Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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Published online: 22 May 2014.

To cite this article: Yasuhiro MIHARA, Kohki ISHIKAWA, Ei-ichiro SUZUKI & Yasuhisa ASANO (2004) Improving the Pyrophosphate-inosine Phosphotransferase Activity of Escherichia blattae Acid Phosphatase by Sequential Site-directed Mutagenesis, Bioscience, Biotechnology, and Biochemistry, 68:5, 1046-1050, DOI: 10.1271/bbb.68.1046

To link to this article: http://dx.doi.org/10.1271/bbb.68.1046

Biosci. Biotechnol. Biochem., **68** (5), 1046–1050, 2004

Improving the Pyrophosphate-inosine Phosphotransferase Activity of *Escherichia blattae* Acid Phosphatase by Sequential Site-directed Mutagenesis

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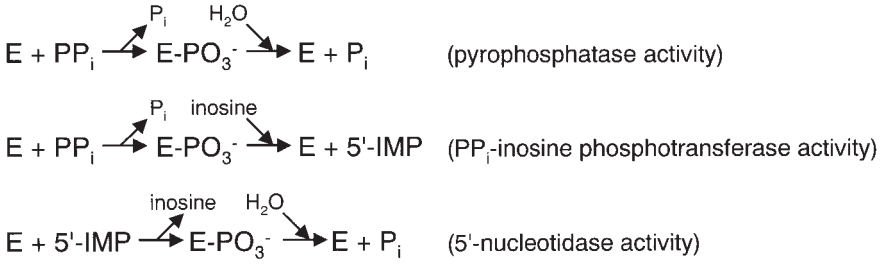
Received October 10, 2003; Accepted January 27, 2004

Escherichia blattae **acid phosphatase/phosphotransferase (EB-AP/PTase) exhibits C-5′-position selective pyrophosphate-nucleoside phosphotransferase activity in addition to its intrinsic phosphatase. Improvement of its phosphotransferase activity was investigated by sequential site-directed mutagenesis. By comparing the primary structures of higher 5′-inosinic acid (5′-IMP) productivity and lower 5′-IMP productivity acid phosphatase/phosphotransferase, candidate residues of substitution were selected. Then a total of 11 amino acid substitutions were made with sequential substitutions. As the number of substituted amino acid residues increased, the 5′-IMP productivity of the mutant enzyme increased, and the activity of the 11 mutant phosphotransferases of EB-AP/PTase reached the same level as that of *Morganella morganii* AP/PTase. This result shows that Leu63, Ala65, Glu66, Asn69, Ser71, Asp116, Thr135, and Glu136, whose relevance was not directly established by structural analysis alone, also plays an important role in the phosphotransferase activity of EB-AP/PTase.**

Key words: *Escherichia blattae*; acid phosphatase; pyrophosphate-inosine phosphotransferase; 5′-inosinic acid

As well as amino acid production, nucleotide production is another key area of interest in biotechnology, because 5′-inosinic acid (5′-IMP) and 5′-guanylic acid (5′-GMP) have a characteristic taste and are used as a flavor potentiator in various foods. Since purine nucleosides such as inosine and guanosine can be efficiently produced by fermentation,[1,2] the progress of large-scale 5′-nucleotide production depends largely on the improvement of the nucleoside-phosphorylation process. Nowadays, there are two main phosphorylation methods. One is a chemical phosphorylation process that uses phosphoryl chloride ($POCl_3$),[3] and the other is an enzymatic phosphorylation process that uses inosine kinase of *E. coli*.[4,5] An enzymatic phosphorylation process is used by Kyowa Hakko Kogyo, and IMP production involves inosine fermentation by *Corynebacterium ammoniagenes* coupled with energy-requiring phosphorylation by guanosine/inosine kinase.

Recently, we have investigated a novel nucleoside phosphorylation process. We found that *Morganella morganii* acid phosphatase exhibits C-5′-position selective pyrophosphate (PP_i)-nucleoside phosphotransferase activity in addition to its intrinsic phosphatase activity, as shown in Scheme 1.[6] Then its phosphotransferase activity was improved by random mutagenesis, and a primary nucleoside phosphorylation process using PP_i as



Scheme 1.

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a phosphate donor was established.[7,8] Next we embarked on research to understand the structure activity relationship of these enzymes and to obtain an even more efficient enzyme with further improved its phosphotransferase activity. We have isolated genes encoding an acid phosphatase with regioselective phosphotransferase activity (AP/PTase) from five genes of enteric bacteria.[9] Among five AP/PTases, we have determined the crystal structure of *Escherichia blattae* AP/PTase at 1.9 Å, and selected the enzyme as the target of rational mutagenesis.[10] We designed several mutations and introduced them into the G74D/I153T mutant, which was generated using knowledge derived from random mutagenesis of the *M. morganii* enzyme, and succeeded in producing an industrially viable mutant enzyme, S72F/G74D/I153T, with a further reduced K_m value for inosine.[11]

The primary structures of *E. blattae* AP/PTase and *M. morganii* AP/PTase were highly homologous, and the G74D/I153T mutations were also effective in both enzymes, but the productivity of the G74D/I153T mutant of *E. blattae* was greatly inferior to that of the G74D/I153T mutant of *M. morganii*. Although we have investigated the improvement of AP/PTases as mentioned above, the reason for such a difference remains to be understood. In the present study, we enhanced the phosphotransferase activity of EB-NSAP by a sequential site-directed mutagenesis. Through this attempt to enhance phosphotransferase activity, the role of some residues that are important for phosphotransferase activity but whose relevance was not fully established by structural analysis, was successfully elucidated.

Material and Methods

Strains, plasmids, and culture conditions. Escherichia coli JM109[12] was used as the host strain. Plasmid, pEAP320 containing the AP/PTase gene *phoC* of *Escherichia blattae*,[10] was used as a template in PCR mutagenesis. Plasmid, pUC18 (Takara Shuzo, Japan), was used as a cloning vector. *E. coli* transformants were cultured aerobically at 37 °C on Luria–Bertani medium containing ampicillin (50 μg/ml).

DNA mutagenesis and purification of mutant AP/PTases. All basic recombinant DNA procedures, such as isolation and purification of DNA and transformation of *Escherichia coli*, were performed as described by Sambrook *et al.*[13] Site-directed mutagenesis was performed on the *E. blattae* acid phosphatase gene, cloned in pUC18[12] using the Quick Change mutagenesis kit (Stratagene) according to its protocol. Mutations were confirmed by sequencing by the dideoxynucleotide chain termination method with a Dye Terminator Cycle sequencing kit (Perkin-Elmer) and a DNA sequencer (model 373A, Perkin-Elmer). Synthesized universal and fragment-specific oligonucleotides for each clone were used as primers.

Each acid phosphatase was purified from harvested cells of *E. coli* JM109 transformants harboring mutant *phoC* by ammonium sulfate fractionation and ion-exchange, and hydrophobic column chromatographies, as described previously.[10] The purity of the recovered samples was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14% polyacrylamide).

Enzyme assay. Phosphotransferase activity was assayed in a standard reaction mixture containing 100 μmol of sodium acetate buffer (pH 5.0), 40 μmol of inosine, 100 μmol of tetrasodium pyrophosphate, and the enzyme solution in a total volume of 1 ml. The reaction mixture was incubated for 10 m at 30 °C and then the reaction was stopped by adding 0.2 ml of 2 N HCl. Quantitative determination of inosine and 5′-IMP was carried out by HPLC as described previously.[8] One unit of phosphotransferase activity was defined as the amount of enzyme that produces 1 μmol of 5′-IMP per minute under the assay conditions. 5′-Nucleotidase activity was assayed in a standard reaction mixture containing 100 μmol of sodium acetate buffer (pH 4.0), 10 μmol of 5′-IMP, and the enzyme solution in a total volume of 1 ml. The reaction mixture was incubated for 5 m at 30 °C. The reaction was stopped by adding 0.2 ml of 2 N HCl, and then released inosine was measured by HPLC. One unit of 5′-nucleotidase activity was defined as the amount of enzyme that produced 1 μmol of inosine per minute under the assay conditions. Kinetic constants for inosine in the transphosphorylation reaction and for 5′-IMP in the dephosphorylation reaction were measured at pH 4.0, at which 5′-IMP synthesis was performed. The initial velocities were determined under the assay conditions described above, and the steady-state kinetic constants were calculated by using a Hanes–Woolf plot. Since the solubility of inosine was limited, kinetic constants for inosine were determined with a substrate concentration ranging from 0.5 to 80 mM.

Synthesis of 5′-IMP by E. coli overproducing mutant PhoC. Cells of each *E. coli* JM109 transformant were cultured in LB medium and harvested from culture broth by centrifugation at 10,000 × *g* for 10 m. The harvested cells were washed with 10 mM potassium phosphate buffer (pH 7.0) and then suspended in the same buffer. Cell growth was estimated turbidimetrically by means of a dry-cell calibration curve for absorbance at 610 nm: 0.24 of dry cell weight per ml was equivalent to 1.0 of optical density at 610 nm. The standard reaction mixture for 5′-IMP synthesis contained various concentrations of inosine, 150 g/l (200 mM) disodium hydrogen pyrophosphate, 1 mM sodium acetate buffer (pH 4.0), and 10 g/l (dry weight) of cells in 10 ml. The reaction was carried out at 30 °C with moderate shaking and stopped by adding 1 ml of 2 N HCl. Synthesized 5′-IMP was calculated as IMP·2Na·7.5H_2O (MW 527).

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Ebl  : 63 LAAEDANLSSGGVANAFSGA 82--- 113 SAKDHYMRIRPFAFYGVSTC NTTEQDKLSKNGSYPSGHTS IGWATALVLA 162
Eae  : 61 LAAEDANLSAGGVANAFSSA 80--- 111 SAKEKYMRIRPFAFYGVSTC NTTEQDKLSKNGSYPSGHTS IGWATALVLA 160
Kpl  : 61 LAAEDANLSAGGVANAFSAA 80--- 111 GAKEKYMRIRPFAFYGVSTC NTTEQDKLSKNGSYPSGHTS IGWATALVLA 160
Mmo  : 61 QAQADADLAAGGVATAFSGA 80--- 111 SAKEHYMRIRPFAFYGTETC NTKDQKKLSTNGSYPSGHTS IGWATALVLA 160
Pst  : 61 QAAKDADLAAGGVANAFSEA 80--- 111 SAKEKYMRIRPFAFYGVATC NTKDQDKLSKNGSYPSGHTA IGWASALVLS 160
           *  ** *  **** *** *          **  ********** ** **  * *** *********  **** ****
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Fig. 1. Amino Acid Alignment of the Deduced Amino Acid Sequences of AP/PTases around Target Residues.

Numbering of the amino acids starts at the amino-terminus of the mature proteins. Asterisks show identical amino acid residues. The positions of target residues are boxed in gray. The Gly74 and Ile153 residues, of which substitutions to Asp and Thr respectively were reported to be effective for the improvement of 5′-IMP productivity in *M. morganii* AP/PTase,[8] are boxed in gray. The sequences shown are: Mmo, *Morganella morganii*; Pst, *Providencia stuartii*; Eae, *Enterobacter aerogenes*; Ebl, *Escherichia blattae*; Kpl, *Klebsiella planticola*. The conserved residues are marked with an asterisk.

Table 1. Plasmids in Which AP/PTase Genes Were Cloned in This Study

Plasmid	Parent	Nucleic acid substitutions	Amino acid substitutions	Mutated enzyme	Reference
pEAP320	—			Wild type	10
pEAP330	pEAP320	T458C	I153T	I153T	11
pEAP340	pEAP330	G221A, G222T	G74D	G74D/I153T	11
pEAP350	pEAP340	T188A, G193C, C194A, A197C	L63Q, A65Q, E66A	L63Q/A65Q/E66A/G74D/I153T	This study
pEAP360	pEAP350	C404A, C405A, G408C	T135K, E136D	L63Q/A65Q/E66A/G74D/T135K/E136D /I153T	This study
pEAP370	pEAP360	A205G, A211G, G212C, A214G, G215C	N69D, S71A, S72A	L63Q/A65Q/E66A/N69D/S71A/S72A/ G74D/T135K/E136D/I153T	This study
pEAP380	pEAP370	T348A	D116E	L63Q/A65Q/E66A/N69D/S71A/S72A/ G74D/D116E/T135K/E136D/I153T	This study

Results and Discussion

Site-directed mutagenesis for an improved PP_i-inosine phosphotransferase activity mutant

We have isolated genes encoding an acid phosphatase with regioselective phosphotransferase activity (AP/PTase) from *Enterobacter aerogenes*, *Escherichia blattae*, *Klebsiella planticola*, *Morganella morganii*, and *Providencia stuartii*, and compared the primary structures and enzymatic characteristics of these enzymes. Although they were highly homologous in primary structure, the 5′-IMP productivities of the *E. aerogenes*, *E. blattae*, and *K. planticola* enzymes were inferior to those of the *M. morganii* and *P. stuartii* enzymes.[9] This suggested that local sequence differences are responsible for the differences in the phosphotransferase reaction. Based on the alignment of the sequences of five AP/PTases, candidate residues for amino acid substitution of EB-AP/PTase were selected as shown Fig. 1, and each mutation was introduced additively on the EB-AP/PTase gene cloned on pUC18. The target residues that located adjacently were mutated at one time. Each mutant plasmid was used as the PCR template of the next generation. Finally, the mutant plasmid coding the mutant enzyme of which 11 amino acid residues were substituted was constructed as shown Table 1. All mutant enzymes were overexpressed in *E. coli* JM109. No changes in the levels of production of mutant enzymes in each *E. coli* transformant were observed under the culture conditions used.

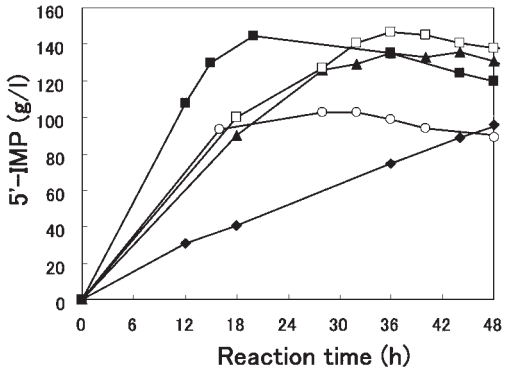

Fig. 2. 5′-IMP Synthesis by *E. coli* Overproducing Each AP/PTase.

The time course of 5′-IMP synthesis by resting cells of *E. coli* JM109 (pEAP340); ◆, *E. coli* JM109 (pEAP350); ○, *E. coli* JM109 (pEAP360); ▲, *E. coli* JM109 (pEAP370); □, and, *E. coli* JM 109 (pEAP380); ■ was measured. The reaction was carried out at pH 4.0 and 30 °C in a reaction mixture consisting of 0.1 M sodium acetate buffer (pH 4.0) containing 40 g/l (148 mM) inosine, 150 g/l (676 mM) disodium hydrogen pyrophosphate and 1 g/l (dry weight) of each type of cell.

Synthesis of 5′-IMP by E. coli overproducing the mutant PhoC acid phosphatase

The time course of 5′-IMP production using *E. coli* JM109 overproducing each mutant enzyme is shown in Fig. 2. As we described previously, substitution of G74D/I153T increases the 5′-IMP productivity of EB-NSAP.[10] In spite of the fact that its productivity was inferior to that of the *M. morganii* G72D/I151T mutant

AP/PTase, 104 g/l 5′-IMP was produced with a molar yield of 52% from inosine using *E. coli* overproducing the G74D/I153T mutant of the EB-AP/PTase. As the number of substituted amino acid residues increased, the productivity of the mutant enzyme increased. Using *E. coli* overproducing the 10 amino acid substituted mutant, the L63Q/A65Q/E66A/N69D/S71A/S72A/G74D/T135K/E136D/I153T mutant EB-AP/PTase, 145 g/l of 5′-IMP was obtained with a molar yield of 74% from inosine. By the mutation of D116E, the phosphotransferase reaction rate increased almost two times, and using *E. coli* overproducing the 11 amino acid substituted mutant, L63Q/A65Q/E66A/N69D/S71A/S72A/G74D/D116E/T135K/E136D/I153T mutant EB-AP/PTase, 156 g/l of 5′-IMP was obtained with a molar yield of 79% in 24 h. This was almost the same productivity as that of the *E. coli* overproducing *M. morganii* G72D/I151T mutant AP/PTase.

Characterization of mutant acid phosphatases

Each mutant enzyme was purified from crude extracts of *E. coli* JM109 transformant and its enzymatic characteristics were investigated. The kinetic constants of these enzymes in the transphosphorylation and dephosphorylation reactions are summarized in Table 2. One key distinction of the improved mutant was a reduction in the K_m value for inosine in the phosphotransferase reaction to approximately one-third of that in the wild type. In spite of the decrease due to the effect of the two mutations, the K_m value for inosine in the phosphotransferase reaction of the G74D/I153T mutant was 109 mM, which was extremely high, and above the achievable inosine concentration of 80 mM. As the number of substituted amino acid residues increased, the K_m value for inosine decreased without affecting the V_{max} value very much, and the 10 amino acid substituted mutant EB-AP/PTase exhibited almost the same K_m value for inosine as that of the *M. morganii* G74D/I153T mutant AP/PTase (43 mM).[8] This result underlines the importance of lower K_m values for efficient nucleotide production. The D116E mutation appeared not to contribute to the decrease in the K_m value for inosine, but the V_{max} value of the 11 amino acids substituted mutant EB-AP/PTase in the phosphotransferase reaction increased approximately two times. EB-AP/PAase also catalysed the dephosphorylation reaction, and a decrease in the 5′-nucleotidase activity was another key distinction of the improved mutant. The V_{max} value of the 5 amino acids substituted mutant in the dephosphorylation reaction decreased to almost half of that of the G74D/I153T mutant. There were only small changes in the kinetic parameters by the 5 subsequential mutations. The V_{max} value in the dephosphorylation reaction was also increased by the D116E mutation.

Although the target residues were selected only for the comparison of primary structures of homologous proteins, all 11 residues were placed on one side of the EB-AP/PTase, where the active site was located. We have demonstrated that Ser72, Gly74, and Ile153, situated near an inosine binding site and that S72A, G74D and I153T contribute to the decrease in the K_m value for inosine.[11] Contrary to our expectations, 7 of 11 amino acid residues, Leu63, Ala65, Glu66, Asn69, Ser71, Thr135, and Glu136, were located far from the substrate binding pocket. It is likely that a local structural change due to the substitutions of these residues had an effect on the structure of the substrate binding site. Since Asn69 and Ser71 are situated comparatively close to the substrate binding pocket, it is possible that the N69D and S71D mutations gave a positive charge and strengthened the interaction with inosine. Thr135 and Glu136 are located comparatively close to the putative phosphate binding site. The mutation of Glu136 to Asp, a shorter side chain than Glu, might reduce repulsion against the phosphate residue. As D116 is situated near an N-terminal residue,

Table 2. Kinetic Constants for Transphosphorylation Reactions

The enzyme activities were assayed as described in the Material and Method section. Initial velocities were determined, and the steady-state kinetic constants were calculated by using a Hanes–Woolf plot.

Enzyme	Activity	Substrate	K_m (μM)	V_{max} (units/mg)
Wild type	Phosphotransferase	Inosine	202,000	1.83
G74D/I153T			109,000	1.39
L63Q/A65Q/E66A/G74D/I153T			85,000	1.03
L63Q/A65Q/E66A/N69D/S71A/S72A/G74D/T135K/E136D/I153T			42,000	1.15
L63Q/A65Q/E66A/N69D/S71A/S72A/G74D/D116E/T135K/E136D/I153T			43,000	2.60
Wild type	5′-Nucleotidase	5′-IMP	180	49.4
G74D/I153T			220	6.00
L63Q/A65Q/E66A/G74D/I153T			340	3.11
L63Q/A65Q/E66A/N69D/S71A/S72A/G74D/T135K/E136D/I153T			150	4.39
L63Q/A65Q/E66A/N69D/S71A/S72A/G74D/D116E/T135K/E136D/I153T			57.0	6.96

it was speculated that a mutation of Asp136 to Glu, a longer side chain than Asp, caused the structural change and reduced possible interruption of the enzymatic reaction by the N-terminal residues.

As described above, the present study was undertaken in order to increase the phosphotransferase activity of EB-AP/PTase, which is a side reaction of the enzyme. By comparing the primary structures of higher productivity and lower productivity enzymes, candidate residues of substitution were selected. Then a total of 11 amino acid substitutions were conducted with sequential substitutions, and we succeeded in enhancing the phosphotransferase activity of EB-AP/PTase to the same level as that of MM-AP/PTase. With this in hand, a number of positions, which might be important for phosphotransferase activity but whose relevance was not established by structural analysis, were elucidated. The result shows that Leu63, Ala65, Glu66, Asn69, Ser71, Asp116, Thr135, and Glu136 also play an important role in the phosphotransferase activity of EB-AP/PTase. We have already found that a substitution of S72F significantly reduced the K_m value for inosine and increased the productivity of 5′-IMP in the EB-AP/PTase.[10] Combining this knowledge, further improvement of the EB-AP/PTase to increase the productivity of 5′-GMP is in progress.

Acknowledgments

The authors thank T. Utagawa and H. Kawasaki for their advice on this work.

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