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A New Subfamily of Polyphosphate Kinase 2 (Class III PPK2) Catalyzes both Nucleoside Monophosphate Phosphorylation and Nucleoside Diphosphate Phosphorylation

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Inorganic polyphosphate (polyP) is a linear polymer of tens to hundreds of phosphate (P) residues linked by “high-energy” phosphoanhydride bonds as in ATP. PolyP kinases, responsible for the synthesis and utilization of polyP, are divided into two families (PPK1 and PPK2) due to differences in amino acid sequence and kinetic properties. PPK2 catalyzes preferentially polyP-driven nucleotide phosphorylation (utilization of polyP), which is important for the survival of microbial cells under conditions of stress or pathogenesis. Phylogenetic analysis suggested that the PPK2 family could be divided into three subfamilies (classes I, II, and III). Class I and II PPK2s catalyze nucleoside diphosphate and nucleoside monophosphate phosphorylation, respectively. Here, we demonstrated that class III PPK2 catalyzes both nucleoside monophosphate and nucleoside diphosphate phosphorylation, thereby enabling us to synthesize ATP from AMP by a single enzyme. Moreover, class III PPK2 showed broad substrate specificity over purine and pyrimidine bases. This is the first demonstration that class III PPK2 possesses both class I and II activities.

Inorganic polyphosphate (polyP), a linear polymer of tens to hundreds of phosphate (P) residues, has been found in all living organisms from bacteria to higher eukaryotes. PolyP has numerous biological functions that include serving as a means of storing energy, a reservoir for Pi, a chelator of metal ions, a buffer against alkali ions, a channel for DNA entry, a regulator of stress and survival, and a supportive component in gene regulation and enzyme function. Polyphosphate kinase 1 (PPK1) is an enzyme that catalyzes the transfer of the terminal P residue of ATP to short-chain polyP, generating long-chain polyP. PPK1 is responsible for the synthesis of most of the cellular polyP. PPK1 also catalyzes polyP-driven ATP synthesis by its reverse reaction. In the case of Escherichia coli PPK1, the order of substrate specificity is ADP > GDP > UDP, CDP (9). Another widely distributed polyphosphate kinase (PPK2), which shows no sequence similarity to PPK1, has been found in Pseudomonas aeruginosa as an enzyme catalyzing GTP synthesis from GDP and polyP. In contrast to PPK1, PPK2 preferentially catalyzes the reverse reaction. The expression of PPK2 increases 100 times in P. aeruginosa during the stationary growth phase, suggesting that PPK2 functions in the generation of GTP to support the synthesis of alginate, an exopolysaccharide essential for its virulence.

Many microbial genomes encode 2 or 3 PPK2 paralogs. Metagenomic analysis of Accumulibacter phosphatis, a dominant polyP-accumulating microorganism in the enhanced biological phosphorus removal (EBPR) system, revealed the presence of five paralogs of PPK2 (12), suggesting that metabolism of polyP by PPK2 is important for its survival in the EBPR system. Nocek et al. found that most of the PPK2 enzymes contain a single domain of ~230 amino acids in length (1-domain PPK2), while some contain two homologous domains (2-domain PPK2), a probable result of a gene duplication event (13). In addition, they demonstrated that 1-domain PPK2 catalyzes nucleoside triphosphate (ATP and GTP) synthesis from the respective nucleoside diphosphates and polyP, whereas the 2-domain PPK2 catalyzes nucleoside diphosphate (ADP and GDP) synthesis from the respective nucleoside monophosphates and polyP (13).

Here, we report that a new subfamily of PPK2, phylogenetically distinct from the previously characterized 1-domain and 2-domain PPK2, catalyzes both nucleoside monophosphate and nucleoside diphosphate phosphorylation. Since this enzyme reaction is different from those reported so far, we propose to divide PPK2 into three subfamilies based on phylogenetic and functional characterization: class I, 1-domain PPK2 group catalyzing phosphorylation of nucleoside diphosphates; class II, mainly 2-domain PPK2 group catalyzing phosphorylation of nucleoside monophosphates; and class III, 1-domain PPK2 catalyzing both nucleoside monophosphate and nucleoside diphosphate phosphorylation.

MATERIALS AND METHODS

Plasmid construction. Meiothermus ruber NBRC 106122 (DSM 1279) was obtained from the NITE Biological Resource Center (Chiba, Japan). A DNA fragment encoding PPK2 (Mrub_2488) was amplified from M. ruber NBRC 106122 chromosomal DNA using the appropriate primer pair mru-1 and mru-2 (see Table S1 in the supplemental material) and then inserted into the BamHI and HindIII sites of pET-21b (Novagen, Madison, WI). The resulting plasmid was designated pET-MrurPPK2.

Expression and purification of M. ruber PPK2. E. coli Rosetta (DE3)pLysS (Novagen) harboring pET-MrurPPK2 was grown in 2 × YT medium (1.6% peptone, 1.0% yeast extract, and 0.5% NaCl) (14) contain-
ing 1% glucose, 50 mg/liter carbenicillin, and 30 mg/liter chloramphenicol at 37°C. When the optical density at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.25 mM, and the cells were cultivated for 12 h at 20°C. The cells were harvested by centrifugation (8,000 × g, 15 min) and disrupted by sonication in 20 mM HEPES-NaOH buffer (pH 7.4) containing protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan) and 12.5 U/ml DNase I (TaKaRa Bio, Shiga, Japan). After centrifugation at 20,000 × g for 30 min, the supernatant was filtered through a 0.45-μm membrane filter and applied onto a Hitrap HP column (GE Healthcare, Buckinghamshire, United Kingdom) equilibrated with 20 mM HEPES-NaOH (pH 7.4) containing 20% glycerol, 0.5 M NaCl, and 25 mM imidazole. The recombinant protein was eluted with a linear gradient of 20 mM HEPES-NaOH (pH 7.4) containing 20% glycerol, 0.5 M NaCl, and 0.5 M imidazole. Mutant pppk genes were constructed by an inverse PCR method with appropriate primers (see Table S1 in the supplemental material). Protein concentrations were measured by the Bradford method with bovine serum albumin as the standard (15).

**Determination of the N-terminal sequence of M. ruber PPK2.** The purified M. ruber PPK2 was subjected to SDS-PAGE, electroblotted onto a polyvinylidene difluoride (PVDF) membrane (FluoroTrans PVDF membrane; Pall, Port Washington, NY), and stained with Coomassie brilliant blue. The band on the PVDF membrane was excised, and the N-terminal amino acid sequence was determined by Edman analysis.

**Enzyme assay.** ATP production by purified PPK2 (40 ng) was measured in 30 μl of 50 mM MOPS (morpholinepropanesulfonic acid)-NaOH (pH 7.0), 10 mM MnCl2, 0.25 mM ADP, and 5 mM polyP65 (a linear chain of about 65 Pi residues) (Sigma, St. Louis, MO, or Bioenex Inc., Hiroshima, Japan). The reaction mixture (40 μl) contained PPK2, 50 mM MOPS-NaOH (pH 7.0), 10 mM MnCl2, 1 mM AMP or ADP, and 0.055 mM 32P-labeled polyP700. The incubation was at 70°C, and 1-μl samples were spotted onto a polyethyleneimine-thin-layer chromatography (PEI-TLC) plate (Merck, Whitehouse Station, NJ) for the time indicated. The TLC plate was developed with 1 M formic acid and 0.6 M LiCl. The plate was dried after development, exposed to a film (Bas-IP; Fujifilm, Kanagawa, Japan), and then analyzed using the Typhoon image analyzer (GE Healthcare). The ratio of the image intensities of the ADP or ATP spot to the total image intensity was determined by Image Analyst (CLSI; Roche Diagnostics, Basel, Switzerland). For radioactive assay of ATP and ADP production, 32P-labeled polyP700 (a linear chain of about 700 Pi residues) was made using E. coli PPK as described previously (9). The reaction mixture (40 μl) contained PPK2, 50 mM MOPS-NaOH (pH 7.0), 10 mM MnCl2, 1 mM AMP or ADP, and 0.055 mM 32P-labeled polyP700. The incubation was at 70°C, and 1-μl samples were spotted onto a polyethylenimine-thin-layer chromatography (PEI-TLC) plate (Merck, Whitehouse Station, NJ) for the time indicated. The TLC plate was developed with 1 M formic acid and 0.6 M LiCl. The plate was dried after development, exposed to a film (Bas-IP; Fujifilm, Kanagawa, Japan), and then analyzed using the Typhoon image analyzer (GE Healthcare). The ratio of the image intensities of the ADP or ATP spot to the total image intensity was determined by Image Analyst (CLSI; Roche Diagnostics, Basel, Switzerland).

**Sequence alignment.** Amino acid sequences of PPK2 homologs were obtained from the NCBI database using the BLAST search and aligned using ClustalW (www.genome.jp/tools/clustalw/). Among PPK2 homologs, Atu0148 of Agrobacterium tumefaciens C58 (13), NcCl2620 of Corynebacterium glutamicum ATCC 13032 (16), MSMEG_0891 of Mycobacterium smegmatis mc² 155 (17), and Rv3232c of Mycobacterium tuberculosis H37Rv (17) were used as the query sequences. For the phylogenetic analysis, a multiple alignment of 160 PPK2 homologous proteins defined as the amount that produces 1 μmol of ATP per minute.

**Phylogenetic analysis of PPK2.** A phylogenetic tree was constructed from 209 PPK2 sequences (see Table S2 in the supplemental material) including 160 PPK2 homologous proteins previously listed in the article by Rao et al. (22). PPK2 seemed to be phylogenetically divided into three subfamilies: class I, consisting of 131 1-domain PPK2s; class II, consisting of 34 2-domain PPK2s and five 1-domain PPK2s; and class III, consisting of 39 1-domain PPK2s (Fig. 1). The first characterized P. aeruginosa PPK2 belongs to the class I subfamily. Twelve proteins of the class I subfamily have been characterized and are known to catalyze nucleoside diphosphate phosphorylation (nucleoside triphosphate synthesis) (11, 13, 16–18). On the other hand, the first characterized PPK2 catalyzing nucleoside monophosphate phosphorylation, known as polyP-AMP phosphotransferase (PAP), of A. johnsonii (13), belongs to the class II subfamily. In class II, three proteins of 2-domain PPK2 including the A. johnsonii PAP and two proteins of 1-domain PPK2 have been characterized and are known to catalyze nucleoside monophosphate phosphorylation (13, 20, 21, 23). Therefore, the prediction of biochemical properties based on the number of domains, i.e., the 1-domain PPK2 catalyzes nucleoside diphosphate phosphorylation and the 2-domain PPK2 catalyzes nucleoside monophosphate phosphorylation (13), is not always true. Rather, the classification based on the phylogenetic analysis appears to provide better prediction of enzyme function. Our phylogenetic analysis also revealed an uncharacterized novel subfamily, “class III” (Fig. 1).

**Cloning and purification of class III PPK2.** To characterize biochemical properties of the class III subfamily, a gene of M. ruber PPK2 (Mrub_2488) was amplified and expressed in an E. coli expression system. The purified protein showed a single band (32 kDa) (see Fig. S1 in the supplemental material), whose N-terminal sequence (MKKYRQVDPDG) was perfectly matched with a sequence starting from the second methionine codon (13 amino acids downstream from the hypothetical N-terminal methionine of M. ruber PPK2) (24). A typical Shine-Dalgarno (SD) sequence (GGGAG) was found 10 bp upstream of the experimentally determined start codon but not upstream of the hypothetical N terminus. These results indicated that M. ruber PPK2 consists of 267 amino acids with a molecular mass of 31.6 kDa.

We first tested whether M. ruber PPK2 synthesizes ATP from ADP and polyP. M. ruber PPK2 synthesized ATP, as did class I

**RESULTS AND DISCUSSION**

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PPK2. The reaction was limited by the amount of *M. ruber* PPK2 (see Fig. S2 in the supplemental material). It is known that class I PPK2 of *P. aeruginosa* requires Mg\(^{2+}\)/H\(^{10001}\) or Mn\(^{2+}\)/H\(^{10001}\) ions for its activity and prefers short-chain polyP (11). On the other hand, *E. coli* PPK1 is activated in the presence of ammonium sulfate (9). *M. ruber* PPK2 required Mn\(^{2+}\)/H\(^{10001}\) rather than Mg\(^{2+}\)/H\(^{10001}\) for its activity (Fig. 2A), preferred polyP of 25 to 50 in chain length (Fig. 2B), and was not activated in the presence of ammonium sulfate (Fig. 2C).

**Mn- and polyP-dependent thermostability of *M. ruber* PPK2.** The optimal temperature for *M. ruber* growth is approximately 60°C (25). Expectedly, *M. ruber* PPK2 showed thermostable characteristics. The optimal temperature of *M. ruber* PPK2 was between 60 and 70°C (Fig. 3A). *M. ruber* PPK2 was stable at 70°C in the presence of both Mn\(^{2+}\) and polyP but immediately lost its activity in the absence of either Mn\(^{2+}\) or polyP (Fig. 3B). In the presence of Mg\(^{2+}\) and polyP, *M. ruber* PPK2 lost its activity within 15 min (Fig. 3B). Ishige et al. found that oligomerization of *P. aeruginosa* PPK2 (class I) is closely related to the enzyme stability (11). It was demonstrated that the PPK2 tetramer is not stable, whereas the addition of polyP stimulates the octamer formation and drastically increases the enzyme stability (11). The requirement of polyP for the stability at high temperature of *M. ruber* PPK2 could be explained by this polyP-induced oligomerization. However, the reason for the preference for Mn\(^{2+}\) over Mg\(^{2+}\) is currently not clear.

**Class III PPK2 showed broad substrate specificity.** When ADP and \(^{32}\)P-labeled polyP were used as the substrates, radioactive ATP was detected (Fig. 4, left). Unexpectedly, radioactive ADP was also detected. No \(^{32}\)PADP or \(^{32}\)PATP was obtained without enzyme or with inactivated enzyme (95°C for 10 min) (data not shown). Assuming that *M. ruber* PPK2 also possesses PAP activity (class II activity), it could synthesize polyP from ADP as reported for *A. johnsonii* PAP (26), which would result in the generation of AMP. In the reverse reaction, the resultant AMP...
Effects of Mg2⁺, Mn2⁺, and polyP on thermostability (B) were studied. (A) The reaction mixture (30 µl) contained 50 mM MOPS-NaOH (pH 7.0), 10 mM MnCl₂, 5 mM polyP65, 0.25 mM ADP, and 40 ng PPK2. (B) Purified PPK2 was incubated in MOPS buffer without additives (open squares), with 0.5 mM polyP65 (closed squares), with 10 mM MgCl₂ (open circles), with 10 mM MnCl₂ (closed circles), with 0.5 mM polyP65 and 10 mM MgCl₂ (open triangles), or with 0.5 mM polyP65 and 10 mM MnCl₂ (closed triangles) at 70°C for the indicated periods of time. The residual activity was measured at 70°C in the reaction buffer described for panel A. The 100% values of the activity were as follows: 2.28 units/mg protein (A) and 1.79 units/mg protein (without additives), 3.63 units/mg protein (with polyP), 1.66 units/mg protein (with MgCl₂), 1.99 units/mg protein (with MnCl₂), 2.89 units/mg protein (with polyP and MgCl₂), and 3.28 units/mg protein (with polyP and MnCl₂) (B). Graphs show means ± standard deviations of two independent experiments.

FIG 3 Thermostability of M. ruber PPK2. Optimal temperature (A) and effects of Mg2⁺, Mn2⁺, and polyP on thermostability (B) were studied. (A) The reaction mixture (30 µl) contained 50 mM MOPS-NaOH (pH 7.0), 10 mM MnCl₂, 5 mM polyP65, 0.25 mM ADP, and 40 ng PPK2. (B) Purified PPK2 was incubated in MOPS buffer without additives (open squares), with 0.5 mM polyP65 (closed squares), with 10 mM MgCl₂ (open circles), with 10 mM MnCl₂ (closed circles), with 0.5 mM polyP65 and 10 mM MgCl₂ (open triangles), or with 0.5 mM polyP65 and 10 mM MnCl₂ (closed triangles) at 70°C for the indicated periods of time. The residual activity was measured at 70°C in the reaction buffer described for panel A. The 100% values of the activity were as follows: 2.28 units/mg protein (A) and 1.79 units/mg protein (without additives), 3.63 units/mg protein (with polyP), 1.66 units/mg protein (with MgCl₂), 1.99 units/mg protein (with MnCl₂), 2.89 units/mg protein (with polyP and MgCl₂), and 3.28 units/mg protein (with polyP and MnCl₂) (B). Graphs show means ± standard deviations of two independent experiments.

would be radiolabeled with [32P]polyP, generating radioactive ADP. Indeed when AMP and [32P]polyP were used as the substrates, radioactive ADP was synthesized (Fig. 4, right). This result indicated that M. ruber PPK2 also possesses PAP activity.

When other nucleoside diphosphates (GDP, CDP, and UDP) were used as the substrates, the respective nucleoside triphosphates (GTP, CTP, and UTP) were detected (Fig. 4, left). When other nucleoside monophosphates (GMP, CMP, and UMP) were used as the substrates, the respective nucleoside diphosphates (GDP, CTP, and UTP) were also detected (Fig. 4, right). M. ruber PPK2 showed broad substrate specificity over purine and pyrimidine bases. Recently, Nahalka and Patoprsty found a PPK2 homolog of Ruegeria pomeroyi (named PPK3) that preferentially catalyzes the synthesis of pyrimidine nucleoside triphosphates (CTP and UTP) from the respective nucleoside diphosphates and polyP (18). Phylogenetic analysis showed that PPK3 belongs to the class I subfamily catalyzing nucleoside diphosphate phosphorylation (Fig. 1). The class III PPK2 is phylogenetically and biochemically distinct from class I and class II PPK2s.

Class III PPK2 synthesizes ATP from AMP and polyP. M. ruber PPK2 catalyzed phosphorylation of both ADP and AMP. These results prompted us to test ATP synthesis from AMP and polyP by a single enzyme. AMP and ADP phosphorylation activities of M. ruber PPK2 were 99.1 ± 0.9 and 1.8 ± 0.2 units/mg protein, respectively (Fig. 5), indicating that the rate-determining step of ATP synthesis from AMP is phosphorylation of ADP. Expectedly, ATP was synthesized from AMP as well as from ADP in the presence of high concentrations of polyP (Fig. 6). M. silvanus PPK2, which is phylogenetically close to M. ruber PPK2 (Fig. 1), also synthesized ATP from AMP and polyP (Fig. 6). Other class III PPK2s of D. geothermalis, D. radiodurans, and T. elongatus, relatively distant from M. ruber PPK2 (Fig. 1), also synthesized ATP from AMP and polyP (Fig. 6). While there have been no reports of ATP synthesis from AMP and polyP by class I and II PPK2s, we also tested the activities of three proteins in these two classes. Class

FIG 4 PolyP-driven nucleoside diphosphate and nucleoside monophosphate phosphorylation of M. ruber PPK2. The reaction mixture (20 µl) containing 50 mM MOPS-NaOH (pH 7.0), 10 mM MnCl₂, 0.055 mM [32P]polyP700, 1 mM NDP (left) or NMP (right), and 500 ng PPK2 was incubated at 70°C for the indicated periods of time. The reaction mixture was separated on a PEI-TLC plate. The product was analyzed as described for Fig. 4. No [32P]ADP or [32P]ATP was obtained without enzyme or with inactivated enzyme (data not shown). These results were reproduced in two independent experiments.

FIG 5 Time course of polyP-driven AMP and ADP phosphorylation of M. ruber PPK2. The reaction mixture (40 µl) contained 50 mM MOPS-NaOH (pH 7.0), 10 mM MnCl₂, 0.055 mM [32P]polyP700, 1 mM AMP or ADP, and the indicated amounts of PPK2. At the indicated times during incubation at 70°C, a 1-µl sample was taken from the reaction mixture and spotted onto a PEI-TLC plate. The product was analyzed as described for Fig. 4. No [32P]ADP or [32P]ATP was obtained without enzyme or with inactivated enzyme (data not shown). These results were reproduced in two independent experiments.
I PPK2 of *P. aeruginosa* (PA0141) and *C. metallidurans* (Rmet_4762) did not synthesize ATP from AMP and polyP but did synthesize it from ADP and polyP (Fig. 6). Class II PPK2 of *A. johnsonii* did not synthesize ATP from either AMP and polyP or ADP and polyP (Fig. 6), while it synthesized ADP from AMP and polyP (data not shown). These results indicated that only class III PPK2 catalyzes both ADP and AMP phosphorylation.

**Conserved amino acids in each class of PPK2.** PPK2 is a member of the P-loop kinases, which are characterized by the presence of two conserved sequence motifs (Walker A and Walker B) and the lid module. The DxxGK motif in Walker A is well conserved in all classes of PPK2, but glutamic acid preceding Walker A is conserved in class I and II PPK2 but changed to glutamine in class III PPK2. This glutamic acid is predicted to contribute to the binding to adenine base (13). The RRKK motif in the lid module is well conserved in all classes of PPK2. The RSxY motif in Walker B is well conserved in all classes of PPK2, but the amino acid following the Walker B appears specific in each class, i.e., asparagine for class I, glycine for class II, and glutamic acid (E126 in *M. ruber* PPK2) for class III. Assuming that this amino acid determines the substrate specificity of PPK2, E126N and E126G mutants of *M. ruber* PPK2 would show only class I (ATP synthesis from ADP) and class II (ADP synthesis from AMP) activities, respectively, and lose class III (ATP synthesis from AMP) activity. We constructed these mutant enzymes (see Fig. S1 in the supplemental material) and tested their activities. ATP synthesis activities from AMP of the E126N and E126G mutants were reduced to 5.0% and 17.5% of the wild type, respectively (Table 1), indicating that E126 is important for class III PPK2 activity. However, ATP synthesis activity from ADP of the E126G mutant was still retained at a level of 23.3% of the wild type (Table 1).

**FIG 6** Comparison of polyP-driven ATP production in class I, II, and III PPK2s. The reaction mixture (30 µl) containing 50 mM MOPS-NaOH (pH 7.0), 10 mM MnCl₂, 5 mM polyP65, 0.25 mM AMP (open circles) or ADP (closed circles), and 100 ng PPK2 was incubated at 30°C (*D. radiodurans* and *C. metallidurans* PPK2), 37°C (*A. johnsonii* PAP and *P. aeruginosa* PPK2), 60°C (*T. elongatus* PPK2), or 70°C (*M. ruber*, *M. silvanus*, and *D. geothermalis* PPK2). The generated ATP was determined using a bioluminescence assay reagent. ATP was stable at least for 30 min even at 70°C (data not shown). These results were reproduced in two independent experiments.

**TABLE 1** ATP synthesis activities of point mutants of *M. ruber* PPK2

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Wild</th>
<th>E126N</th>
<th>E126G</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP to ATP</td>
<td>1.6 ± 0.2</td>
<td>0.08 ± 0.01</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>ADP to ATP</td>
<td>2.7 ± 0.3</td>
<td>0.37 ± 0.05</td>
<td>0.63 ± 0.12</td>
</tr>
</tbody>
</table>

*One unit of PPK2 activity was defined as the amount that produces 1 µmol of ATP per minute. Data are presented as means ± standard deviations (n = 3).
E126N and E126G mutants were not much different from that of the wild type (data not shown). These results indicated that E126 alone does not determine the substrate specificity of PPK2.

**Computer simulation of M. ruber PPK2 structure.** We attempted to find clues to the broad substrate specificity of M. ruber PPK2 by modeling its structure using the SWISS-MODEL protein structure homology-modeling server (http://swissmodel.expasy.org/). The proposed structure of M. ruber PPK2 (see Fig. S3 and S4 in the supplemental material) was based on *Arthrobacter aurescens* PPK2 (PDB ID: 3RHF), whose sequence shares the highest homology (43% identity, 62% similarity) with that of M. ruber PPK2 among the structure-determined PPK2 enzymes. Nocce et al. revealed the structures of PPK2 (classes I and II according to our classification) and proposed functions of important conserved amino acids (13). They also modeled the position of the ligand (bis-adenosine-5'-pentaphosphate) for the class I PPK2. Using their model, we compared the structures of presumed polyP-, ribose-, and base-binding sites of classes I (SMc02148 from *S. meliloti*), II (PA3455 from *P. aeruginosa*), and III (simulated *M. ruber* PPK2).

As for polyP- and ribose-binding sites, K97, R205, and R209 (class I) and K311, R419, and R423 (class II) were predicted to interact with polyP oxygens (13). R149 (class I) and R363 (class II) were proposed to interact with the terminal P residue of the nucleotide and polyP in concert with Mg²⁺. D93 (class I) and D307 (class II) were proposed to act as a general base attracting the 1-hydroxy proton from the terminal P, residue of the nucleotide and preparing it to attack the terminal P, of polyP (13). These amino acids are well conserved in class I and II PPK2s. The corresponding amino acids of class III PPK2 (K70, R178, R182, R122, and D66) were also conserved, although the orientation of the side chain of R122 in class III is somewhat different (see Fig. S3 in the supplemental material). Consequently, the distance between R122 and D66 of class III is relatively close compared to that between the corresponding amino acids of class I and II PPK2s. E126, the important amino acid for class III PPK2 activity (Table 1), was localized near R122 in class III (see Fig. S3). D148 (class I) and D562 (class II) are important for enzyme activity and are conserved among class I and II enzymes (13). This amino acid was replaced with N121 in class II (see Fig. S3). D93 and Y233 (class I) and D307 and Y447 (class II) were proposed to interact with the 3'- and 2'-hydroxyl groups of ribose, respectively (13). This structural relationship was also conserved in class III PPK2 (see Fig. S3).

As for the base-binding site, it was previously proposed that the adenine is sandwiched between the side chains of V157 and F163 and coordinated through hydrogen bonding with E90, Y130, R133, Y134, and E158 (class I) (13). The position of these amino acids and the orientation of their side chains are well conserved in class II (13) but not in class III (see Fig. S4 in the supplemental material). Interestingly, two amino acids with bulky aromatic side chains in class I (Y134 and F163) and class II (F348 and F377) were replaced by amino acids with smaller aliphatic side chains (V107 and L136) in the class III enzyme (see Fig. S4). The presumed binding pocket of nucleotide bases in class III seemed looser than those in classes I and II, suggesting a broader nucleoside specificity of class III PPK2. On the other hand, greater conformational changes that are necessary to accommodate both nucleoside diphosphates and nucleoside monophosphates could involve a number of amino acids beyond the substrate binding sites. Unfortunately, modeling of conformational changes of class III PPK2 is highly complicated and uncertain and is beyond the scope of this study.

**The class III subfamily is closest to a PPK2 ancestor.** Evolutionary study of P-loop kinases has indicated that thymidylate kinases, which catalyze an essential step in the biosynthesis of DNA precursors, have evolved from the common P-loop kinase ancestor with PPK2 (27). Inclusion of the thymidylate kinase cluster in the phylogenetic tree of PPK2 enabled us to infer the probable sequence of PPK2 evolution and diversification. This analysis indicated that the first diversification occurred with the emergence of class III PPK2 and an ancestor of class I and II PPK2 (see Fig. S5 in the supplemental material), followed by separation of class I and class II PPK2s. Broad specificity is believed to be a property of primordial enzymes, which later evolved into highly specific and efficient enzymes (28). Both the phylogenetic analysis and the broad substrate specificity of class III PPK2 suggested that class III is probably closest to a PPK2 ancestor.

While we did not investigate the functions of class III PPK2, many researchers have concluded that polyP-driven nucleotide phosphorylation is important for the survival of microbial cells under conditions of stress or stationary phase (11, 17, 29). For instance, *ppk2* knockout in *Mycobacterium* species affects intracellular nucleotide pools and impairs cell survival in macrophages (17). It was also reported that the *ppk2* mutant of *Campylobacter jejuni* exhibits a significant survival defect under osmotic, nutrient, aerobic, and antimicrobial stresses (29). The maintenance of intracellular nucleotide pools is crucial for many physiological functions. Since class III PPK2 is able to phosphorylate a wide range of nucleotides, it might also contribute to the maintenance of intracellular nucleotide pools and cell survival under conditions of stress or stationary phase.

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**REFERENCES**


