Polyphosphate:AMP Phosphotransferase as a Polyphosphate-Dependent Nucleoside Monophosphate Kinase in *Acinetobacter johnsonii* 210A

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We have cloned the gene for polyphosphate:AMP phosphotransferase (PAP), the enzyme that catalyzes phosphorylation of AMP to ADP at the expense of polyphosphate [poly(P)] in *Acinetobacter johnsonii* 210A. A genomic DNA library was constructed in *Escherichia coli*, and crude lysates of about 6,000 clones were screened for PAP activity. PAP activity was evaluated by measuring ATP produced by the coupled reactions of PAP and purified *E. coli* poly(P) kinases (PPKs). In this coupled reaction, PAP produces ADP from poly(P) and AMP, and the resulting ADP is converted to ATP by PPK. The isolated *pap* gene (1,428 bp) encodes a protein of 475 amino acids with a molecular mass of 55.8 kDa. The C-terminal region of PAP is highly homologous with PPK2 homologs isolated from *Pseudomonas aeruginosa* PAO1. Two putative phosphate-binding motifs (P-loops) were also identified. The purified PAP enzyme had not only strong PAP activity but also poly(P)-dependent nucleoside monophosphate kinase activity, by which it converted ribonucleoside monophosphates and deoxyribonucleoside monophosphates to ribonucleoside diphosphates and deoxyribonucleoside diphosphates, respectively. The activity for AMP was about 10 times greater than that for GMP and 770 and about 1,100 times greater than that for UMP and CMP.

Inorganic polyphosphate [poly(P)], a linear phosphate polymer linked by high-energy phosphoanhydride bonds, is found in all cells in nature (12, 13). Many enzymes that phosphorylate nucleotides using poly(P) as a phosphate donor (12, 13) have been identified. Identified enzymes include poly(P) kinases (PPKs) (PPK1 in *Escherichia coli* and PPK2 in *Pseudomonas aeruginosa*), which generate nucleoside triphosphate (1, 6, 8, 11, 14, 20), and polyphosphate:AMP phosphotransferase (PAP), which generates ADP (3, 4; T. Shiba and A. Kornberg, unpublished data). Therefore, one of the main physiological functions of poly(P) in microorganisms might be as an energy or phosphate reservoir to supply a wide variety of phosphorylated compounds in cells.

PAP was first identified in *Corynebacterium xerosis* (4) and was also found in *Myxococcus xanthus* (Shiba and Kornberg, unpublished) and *Acinetobacter johnsonii* 210A as a major poly(P)-utilizing enzyme (3). PAP catalyzes phosphoconversion of poly(P) to AMP, forming ADP. In *A. johnsonii* 210A, it has been reported that a large amount of poly(P) (300 mg of P_i per g of dry cells) is accumulated during growth and that poly(P) is degraded and P_i is released into the medium when energy generation is no longer possible (18, 19). PAP makes it possible to conserve the energy liberated from the cleavage of poly(P) coupling with adenylate kinase, which mainly catalyzes ATP and AMP formation from two molecules of ADP, to use poly(P) as a source of ATP generation (18, 19). In a previous study, PAP was purified more than 1,500 fold from a crude

lysate of *A. johnsonii* 210A, and its basic properties were analyzed (3).

To further investigate biochemical characteristics of PAP, we cloned a gene encoding PAP from *A. johnsonii* 210A. This is the first study to reveal the primary structure of the *pap* gene as well as some novel characteristics of the PAP enzyme.

Nucleotide sequence accession number. The DNA sequence determined in this study has been assigned GenBank accession number AB092983.

Molecular cloning of *pap* gene. Genomic DNA extracted from *A. johnsonii* 210A was partially digested with Sau3AI, and 7- to 10-kb DNA fragments were purified by glycerol gradient centrifugation. The purified DNA fragments were ligated into the BamHI site of the pBluescript II SK(+) cloning vector (Toyobo, Osaka, Japan). The ligated DNA was introduced into *E. coli* DH5 α , and recombinant *E. coli* was selected by ampicillin (100 μ g/ml). Crude lysates of about 6,000 ampicillin-resistant colonies were screened by the coupled reaction of PAP and purified *E. coli* PPK1 (Fig. 1A) producing ATP from AMP in the presence of poly(P) (10). The amounts of ATP generated by the PAP-PPK coupling reaction were determined with the ATP Bioluminescence Assay kit CLSII (Roche Diagnostics, Mannheim, Germany). When a mixture of lysate that produced a relatively high level of ATP had been found, the PAP activity of each clone was directly measured using [³²P]poly(P) as a substrate, with an average chain length of around 750 phosphate residues, synthesized with [γ -³²P]ATP and purified *E. coli* PPK1 as described previously (1). Products were separated on polyethyleneimine cellulose–thin-layer chromatography (TLC) plates (Merck) in 0.75 M KH₂PO₄ (pH

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FIG. 1. Screening of PAP activity. (A) A schematic protocol of the PAP-PPK coupling reaction used in screening for the *pap* gene. ADP was formed from AMP by PAP activity in a mixture of crude lysates, and the resultant ADP was converted to ATP by an excess amount of purified PPK1 in the presence of poly(P). The amount of ATP formed by this coupling reaction is dependent on the amount of PAP in the crude lysates. ATP was quantified by a bioluminescence assay (15, 16). (B) Determination of a *pap*-positive clone by measuring the amount of ADP formed from AMP and [³²P]poly(P). PAP activity of crude lysate, prepared from the positive clone, was confirmed by detecting phosphoconversion from [³²P]poly(P) to AMP. [³²P]ADP formation was detected by TLC analysis of the reaction mixture containing lysate from a positive clone (lane A). Lanes: PP, [³²P]poly(P); A, PAP-PPK coupling reaction mixtures containing crude lysates of positive clone; N, reaction mixtures including *E. coli* lysates that have a vector plasmid, pBluescript SK(+); P, reaction mixtures including partially purified PAP of *M. xanthus* as a positive control (10).

3.5), and the radioactive spots were analyzed by using a BAS 2000 radioimage analyzer (FUJIX, Kyoto, Japan).

A plasmid containing the *pap* gene, designated pPAP2, was obtained from the genomic library of *A. johnsonii* 210A. A

lysate prepared from a clone harboring pPAP2 was found to have strong PAP activity (Fig. 1B). The entire region of the inserted DNA fragment of pPAP2 (9.6 kb) was sequenced. Three putative open reading frames (ORFs) were found in the inserted DNA fragment. Each putative ORF was subcloned, and the encoded protein was separately expressed. A crude lysate prepared from *E. coli* expressing one subcloned ORF had PAP activity. The ORF that encodes the PAP encodes 475 amino acids with a calculated molecular mass of 55.8 kDa. A putative Shine-Dalgarno sequence was found 6 bp upstream from the start codon of PAP. A palindromic sequence, which is a putative transcriptional termination signal, was also found 11 bp downstream of the termination codon. A BLAST search (2) of GenBank databases with the amino acid sequence of PAP revealed significant similarities with the PPK2 homolog 2 (41.3% identical). The C-terminal region of PAP (200 amino acid residues) is homologous with both PPK2 (15.8% identical) and PPK2 homolog 1 (17.5% identical) (Fig. 2) (20). PPK2 has been isolated as a poly(P)-dependent GDP kinase from *P. aeruginosa* PAO1 and has nearly 100-fold-greater poly(P)-utilizing activity (GDP kinase activity) than it has poly(P) synthetic activity (GTPase activity) (7). The similarity between PAP and these PPK2 homologs indicates that there is a common mechanism by which these enzymes use poly(P) for phosphorylation of nucleosides. The C-terminal region of PAP, including one P-loop (amino acids 286 to 292), is thought to be essential for poly(P)-dependent nucleotide phosphorylation. An additional P-loop was found in the N-terminal region (amino acids 45 to 51) of PAP, and this region is also highly homologous with PPK2 homolog 2, suggesting that this second P-loop may play a crucial role in binding with nucleoside monophosphate. Since PPK2 homologs have been found in many bacteria (20), PAP could also be distributed in a wide variety of bacteria. PAP homologs were also found in archaea such as *Methanosarcina acetivorans* and *Methanosarcina mazei*. No PAP homologs have been found in *Saccharomyces cerevisiae* or other eukaryotes (20).

Purification and complex formation of PAP. The standard assay for PAP activity was performed by measuring the amount of ADP produced from AMP and poly(P) by high-performance liquid chromatography (HPLC) according to our previously published paper (9). The reaction mixture (50 μ l) contained 50 mM Tris-HCl (pH 8.0), 100 mM MgCl₂ (20 mM MgCl₂ for the assay in purification), 10 mM poly(P) with an average chain length of 18 phosphate residues (Sigma), and 5 mM AMP. All poly(P) concentrations are presented in terms of phosphate residues. One unit of enzyme is defined as the amount of PAP that produces one micromole of ADP per minute at 37°C. For overproduction of PAP, *E. coli* JM109 containing pPAP2 was employed because this strain produces a large amount of PAP with a high level of activity (Fig. 3A, lane I). The cells were cultivated at 30°C for 20 h in 1 liter of Luria-Bertani medium containing 100 μ g of ampicillin/ml. Cells were harvested and washed twice with 40 ml of 50 mM Tris-HCl (pH 7.5) and then sonicated for 10 min at 300 W of output with an ultrasonic homogenizer VP-30S (Taitec Co., Tokyo, Japan) in 20 ml of the same buffer. The intact cells and debris were removed by centrifugation (23,000 $\times$ g; 15 min), and the supernatant was pooled as a crude extract. PAP was purified from the crude extract by successive steps of the following operations: ammo-

FIG. 2. Alignment of the amino acid sequences of PAP homologs in *P. aeruginosa* PAO1. Alignment was done with GENETYX software (Software Development, Tokyo, Japan). Amino acid sequences of *P. aeruginosa* PPK2 and their homologs (20) were obtained from GenBank under accession numbers AY168003 (PPK2), NP_251118 (PPK2-Hom1), and NP_252145 (PPK2-Hom2). Residues identical to PAP are shown in black boxes, and similar residues are shown in gray boxes. Two putative P-loops (ATP/GTP-binding site motif) are indicated with asterisks (5).

nium sulfate precipitation, DEAE-Toyopearl anion-exchange column (1.4 by 10 cm; Tosoh) chromatography, CHT-II column (1.35 by 3.5 cm; Bio-Rad) chromatography; and Hiload 16/60 Superdex 200 pg gel filtration chromatography (GFC) (Amersham Pharmacia Biotech) (Fig. 3A). The scheme for purification of PAP is shown in Table 1. Two protein peaks that had PAP activities were obtained by GFC (Hiload 16/60, Superdex 200 pg) (Fig. 3B). They were electrophoretically homogeneous in the presence of sodium dodecyl sulfate (SDS) and appeared to be composed of only one polypeptide with a molecular mass of 56 kDa (Fig. 3A), which is in good agreement with the molecular mass deduced from the DNA sequence. This indicates that the enzyme forms both a tetramer and a dimer. Since the specific activity of the PAP dimer was

2.2-fold higher than that of the tetramer, the dimer fraction was used as purified PAP for the following experiments. The PAP dimer, purified 22.1 fold from crude extract with a 26.5% yield, gave an apparently homogeneous protein (Table 1 and Fig. 3A). The N-terminal amino acid sequences of the purified PAP dimer and tetramer were MDTETIASAV and MDTETIAS, respectively. These sequences are identical to those of the N-terminal peptides predicted by the nucleotide sequence of the gene.

PAP as a poly(P)-dependent NMP kinase. Since we found in a previous study that PAP in *M. xanthus* has poly(P)-dependent GDP kinase activity (10), we carried out an experiment to determine whether purified PAP also has poly(P)-dependent nucleoside monophosphate kinase activity. Substrate specific-

FIG. 3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and GFC analysis of PAP. (A) SDS-PAGE analysis. A gradient gel (8 to 16% PAGE) was used for SDS-PAGE. Lanes: M, molecular mass markers; C, whole-cell extract of *E. coli* harboring pBluescript SK(+) (vector); I, whole-cell extract of *E. coli* harboring pPAP2; II, a pooled fraction after ammonium sulfate fractionation; III, DEAE-Toyopearl chromatography; IV, CHT-II chromatography; V, Hiload 16/60 Superdex 200 pg chromatography. (B) GFC analysis. GFC analysis was performed with a TSKgel Super SW3000 column. Arrows indicate the elution position of PAP activity.

ities of PAP on AMP, GMP, UMP, CMP, IMP, dAMP, dGMP, dCMP, and TMP were examined by measuring phosphoconversion from [³²P]poly(P) to ribonucleoside monophosphates (NMPs) or deoxyribonucleoside monophosphates. As shown in Fig. 4, poly(P)-dependent phosphoconversion was observed with all substrates, although the activity in pyrimidine nucleotides such as UMP, CMP, dUMP, dCMP, and TMP was weak. When an excess amount of the enzyme (500 ng) was added to the reaction mixture, formation of labeled UDP, CDP, dUDP, dCDP, and TDP was clearly observed. The migration positions of labeled products in TLC are consistent with the positions of

nucleoside diphosphates. To compare the activities of PAP on NMPs and deoxyribonucleoside monophosphates (dNMPs), relative activities on 5 mM substrates were measured by HPLC (9) with 10 mM poly(P) with an average chain length of 18 phosphate residues (Table 2). The activity was 10 times lower for GDP kination and around 1,000 times lower for pyrimidine nucleotide (UMP and CMP) kination than the efficiency of AMP kination. The activities on deoxyribonucleotides were around four to six times less than that on ribonucleotides, and the activity on deoxypyrimidine was also about 1,000 times lower than that of dAMP.

TABLE 1. Purification of PAP

Fraction no., column contents	Vol (ml)	Activity (U/ml)	Protein (mg/ml)	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Fold	Yield (%)
I, crude extract	20	28.3	11.4	566	228	2.48	1	100
II, ammonium sulfate fractionation	10	54.5	6.5	545	65	8.38	3.38	96.3
III, DEAE-Toyopearl 650 M anion exchange	75	6.81	0.23	511	17.4	29.4	11.8	90.2
IV, CHT-II Econo-Pac hydroxyapatite	10	26.4	0.87	264	8.67	30.4	12.3	46.6
V, Hiload Superdex 200 pg gel filtration	1.5 (Dimer)	100	1.82	150	2.74	54.9	22.1	26.5
	1.5 (Tetramer)	35.8	1.44	53.6	2.16	24.9	10	9.47

K_m and V_{max} values of the PAP dimer on these substrates were determined by HPLC analysis (9) of PAP activity in reaction mixtures containing 100 mM $MgCl_2$ and 10 mM poly(P) with an average chain length of 18 phosphate residues at 37°C. These values were calculated by the method of least squares with Taylor expansion (17). K_m values for AMP and GMP were 0.27 ± 0.02 and 4.4 ± 0.3 mM, respectively (Table 3). The V_{max} , k_{cat} , and k_{cat}/K_m values of PAP for AMP were $180 \pm 6 \mu\text{mol mg}^{-1} \text{min}^{-1}$, $10,000 \pm 300 \text{ min}^{-1}$, and $3.7 \times 10^4 \text{ min}^{-1} \text{ mM}^{-1}$, respectively. These kinetic parameters are also consistent with the substrate preference of PAP. Since maximum PAP activity was obtained in the reaction mixture containing 100 mM $MgCl_2$ and in that containing both 20 mM $MgCl_2$ and 50 mM $(NH_4)_2SO_4$, we employed both reaction conditions to measure the kinetic parameters (Table 3). Other metal ions such as Mn^{2+} , Fe^{2+} , Ca^{2+} , Cu^{2+} , Zn^{2+} , and Co^{2+}

could not enhance the activity, even though the reaction mixture contained $(NH_4)_2SO_4$ (data not shown). Since a lower K_m value was observed with a high concentration of $MgCl_2$, all other assays were performed in the presence of 100 mM $MgCl_2$. In *A. johnsonii* 210A, Mg^{2+} is always released and taken up simultaneously with phosphate, since Mg^{2+} is necessary as a major counterion for poly(P) accumulated in the cells (19). Thus, cellular Mg^{2+} concentration could be high when a large amount of poly(P) accumulates. For this reason, PAP may require relatively high Mg^{2+} concentrations to obtain its maximum activity.

Although the K_m value of PAP for AMP is almost the same as that of *E. coli* PPK1 for ADP, the k_{cat} value of PAP is about 16 times higher than that of PPK1 (14). This means that PAP is much more efficient at phosphoconversion than is PPK1. *P. aeruginosa* PPK2 also has a higher level of phosphoconversion activity than does *E. coli* PPK1. The V_{max} value of PPK2 is of the same order as that of PAP and is 51 times higher than that of PPK1.

To confirm that purified NMP kinase activity is not derived from contamination of other enzymes in the purified PAP fraction, the correlation between a protein peak of GFC and NMP kinase activity was examined. As shown in Fig. 5, all of the activities were found in the dimer and tetramer, and the elution profile of the protein obtained by GFC with a Hiload 16/60 Superdex 200 pg (Fig. 5A and B) completely matched between the elution profiles of PAP (Fig. 5C) and GMP kinase activity (Fig. 5D). Other NMP kinase activities were also matched with the elution profiles of PAP (data not shown).

FIG. 4. Poly(P)-dependent nucleoside monophosphate kinase activities of PAP. Reaction mixtures (each, 20 μl) containing 100 ng (a) or 500 ng (b) of purified PAP, 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 5 mM NMP or dNMP, and 0.142 mM $[^{32}\text{P}]$ poly(P) were incubated at 37°C for 4 h. Reaction products were separated on a polyethyleneimine cellulose-TLC plate (Merck), and radioactive spots were visualized with a BAS 2000 radioimage analyzer.

TABLE 2. Substrate specificities of PAP^a

Substrate	Relative activity (%)
AMP.....	100
GMP.....	10
UMP.....	0.13
CMP.....	0.09
IMP.....	2.2
dAMP.....	18
dGMP.....	2.6
dCMP.....	0.008
TMP.....	0.012

^a Reaction mixtures (each, 50 μl) containing purified PAP, 50 mM Tris-HCl buffer (pH 8.0), 100 mM $MgCl_2$, 10 mM poly(P), and 5 mM NMP or dNMP were incubated at 37°C. The amount of the product ribonucleoside diphosphate or deoxyribonucleoside diphosphate was measured by HPLC (9).

TABLE 3. K_m and k_{cat} values of PAP

NMP	Salt	K_m (mM)	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{min}^{-1}$)
AMP	100 mM MgCl_2	0.27 ± 0.02	180 ± 6	$10,000 \pm 300$	3.7×10^4
AMP	20 mM MgCl_2 , 50 mM $(\text{NH}_4)_2\text{SO}_4$	0.45 ± 0.04	160 ± 5	$9,100 \pm 300$	2.0×10^4
GMP	100 mM MgCl_2	4.4 ± 0.3	37 ± 1	$2,100 \pm 50$	4.7×10^2

FIG. 5. Consistency of protein peaks of GFC with NMP kinase activities of PAP. (A) Elution profiles of GFC monitored by protein concentration. Purified PAP (550 μg ; fraction V in Table 1) was applied to a Hiload 16/60 Superdex 200 pg column. (B) SDS-PAGE analysis of each fraction obtained from GFC. Each fraction (20 μl ; fraction numbers 51 to 74) was analyzed with SDS-10% PAGE gels. TLC analysis of poly(P)-dependent mononucleoside kinase activity of PAP with AMP (C) or GMP (D) as a substrate. The reaction mixtures containing each fraction obtained by GFC (fraction numbers 51 to 74) were incubated at 37°C with 50 mM Tris-HCl (pH 8.0), 20 mM MgCl_2 , 5 mM NMP, and 0.142 mM [^{32}P]poly(P). Volumes of GFC fractions added to the reaction mixtures varied: 0.2 μl (C) and 1.0 μl (D). The reaction times also varied: 3.0 h (C) and 1.0 h (D).

Thus, purified PAP is a poly(P)-dependent nucleoside monophosphate kinase.

Other basic characteristics of PAP. The optimum pH of PAP was between 8.0 and 9.0, and the PAP was stable between pH 7 and 11 (data not shown). Optimum temperature for PAP was 50°C, and the level of activity at 50°C was 3.2- and 1.8-fold higher than that at 30 and 37°C, respectively (data not shown). PAP was stabilized in the presence of poly(P). PAP retained about 40% of its original activity after being heated at 45°C for 10 min without poly(P). However, in the presence of poly(P) (10 mM), the remaining PAP activity was 85% of the original activity (data not shown).

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