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Note

Use of *Escherichia coli* Polyphosphate Kinase for Oligosaccharide Synthesis

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The *Escherichia coli* polyphosphate kinase (PPK) has been known to catalyze the reversible transfer of phosphate molecules between ATP and polyphosphate (poly(P)). It has also been found that the PPK catalyzes the kination of not only ADP but also other nucleoside diphosphates (NDPs) using poly(P) as a phosphate donor, yielding nucleotide triphosphates (NTPs). We used the PPK and poly(P) in place of pyruvate kinase and phosphoenol pyruvate for NTP regeneration followed by synthesis of sugar nucleotides in a cyclic synthesis system for oligosaccharides. It was confirmed that the PPK efficiently catalyzed the UTP regeneration in the cyclic system of *N*-acetyllactosamine synthesis. This novel activity of PPK enables us to perform the practical synthesis of oligosaccharides.

Key words: polyphosphate; polyphosphate kinase; glycosyltransferase; oligosaccharides; *N*-acetyllactosamine

Recently biologically active oligosaccharides have been enzymatically synthesized using sugar nucleotides and glycosyltransferases. However, the enzymatic synthesis of oligosaccharides is not popular, mainly due to the expense of sugar nucleotides. Therefore, the Scripps Research Institute group has developed a cyclic system for enzymatic synthesis of oligosaccharides with no use of expensive sugar nucleotides.[1-3] Their system consists of an enzymatic synthesis of sugar nucleotides, a glycosyltransfer reaction catalyzed by glycosyltransferases, and an enzymatic regeneration of nucleoside triphosphates (NTPs). Since nucleoside diphosphates (NDPs) generated through the glycosyltransfer reaction inhibit the further glycosyltransfer reaction, it is essential to eliminate the accumulated NDPs by dephosphorylation or convert NDPs to NTPs by kination. The Scripps group has proposed the use of pyruvate kinase for kination of NDP and phosphoenole pyruvate (PEP) as a phosphate donor in their NTP regenaration. The problem of their system for practical production of oligosaccharides is the commercial unavailability and the expense of PEP.

Escherichia coli polyphosphate kinase (PPK) has been known to catalyze the reversible transfer of phosphate molecule from adenosine triphosphates (ATP) to polyphosphate (poly(P)) in the following way.[4-7]

$$ATP + poly(P)_n \rightleftarrows ADP + poly(P)_{n+1}$$

The PPK reaction has been shown to be promising as an ATP regeneration system for the kination reaction using ATP as a phosphate donor.[8] Recently Kuroda and Kornberg,[9] and we independently found that it has a novel activity. The PPK can irreversibly tranfer the phosphate molecule from poly(P) to another nucleoside diphosphate (NDP), yielding nucleoside triphosphate (NTP) as follows:

$$NDP + poly(P)_n \rightarrow NTP + poly(P)_{n-1}$$

Poly(P) is a quite cheap material compared to PEP as a phosphate donor for NTP regeneration. Thus we examined the possibility that the combination of PPK and poly(P) can be substituted for that of pyruvate kinase and PEP in the oligosaccharides synthesis system coupled with the enzymatic regeneration of NTPs and sugar nucleotides.

Overproduction and preparation of PPK. The PPK-coding region of the *E. coli ppk* gene was amplified by polymerase chain reaction (PCR) using 2 primers (5′-TACCATGGGTCAGGAAAAGCTATA-3′ and 5′-ATGGATCCTTATTCAGGTTGTTCGAGTGA-3′) designed on the basis of its nucleotide sequence.[7] The amplified DNA was digested with *Nco*I and *Bam*HI, and introduced into *Nco*I-*Bam*HI sites in pTrc99A[10] purchased from Pharmacia Co., to give pTrc-PPK. *E. coli* JM109[11] harboring the pTrc-PPK was cultivated in 2×YT medium[11] with 100 μg/ml ampicillin at 30°C until it reached 2×10^8 cells/ml and then IPTG was added to induce the expression of the inserted *ppk* gene driven by the *trc* promoter.[10] After 3 h of induction, cells were harvested by centrifugation and resuspended in a buffer of 50 mM Tris-HCl (pH 7.8) containing 1 mM EDTA. A cell free extract was prepared by sonic oscillation and the PPK was purified from the cell-free extract as described by Akiyama and Kornberg.[7]

The enzyme activity was measured as follows: the reaction mixture (1.0 ml) contained 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 10 mM ADP, and poly(P) (150 mM as phosphate, chain length; 15–20, obtained from Sigma Co.). The reaction was started by addition of enzyme, incubated at 37°C, and stopped by addition of 0.1 ml of 0.2 N acetate. The amount of ATP generated in the reaction solution was measured by HPLC. One unit of PPK was defined as the amount

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; GlcNAc, *N*-acetyglucosamine; PCR, polymerase chain reaction; poly(P), polyphosphate; PPK, polyphosphate kinase; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; UDP, uridine diphosphate; UDP-Gal, UDP-galactose; UDP-Glc, UDP-glucose; UTP, uridine triphosphate.

Table 1. Kination of NDPs by *E. col* PPK

The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM NDP, 75 mM poly(P) (as phosphate), and 0.2 units/ml PPK, and was incubated at 37°C for 16 h.

Substrate	Kination efficiency (%)
ADP	52.3
CDP	31.1
GDP	43.5
UDP	49.6

producing one μmole of ATP from ADP per min under these conditions. The PPK activity of the cell-free extract from JM109 carrying pTrc-PPK and that of purified protein were 0.10 and 0.92 units/mg protein, respectively.

Kination of NDP by PPK. We first examined the ability of PPK for synthesis of NTPs from NDPs. As shown in Table 1, almost half of the NDP initially added to the reaction solution was converted to NTP. Without poly(P), no kination of NDP was observed (data not shown). Further incubation did not improve the conversion rate even in the presence of excess poly(P), although the NDP-kination reaction (except ADP-kination) by PPK is irreversible.[9] Even though PPK could not catalyze the complete kination of NDP, this kination activity is available for practical regeneration of NDP.

Synthesis of UDP-glucose from UDP with PPK. Next we made an attempt to synthesize UDP-glucose (UDP-Glc) from UDP with PPK, poly(P), and UDP-Glc pyrophoshorylase that catalyzes the reversible transfer of uridine monophosphate (UMP) between glucose 1-phosphate and UTP as diagrammed in Fig. 1A.

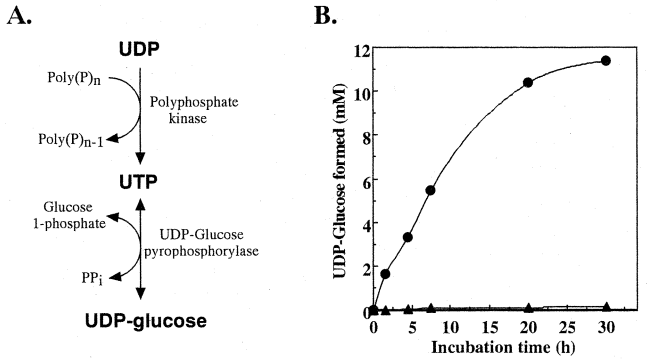

Fig. 1. Synthesis of UDP-Glc from UDP and Glucose 1-Phosphate with PPK.

A. The scheme of UDP-Glc synthesis. PPK catalyzes the poly(P)-dependent kination of UDP, and UDP-Glc pyrophosphorylase synthesizes UDP-Glc from UTP and glucose 1-phosphate.

B. Poly(P) dependent UDP-Glc formation. Reaction mixture contained 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM UDP, 20 mM glucose 1-phosphate, 0.15 units/ml PPK, 2.0 units/ml UDP-Glc pyrophosphorylase, and poly(P) (75 mM as phosphate, chain length; 15–20), and was incubated at 37°C. Closed circles and triangles indicate the concentrations of UDP-Glc formed in the presence of poly(P) and in the absence of it, respectively.

The *E. coli galU* gene[12] encoding UDP-Glc pyrophosphorylase was amplified by PCR with 2 primers (5′-GCGAATTCTGATATACTGGGATGCGATAC-3′ and 5′-ACGTCGACACCGATACGGATGTATCTT-3′), digested with *Eco*RI and *Sal*I, and introduced into *Eco*RI-*Sal*I sites in pTrc99A, to give pTrc-ugpp. A cell-free extract containing UDP-Glc pyrophosphorylase was prepared from JM109 cells carrying pTrc-ugpp in the same way as PPK-preparation. The UDP-Glc pyrophosphorylase activity was measured and defined as described by Weissborn *et al.*,[12] except that 6 mM each of UTP and glucose 1-phosphate were used as substrates and the amount of UDP-Glc produced was measured by HPLC. The UDP-Glc pyrophosphorylase activity in the cell-free extract was found to be 20.4 units/mg protein, and the cell free extract was used as the enzyme solution.

As shown in Fig. 1B, without poly(P) no significant synthesis of UDP-Glc was observed. On the contrary, with poly(P) over 10 mM UDP-Glc was accumulated after 20 hr of reaction, demonstrating that PPK efficiently catalyzed the kination of UDP using poly(P) as a phosphate donor.

Synthesis of N-acetyllactosamine coupled with UTP-regeneration by PPK. We finally synthesized *N*-acetyllactosamine (Gal(β1–4)GlcNAc) using the novel activity of PPK. The synthesis scheme is shown in Fig. 2. The enzyme, UDP-Glc 4-epimerase, that catalyzes the isomerization of UDP-Glc to UDP-galactose (UDP-Gal), was also prepared by essentially the same method as described in the previous section. The *E. coli galE* gene[13] encoding UDP-Glc 4-epimerase was amplified by PCR with 2 primers (5′-TAGAATTCATACCATAAGCC-TAATGGA-3′ and 5′-TAGGATCCTTAATCGGGA-TATCCCTGT-3′), digested with *Eco*RI and *Bam*HI, and cloned into *Eco*RI-*Bam*HI sites in pTrc99A to give pTrc-galE. The epimerase was overproduced in JM109 cells harboring pTrc-galE cultivated with IPTG. The en-

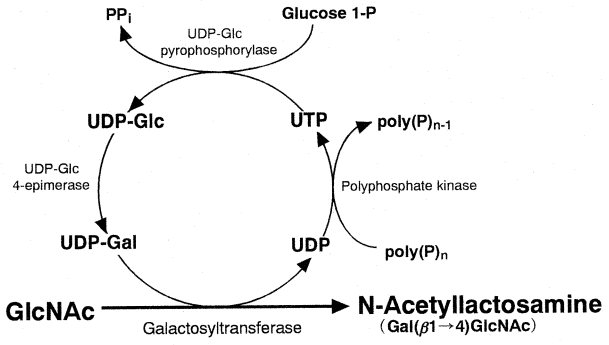

Fig. 2. The Representative of Cyclic Synthesis of *N*-Acetyllactosamine with UTP-Regeneration by PPK.

The UDP-Glc pyrophosphorylase and UDP-Glc 4-epimerase catalyze the synthesis of UDP-Glc from UTP and glucose 1-phosphate and the isomerization of UDP-Glc to UDP-Gal, respectively. The galactosyltransferase transfers galactose from UDP-Gal to *N*-acetylglucosamine resulting in *N*-acetyllactosamine synthesis. UDP formed through the galactose-transfer reaction should be regenerated to UTP by the action of PPK in the presence of poly(P).

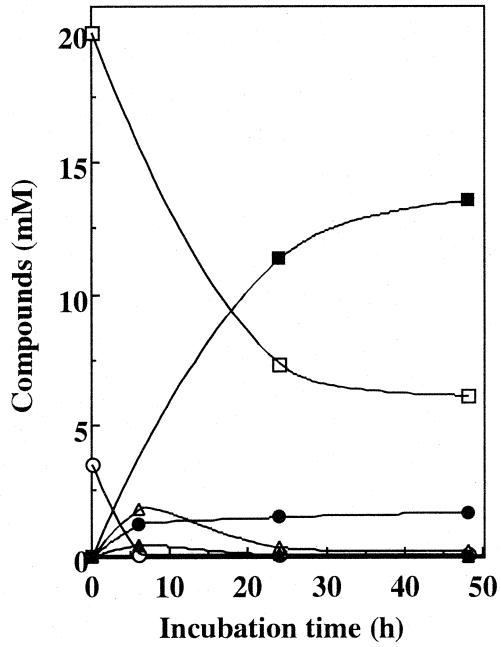

Fig. 3. Synthesis of *N*-Acetyllactosamine with PPK.

Reaction mixture contained 150 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 20 mM *N*-acetylglucosamine, 4 mM UTP, 30 mM glucose 1-phosphate, poly(P) (75 mM as phosphate, chain length; 15-20), 0.1 units/ml PPK, 1.0 unit/ml UDP-Glc pyrophosphorylase, 1.0 unit/ml UDP-Glc 4-epimerase, and 0.5 units/ml galactosyltransferase (from bovine milk, obtained from Sigma Co.), and was incubated at 37°C. Symbols are: open squares, *N*-acetylglucosamine; closed squares, *N*-acetyllactosamine; open circles, UTP; closed circles, UDP; open triangles, UDP-Glc; closed triangles, UDP-Gal. Product formation was monitored by High Performance Anion Exchange Chromatography (Dionex Co., California, USA).

zyme activity in the cell-free extract from the harvested cells was measured and defined by the method of Wilson and Hogness,[14] except that the amount of UDP-Gal formed was measured by HPLC, and was found to be 26.6 units/mg protein. The cell-free extract was used as the epimerase solution for further experiments.

As shown in Fig. 3, 14 mM *N*-acetyllactosamine was accumulated after 48 hr of reaction even though only 4 mM UTP was initially added, demonstrating that UDP generated through the galactose-transfer reaction was converted to UTP and reused for UDP-Glc synthesis by the action of PPK.

It is obvious that PPK and poly(P) can replace the pyruvate kinase and PEP in the enzymatic oligosaccharide synthesis of not only *N*-acetyllactosamine but also other oligosaccharides. We expect that oligosaccharides could be practically produced using our improved economical method.

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