrane integrity or proton leakiness. Therefore, the increment in the coupling ratio might be due to the mutation of the enzyme not the membrane properties.

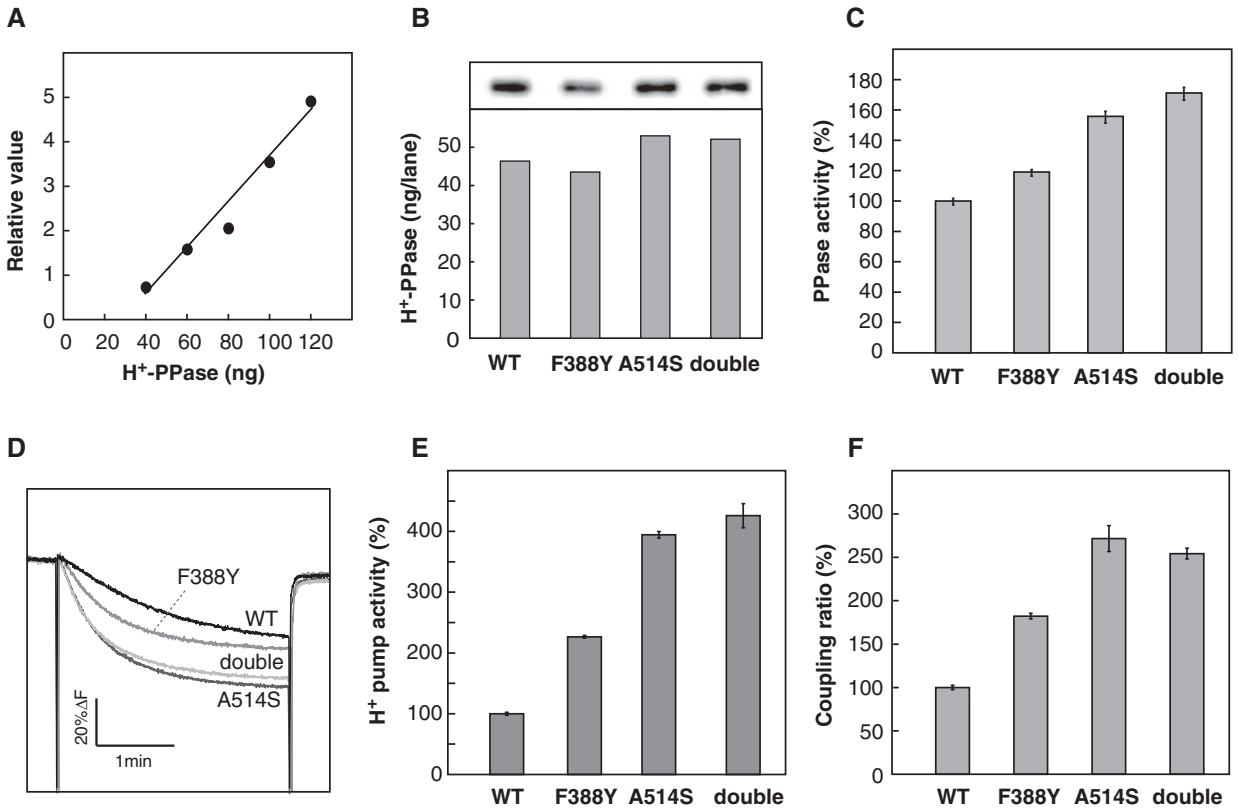

Fig. 2. **Activities of high-pumping ScPP mutants.** Membrane fractions of wild type, F388Y, A514S and a double F388Y/A514S mutant were newly prepared from *E. coli* cells. (A) Calibration curve of H⁺-PPase purified from mung bean in immunoblot analysis. The purified enzyme was subjected to immunoblotting with anti-H⁺-PPase and the intensity of immunostained band was plotted. Standard curve for H⁺-PPase content was prepared from the intensity of each amount. (B) Protein amount of ScPP accumulated in *E. coli* membranes. Aliquots of the membrane preparation (5 μg) were subjected to SDS–PAGE and subsequent immunoblotting with anti-H⁺-PPase. The antigen protein levels were calculated using the standard curve. (C) PPase activity. (D and E) H⁺-pump activity. Both enzyme activities were assayed under the same medium at 30°C and expressed relative to that of wild type. (F) The coupling ratio calculated from PPase and H⁺-pump activities. Values are shown as percentage to that of WT ScPP.

For further characterization of these highly efficient ScPPs, we determined their affinities for the substrate. In this experiment, we used a reaction period of 30 min to obtain the activity at low substrate concentrations. The substrate–saturation curve of the WT ScPP was well consistent with the previous report (*14*, *15*). The WT and mutant ScPPs showed the maximal activity at 0.2 mM PPi (Fig. 3). Thus, PPi at a concentration of 0.4 mM was adequate for measuring enzyme activity as the standard condition. The substrate concentrations of Mg_2PPi, an actual substrate (*1*, *19*), that gave the half-maximal velocity ($K_{0.5}$) were 6.4, 2.4, 6.7 and 9.4 μM for WT, F388Y, A514S and double F388Y/A514S mutant ScPPs, respectively. Thus, there was no marked difference in the values between the WT and highly efficient mutant ScPP, suggesting no change in the apparent affinity of the mutant ScPPs for Mg_2PPi.

Comprehensive mutational analysis of ScPP revealed that F388Y and A514S are highly efficient enzymes with a coupling ratio approximately two-fold that of the WT ScPP. H⁺-PPase hydrolyzes PPi in the cytoplasmic side and then actively translocates H⁺ across the membrane. The catalytic site of PPi hydrolysis is located in the cytoplasmic domain of the enzyme. Hydrolysis of PPi may induce conformational change of the catalytic domain and the membrane helices. In the membrane-topology model of the enzyme, highly conserved, functional motifs are located mainly in loops *e* and *k* and many functional residues are located in TM5, TM9, TM10 and TM11 (*13*, *15*). Phe-388 is located at the interface between TM9 and loop *i*, and Ala-514 at the interface between the loop *k* and TM12 (for details, Fig. S3).

Next, we discuss the increased PPase activity of the A514S mutant enzyme and the enhancement of the coupling efficiency of F388Y, A514S and double mutant ScPPs. Ala-514 is one of the conserved residues of various H⁺-PPases (*15*) and its substitution with Ser, Gly or Thr enhanced the PPase activity (data not shown). There are two possible explanations. First, the amino acid substitution of Ala-514 may accelerate the PPase catalytic process, such as release of inorganic phosphate from the enzyme after PPi hydrolysis. Second, the substitution may lower the energy barrier from the PPi hydrolysis to active proton pump and consequently accelerate the rate of PPi hydrolysis. H⁺-PPase probably keeps energy from PPi hydrolysis as a transient

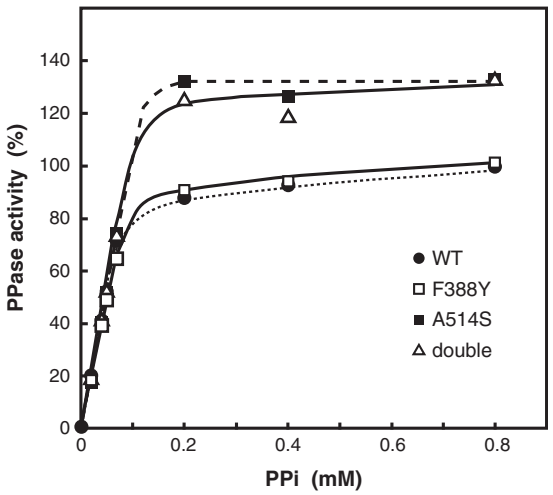

Fig. 3. **Substrate-activity curves of H$^+$-PPase mutants.** PPase activities of WT, F388Y, A514S and a double F388Y/A514S mutant were measured in various concentrations of PPi. The reaction period was 30 min to obtain reliable values of activity at low substrate concentrations. Substrate–saturation curves of WT ScPP (closed circles), F388Y (open squares), A514S (closed squares) and the double mutants (open triangles). These results are expressed as the ratio to the activity of WT enzyme at 0.4 mM of PPi.

conformational change of protein structure, especially in the catalytic domain, and then transfers the energy to the H$^+$-transport process through the TMs. The amino acid substitution may promote the conformational change involved in the energy conversion. It should be noted that each mutation of F388Y and A514S was an addition of a hydroxyl group to the original residue. This small change may not disturb the enzymatic functionality and may provide a benefit for energy conversion.

We prepared a double mutant F388Y/A514S to examine the synergic effect of dual-site substitution. The double mutant enzyme showed only a small additive effect on the PPase activity (Fig. 2C) and the same H$^+$-pump activity as A514S (Fig. 2D and E). This result suggests the functional significance of Ala-514 in the energy conversion and/or H$^+$ pump when compared with Phe-388. Ala-514 is highly conserved among the H$^+$-PPases of various organisms (*15*). On the other hand, the residue corresponding to Phe-388 varies with the organism. F388Y is an intriguing mutant from an evolutional viewpoint. H$^+$-PPases are divided into two groups according to their K$^+$ requirement: one (type I) requires K$^+$ for maximal activity, whereas the other does not (type II) (*2*, *10*). H$^+$-PPase of *S. coelicolor* A3(2) belongs to type II and the plant vacuolar enzyme to type I. The residue corresponding to Phe-388 is occupied by Phe in the type II enzymes and by Tyr in the type I enzymes. Since the specific activity of the purified ScPP is lower than that of type I enzymes such as mung bean H$^+$-PPase (*16*, *18*), the substitution of Phe-388 to Tyr may be regarded as evolutional improvement of H$^+$-PPase from type I to type II.

In relation to the highly efficient mutants, two mutants E229D and E427D of *A. thaliana* H$^+$-PPase type I have been reported to have a high coupling ratio (*4*). The residue corresponding to Glu-229 of *A. thaliana* enzyme is not conserved in ScPP (*14*), and the residue corresponding to Glu-427 is conserved in most H$^+$-PPases. However, this position is occupied by Gly at 386 in ScPP (*15*). G386E mutant ScPP reportedly has low PPase activity, but a high coupling ratio compared with the WT ScPP (*15*). Thus, the positions corresponding to Glu-427 of *A. thaliana* H$^+$-PPase and Gly-386 and Phe-388 of ScPP is a topological key site for energy coupling.

In the present study, we assayed the PPase and H$^+$-pump activities under the same conditions and observed a coupling ratio two-fold that of the WT ScPP. This is the first observation of mutant H$^+$-PPases with a high ratio of H$^+$ pump activity to PPase activity with increased level of PPase activity. The H$^+$/PPi ratio of plant vacuolar H$^+$-PPase has been reported to be 1 : 1 (*22*). Therefore, the H$^+$/PPi stoichiometry of F388Y and A514S mutants may be two-fold that of the WT ScPP. If so, the energy-coupling efficiency may be two-fold that of the WT ScPP. Further detailed analysis will be needed to obtain the H$^+$/PPi ratio of F388Y and A514S mutants of H$^+$-PPase. How the H$^+$-PPases in living cells are kept at the H$^+$/PPi ratio of 1 : 1 remains an issue for the future.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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