Polyphosphate Synthetic Activity of Polyphosphate:AMP Phosphotransferase in Acinetobacter johnsonii 210A

Hiromichi Itoh and Toshikazu Shiba*

Frontier Research Division, Fujirebio, Inc., Hachioji, Tokyo 192-0031, Japan

Received 16 January 2004/Accepted 5 May 2004

Polyphosphate:AMP phosphotransferase (PAP) has been identified as an enzyme that catalyzes the phosphorylation of AMP with inorganic polyphosphates [poly(P)] as phosphate donors. We found that the purified PAP of Acinetobacter johnsonii 210A has poly(P) synthetic activity. The PAP catalyzes the dephosphorylation of ADP and processively synthesizes poly(P) of 200 to 700 residues. Comparatively lower concentrations of MgCl₂ (20 mM) were required to obtain optimum poly(P) synthetic activity, whereas higher concentrations of MgCl₂ (100 mM) were necessary for optimum PAP activity. ADP is preferred over GDP as a phosphate donor for poly(P) synthesis. The Km and Vmax values for ADP in the poly(P) synthetic activity of PAP were 8.3 mM and 55 μmol min⁻¹ mg⁻¹, respectively. We concluded that the PAP of A. johnsonii 210A is a novel type of poly(P) kinase that uses ADP and GDP as substrates.

Polyphosphate kinase (PPK; EC 2.7.4.1) and polyphosphate:AMP phosphotransferase (PAP) are enzymes that can catalyze the phosphorylation of nucleotides by using inorganic polyphosphates [poly(P)] as phosphate donors (1–3, 6–13, 18; T. Shiba, H. Itoh, A. Kameda, K. Kobayashi, Y. Kawazoe, and T. Noguchi, unpublished data; T. Shiba and A. Kornberg, unpublished data). PPK can transfer the terminal phosphate residue of poly(P) to ADP to yield ATP. PPK also catalyzes the processive synthesis of poly(P) from ATP. Two types of PPKs, PPK1 and PPK2, have been identified and are widely found in the bacterial genomes (18). PPK1, which was first isolated from Escherichia coli, prefers poly(P) synthesis to poly(P) utilization. In contrast, PPK2, which was recently identified in Pseudomonas aeruginosa PAO1, prefers poly(P) utilization, resulting in nucleotide triphosphate formation (9, 18). PPK1 and PPK2 have no similarity in their amino acid sequences and were also distinguished with biochemical characteristics such as kinetics, ADP/GDP selectivity as substrates, requirement of metal ions, and their active conformations (9, 18).

PAP catalyzes the conversion of the terminal phosphate residue of poly(P) to AMP, resulting in the synthesis of ADP. Its activity was first discovered in Corynebacterium xerosis (6), and the enzyme was partially purified and characterized in Acinetobacter johnsonii 210A (3). Its marked activity was also found in Myxococcus xanthus, and the activity in the membrane fraction was characterized (10; Shiba and Kornberg, unpublished). Weak PAP activity was detected in E. coli, although this activity may be the consequence of the combined action of PPK and adenylate kinase (ADK) (7). Recently, we cloned the pap gene from A. johnsonii 210A and found that the amino acid sequence of PAP (GenBank accession no. AB092983) was highly homologous with those of PPK2 (GenBank accession no. AY168003) and its homologues (GenBank accession no. NP_251118 and NP_252145) isolated from P. aeruginosa PAO1 (Shiba et al., unpublished).

It has been incorrectly considered that PAP has only a poly(P)-utilizing activity (forward reaction) because the poly(P) synthetic activity of PAP in both the crude extract and partially purified PAP enzyme had not been reported (3, 14). Thus, the PAP reaction has been believed to be irreversible (3). However, discoveries of extensive homology between PAP and PPK2 encourage us to consider the possibility that PAP has poly(P) synthetic activity as its reverse reaction. The forward and reverse reactions were as follows: poly(P)n + AMP ⇌ poly(P)n−1 + ADP. In order to investigate whether or not PAP catalyzes the poly(P) synthetic reaction, we evaluated the reverse reaction of PAP by using a highly purified PAP enzyme.

AMP formation by PAP. To examine the reversibility of the PAP reaction, we monitored the dephosphorylation of ADP by detecting AMP formation under the optimum Mg²⁺ concentration (100 mM) of the PAP forward reaction at 37°C, as shown in Fig. 1. The AMP and ADP concentrations in the reaction mixture were then measured by using an high-pressure liquid chromatography system equipped with a TSK gel ODS-120T column (Tosoh, Tokyo, Japan) at 25°C. The mobile phase was 30 mM diethylaminoethanol, 20 mM phosphate, and 2% (wt/vol) methanol in water, which was delivered at a flow rate of 1.0 ml min⁻¹. The eluent was monitored by UV detection at 260 nm. In the presence of both AMP (5 mM) and poly(P) (20 mM, in terms of phosphate residues) in the reaction mixture, 93.9% of AMP was converted to ADP by the forward reaction of PAP. On the other hand, when only ADP (5 mM) was added in the reaction mixture, 5.1% of ADP was converted to AMP. These results suggest that both reactions reached the equilibrium state in the phosphate conversion between AMP and ADP and that PAP catalyzed the reverse reaction (presented above), which produced poly(P) from ADP. The equilibrium ratio for the AMP and ADP formation by PAP was 1:19 in the presence of 100 mM MgCl₂ and was changed to 1:9.6 (9.4% of AMP [5 mM] was converted to AMP) in the presence of 20 mM MgCl₂ (Fig. 1). Therefore, we examined poly(P)-synthetic activity of PAP with 20 mM MgCl₂ in the reaction mixture.
Poly(P) synthesis by reverse reaction of PAP. In order to prove that PAP has poly(P)-synthetic activity, the product of the reverse reaction was analyzed by gel electrophoresis. ADP at 100 mM was incubated at 37°C with purified PAP (4.6 U ml⁻¹) in the presence of 20 mM MgCl₂, and the resultant reaction products were also analyzed by using polyacrylamide gel electrophoresis (Fig. 2A). Then, 10 μl of the reaction mixtures was loaded with 5 μl of loading dye solution (40 mM Tris-HCl buffer [pH 8.3], 1 mM EDTA, 20% glycerol, 0.05% bromophenol blue) on a 15% polyacrylamide gel (270 mm high by 165 mm wide and 1.0 mm thick). Next, 50 μg of standard poly(P)s (sodium phosphate glass type 65 [average chain length is ca. 61; Sigma] and sodium phosphate glass type 75+ [average chain length is ca. 84; Sigma]) were loaded as poly(P) size markers. Poly(P) synthesized by purified E. coli PPK (average chain length is ca. 750) (1) was also loaded as another size reference. Gel was stained by a 0.05% toluidine blue containing 0.5% glycerol for 5 min and was then destained by water. Compared to the poly(P) synthesized by E. coli PPK, PAP catalyzes the synthesis of a shorter poly(P) ranging from 50 to 700 residues. The average chain length of poly(P) synthesized by PAP was ca. 200 to 500. Since almost all of the products of PAP were concentrated at 200 to 500 poly(P) chain lengths, poly(P) synthesis by PAP seems to be processive.

To further confirm poly(P) synthetic activity, the amount of poly(P) synthesized by PAP was estimated by using the determination of released inorganic phosphate after the hydrolysis of the poly(P) by yeast recombinant exopolyporphatase (rPPX1) (17) (Fig. 2B). The released orthophosphate concentration was measured by using the method of Chen et al. (4). At the same time, the AMP and ADP concentrations in the same PAP reaction mixture were also measured by using an
high-pressure liquid chromatography system equipped with a TSK gel ODS-120T column. Figure 2B shows the amount of AMP and poly(P) in the reaction mixture. The orthophosphate concentration released from poly(P) by rPPX1 treatment is in agreement with the increase of AMP concentration. This suggests that poly(P) synthesis depends on the dephosphorylation of ADP resulting in the formation of AMP.

**Optimizations of MgCl₂ concentration and pH for AMP formation by PAP.** To determine the optimum concentration of MgCl₂ for dephosphorylation of ADP, we evaluated the reverse reaction of PAP by measuring AMP formation at various concentrations of MgCl₂. The highest activity for AMP formation was observed in 20 mM MgCl₂, whereas the optimum MgCl₂ concentration for poly(P) degradation (ADP synthesis) was 100 mM (Shiba et al., unpublished) (Fig. 3). No precipitation was observed in the reaction mixture containing up to 100 mM MgCl₂ in the presence of 10 mM poly(P), although other metal ions such as Mn, Co, and Fe caused precipitation with 10 mM poly(P) even at lower concentrations (<20 mM). This indicates that Mg could be a preferable metal ion that enhances PAP activity without causing of precipitation and that Mn cannot replace Mg for the activation of PAP activity (Shiba et al., unpublished). We confirmed the Mg specific enhancement of the poly(P)-utilization activity by replacing Mg with Mn and found that only 7.9% of maximum activity was detected when the optimum concentration of Mn (5 mM) was added to the reaction mixture (Shiba et al., unpublished).

One hypothesis is that the equilibrium ratio between ADP and AMP formation was changed by MgCl₂ concentration in the reaction mixture, as shown in Fig. 1. This means that Mg²⁺ may play a crucial role in controlling the balance between poly(P) synthesis and utilization. *A. johnsonii* 210A was isolated as a strain that can accumulate up to 300 mg of poly(P) per g (dry weight) (16). When the energy generation is repressed (as, for example, in the absence of oxygen or an electron donor), poly(P) is degraded and inorganic phosphate is released into the medium (15, 16). In such energy-starved conditions, as in the stationary

---

TABLE 1. Comparison of poly(P) synthetic and utilization activities of PAP, PPK1, and PPK2

<table>
<thead>
<tr>
<th>Enzyme (strain)</th>
<th>Process</th>
<th>Substrate</th>
<th>Optimal metal ion (concn)</th>
<th>Vₘₐₓ (µmol min⁻¹ mg⁻¹)</th>
<th>Kₘ (mM)</th>
<th>Processivity Synthesis/utilization ratio (Vₘₐₓ ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP (A. johnsonii 210A)</td>
<td>Poly(P) synthesis</td>
<td>ADP &gt; GDP</td>
<td>Mg::&lt;sup&gt;2⁺&lt;/sup&gt;(20)</td>
<td>8.3 (ADP)</td>
<td>0.27 (ADP)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Poly(P) utilization</td>
<td>GDP &gt; CDP</td>
<td>Mg::&lt;sup&gt;2⁺&lt;/sup&gt;(100)</td>
<td>55 (ADP)</td>
<td>8.5 (ADP)</td>
<td>0.31 (ADP-AMP)</td>
</tr>
<tr>
<td>PPK1 (E. coli)</td>
<td>Poly(P) synthesis</td>
<td>ATP &gt; GDP</td>
<td>Mg::&lt;sup&gt;2⁺&lt;/sup&gt;(5)</td>
<td>2 (ATP)</td>
<td>0.25 (ADP)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Poly(P) utilization</td>
<td>GDP &gt; CDP</td>
<td>Mg::&lt;sup&gt;2⁺&lt;/sup&gt;(2)</td>
<td>51 (ATP)</td>
<td>3.1 (ADP)</td>
<td>5.6 (ATP-ADP)</td>
</tr>
<tr>
<td>PPK2 (P. aeruginosa)</td>
<td>Poly(P) synthesis</td>
<td>GDP &gt; ADP</td>
<td>Mg::&lt;sup&gt;2⁺&lt;/sup&gt;(10)</td>
<td>7.6 (ATP), 6.7 (GTP)</td>
<td>0.5 (ATP), 0.68 (GTP)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Poly(P) utilization</td>
<td>CDP &gt; GDP</td>
<td>Mg::&lt;sup&gt;2⁺&lt;/sup&gt;(10)</td>
<td>500 (GTP), 660 (ADP)</td>
<td>0.2 (GTP), 0.75 (ADP)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

a Shiba et al. (unpublished).
b Ahn and Kornberg (1).
c Kuroda and Kornberg (12).
d Zhang et al. (18).
e Ishige et al. (9).
phase, the concentration of free Mg$^{2+}$ available for the PAP reaction might be different from that in log-phase cells. Furthermore, for poly(P) utilization, we also observed maximum activity in 20 mM MgCl$_2$ together with 50 mM NH$_4$SO$_4$ in the reaction mixture (Shiba et al., unpublished), whereas no enhancement of poly(P) synthetic activity was observed upon the addition of NH$_4$SO$_4$ (data not shown). The alterations of not only the Mg concentration but also combinations of Mg and other salt concentrations may be able to control the poly(P) level in the cells depending on the equilibrium ratio of the PAP reaction.

We examined the optimum pH for the reverse reaction of PAP by using 50 mM concentration of maleate buffer (pH 5.0 to 7.0), Tris-HCl buffer (pH 7.0 to 9.0), and glycine buffer (pH 9.0 to 11.0) by monitoring the ADP hydrolysis activity of PAP (data not shown). We found that the optimum pH for the PAP reverse reaction was 9.0, which was in the same range as for the forward reaction (8.0 to 9.0) (Shiba et al., unpublished).

**Comparison of poly(P) synthetic activities among PAP, PPK1, and PPK2.** We compared the characteristics of PAP for poly(P) synthesis with other poly(P) kinases, PPK1 from *E. coli* (1, 2, 12) and PPK2 from *P. aeruginosa* (9, 18), and the results are summarized in Table 1. The C-terminal region of PAP (amino acid numbers 236 to 475 in GenBank accession no. AB092983) has a 60.5% identity with the similar region of PPK2 (amino acid number 71-256 in GenBank accession no. AA168003) in *P. aeruginosa*, even though the two proteins are only 15.8% identical when the entire sequences are aligned. PAP is different from other PPKs because the PAP synthesizes poly(P) by using the nucleoside “di”-phosphate and not by using the “tri”-phosphate. As substrates, PPK1 can only use ATP, but PPK2 uses both ATP and GTP (9, 18). In the case of PAP, the poly(P) synthetic activity from GDP (5 mM) was also observed, but the level was only 1.1% of that from ADP (5 mM) (data not shown). The kinetic parameters of PAP for poly(P) synthesis from ADP were also determined by the Line-Weaver-Burk plot. The $K_m$ value of PAP for ADP is 8.3 mM. The $V_{max}$ value of PAP for poly(P) synthetic activity is 55 mmol min$^{-1}$ mg$^{-1}$, which is 1.1- and 7.2-fold greater than those for PPK1 and PPK2, respectively. PAP and PPK2 have strong poly(P) utilization activity ($V_{max}$), and the activity of PAP is 20-fold higher than that in PPK1, even though the $K_m$ values for utilization of all three enzymes are almost the same. This implies that PAP and PPK2 work mainly for poly(P) utilization in the cell. However, PAP has the same level of poly(P) synthetic activity ($K_m$ and $V_{max}$) as PPK1, and this suggests that PAP also works for poly(P) synthesis at a significant level. Combining these results, we can conclude that PAP itself has the ability to work both in poly(P) utilization and synthesis, whereas PPK1 and PPK2 work mainly for poly(P) synthesis and utilization, respectively. To determine the reaction direction (synthesis or utilization) of PAP, intracellular concentrations of ADP, AMP, and poly(P), as well as Mg and other salt concentrations might be critical.

Although there is no report about the existence of ppk1 in the *A. johnsonii* genome, Zhang et al. (18) reported the existence of ppk1 in the genomes of *Acinetobacter* spp., including *Acinetobacter baumanii*, *A. calcoaceticus*, and *Acinetobacter* sp. ADP1. Thus, we suppose that *A. johnsonii* also has ppk1. PAP and PPK1 may function in poly(P) synthesis depending on the condition. For example, PPK1 can synthesize poly(P) in energy-rich aerobic growth condition, whereas PAP may work in ADP-rich conditions in which the energy state is not preferable for accumulating ATP. This is consistent with PAP is high $K_m$ value for ADP (8.3 mM). PAP can degrade poly(P) under low-energy conditions (i.e., stationary phase or anaerobic condition) when excess AMP and poly(P) accumulates.

One possibility is that *A. johnsonii* has an efficient phosphate recycling system involving poly(P), AMP, ADP, and ATP. PAP could contribute to this recycling system by making poly(P) from ADP, in addition to making ADP from poly(P) and AMP. The poly(P) utilization (ADP synthetic) activity of PAP in combination with ADK also makes it possible to synthesize ATP from AMP. PAP might therefore be a key enzyme for intracellular phosphate recycling in *A. johnsonii* 210A.

We thank T. Noguchi (YAMASA corporation) for sending highly purified GDP. We also thank A. Kornberg and K. Ishige for valuable suggestions.

This study was supported by a Grant-in-Aid for the Creation of Innovations through the Business-Academic-Public Sector Cooperation and a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

**REFERENCES**