Strictly Polyphosphate-Dependent Glucokinase in a Polyphosphate-Accumulating Bacterium, *Microlunatus phosphovorus*

Shotaro Tanaka,[1] Sun-Og Lee,[1,2] Kazuhiro Hamaoka,[1] Junichi Kato,[1] Noboru Takiguchi,[1]
Kazunori Nakamura,[3] Hisao Ohtake,[1] and Akio Kuroda[1,2]*

*Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530,[1] PRESTO,
Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012,[2] and Institute for
Biological Resources and Functions, AIST, Ibaraki 305-8566,[3] Japan*

ATP-dependent glucokinase is suggested to have evolved from a hypothetical polyphosphate (polyP)-dependent glucokinase (polyP-GK) via a bifunctional polyP/ATP glucokinase (polyP/ATP-GK). Here we showed that polyP-GK is present in a polyP-accumulating bacterium, *Microlunatus phosphovorus*. The polyP-GK produced glucose-6-P_i from glucose and polyP, but it could not phosphorylate glucose with ATP. The polyP-GK was most closely related to the polyP/ATP-GK of *Mycobacterium tuberculosis*.

Inorganic polyphosphate (polyP) is a linear polymer of tens or hundreds of orthophosphate (P_i) residues linked in the same manner as the two high-energy phosphoanhydride bonds in ATP (4, 5). PolyP is readily formed by dehydration of P_i and found in abundance in volcanic condensates and deep-oceanic steam vents (14). Hence, ancient organisms may have utilized polyP instead of ATP in their metabolic reactions (4, 9, 14).

Glucokinases that use ATP as the sole phosphoryl donor to catalyze the phosphorylation of glucose (ATP-GKs) have been present in all contemporary organisms examined (2). Bifunctional glucokinase (polyP/ATP-GK), which utilizes polyP or ATP as the phosphoryl donor to phosphorylate glucose, was found first in *Mycobacterium phlei* (10) and then in many other bacteria, including *Corynebacterium diphtheriae* (11), *Mycobacterium tuberculosis* (12), and *Propionibacterium shermanii* (13). The polyP/ATP-GK of *M. tuberculosis* also utilizes GTP, UTP, and CTP as phosphoryl donors (12). PolyP is recognized as one of the earliest biopolymers and is most likely a prominent precursor in prebiotic evolution (4). Thus, it has been hypothesized that glucose phosphorylation was originally mediated by polyP and that when ATP became available in the environment, a transition was made by the GKs to utilize the latter phosphoryl donor (9). However, nobody has discovered the strictly polyP-dependent glucokinase that utilizes polyP as the sole phosphoryl donor (polyP-GK).

Microlunatus phosphovorus strain NM-1 is a gram-positive, coccus-shaped, non-spore-forming bacterium (7). Strain NM-1, which was originally isolated with an enhanced biological phosphorus removal process, accumulates large amounts of polyP (a maximum of approximately 48% of its dry weight as phosphate [P_i]) in a glucose medium (7). We discovered the existence of polyP-GK in *M. phosphovorus* strain NM-1.

M. phosphovorus strain NM-1 was grown for 2 days under aerobic conditions at 27°C in the glucose medium (7). The cells harvested at the mid-logarithmic phase of growth were dis-

rupted by using Bead-beater (Biospec), and the polyP-GK was precipitated by adding 70% ammonium sulfate. The polyP-GK was further purified by using a DEAE-cellulose (Whatman), a phenyl-Sepharose (Amersham Biosciences), a PE hydrophobic (Poros), and two anion exchange (HQ [Poros] and miniQ [Amersham Biosciences]) columns. The polyP-GK was purified 253-fold (Fig. 1A), and the recovery of polyP-GK activity was 6.7%. The polyP-GK migrated as a 32-kDa protein on sodium dodecyl sulfate (SDS)-polyacrylamide gels, while it fractionated as a 64-kDa protein by gel filtration, suggesting that it was a dimer. The purified enzyme produced glucose-6-P_i from glucose and [^{32}P]polyP (Fig. 1B). The polyP-GK does not catalyze the reverse reaction (data not shown).

The production of glucose-6-P_i was confirmed by oxidizing it to glucose-6-phosphonate with glucose-6-P_i dehydrogenase and NADP. The polyP-GK could not phosphorylate glucose with [γ-^{32}P]ATP even at concentrations as high as 5 to 10 mM (the usual intracellular levels of ATP) (Fig. 1B). The polyP-GK utilizes neither pyrophosphate nor ADP as a phosphoryl donor. The polyP-GK does not degrade polyP in the absence of glucose (data not shown). The K_m values for a long-chain polyP with 700 P_i residues and two short-chain polyPs with 30 and 8 P_i residues were 0.06, 3.8, and 12 μM, respectively. The k_{cat} values for these polyPs were 183, 270, and 87 s^{-1}, respectively. Thus, the polyP-GK prefers a long-chain polyP as a phosphoryl donor. The polyP-GK was able to phosphorylate glucosamine at a rate similar to that of glucose and was able to slowly phosphorylate mannose. The polyP-GK activity required 1 to 10 mM Mg^{2+}. Mn^{2+}, Co^{2+}, and Zn^{2+} (in that order) were also effective. The optimal temperature and pH were 30°C and 5.5, respectively.

The N-terminal amino acid sequence of the purified polyP-GK was determined by using a peptide sequencer (Applied Biosystems). After the enzyme was digested with trypsin, the internal amino acid sequences were also determined by using a mass spectrometer (Micromass). On the basis of the amino acid sequences, the DNA primers T(ACGT)TT(CT)G CIGC(ACGT)GA(AG)(AC)G (containing an inosine [I]) and GC(AG)TC(ACGT)GC(AG)TC(AG)TTCAT were designed for amplification of a DNA fragment containing the gene

* Corresponding author. Mailing address: Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530, Japan. Phone: (81) 824-24-7758. Fax: (81) 824-24-7047. E-mail: akuroda@hiroshima-u.ac.jp.
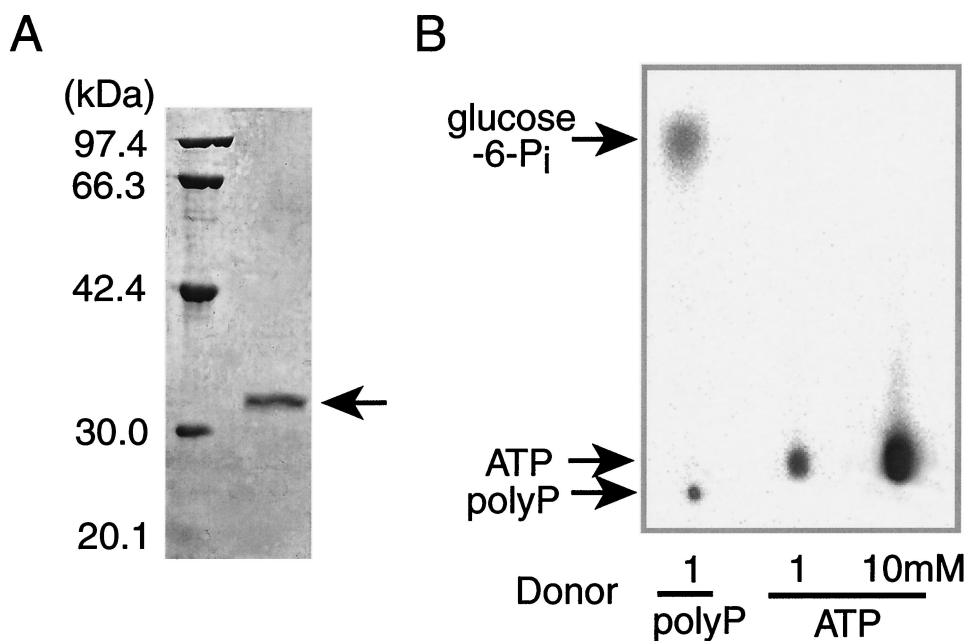
FIG. 1. SDS-polyacrylamide gel electrophoresis (PAGE) of polyP-GK and analysis of polyP-GK activity. (A) The purified polyP-GK was analyzed by SDS–10% PAGE. (B) [^{32}P]polyP (6) and [γ-^{32}P]ATP were used for the phosphorylation of 1 mM glucose by 0.1 μg of the purified polyP-GK. The reaction was performed in the presence of TMB buffer (0.1 M Tris, 0.1 M boric acid, 0.1 M maleic acid [pH 5.5])–50 mM KCl–4 mM MgCl$_2$ at 30°C. After 1 h of incubation, the reaction mixture was plotted onto a polyethyleneimine-cellulose plate and the plate was developed with 1 M formic acid and 0.4 M LiCl. The ^{32}P-labeled products were visualized using BAS1000 (Fujifilm).

(*ppgk*) that encodes polyP-GK. Using the amplified DNA as a probe, a 1.8-kb *Sph*I fragment which contains the entire *ppgk* gene was cloned from its chromosomal DNA. The *M. phosphovorus ppgk* gene encoded a putative polypeptide of 266 amino acids. The deduced amino acid sequence of the polyP-GK showed 50% identity (64% similarity) to that of the *M. tuberculosis* polyP/ATP-GK (Fig. 2). The polyP-GK contained regions that are homologous to common motifs interacting with the ATP molecule that are conserved in the ATP-

and polyP/ATP-GKs from different sources (Fig. 2). These regions are the Phosphate-1 and Phosphate-2 motifs, which contact the β- and γ-P$_i$ of ATP (1). The highly conserved residues in these motifs are Asp and Gly (in Phosphate-1) and Gly and Thr (in Phosphate-2) (1). Two hinge regions at the interface between the two P$_i$-binding domains, the Connect-1 and Connect-2 motifs, were also identified in the polyP-GK.

In addition, the polyP-GK was found to possess a putative polyP-binding region which was homologous to that proposed
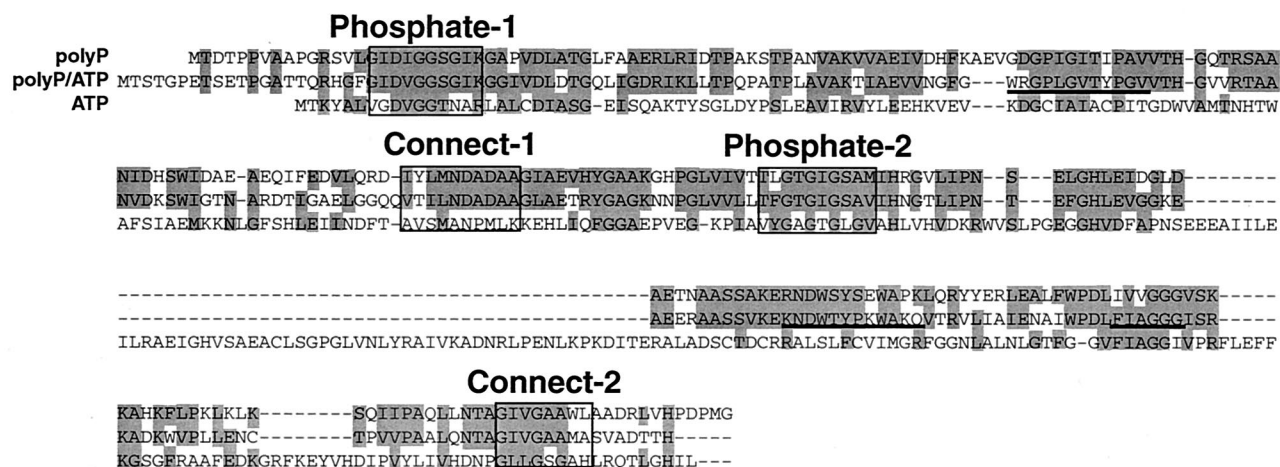

FIG. 2. Comparison of the deduced amino acid sequences of the *M. phosphovorus* polyP-GK, the *M. tuberculosis* polyP/ATP-GK, and the *E. coli* ATP-GK. The shaded amino acids were conserved among the GKs (CLUSTALW analysis; DDBJ). The Phosphate-1, Phosphate-2, Connect-1, and Connect-2 motifs are boxed. The underlined sequences (WRGPLGVTYPGV, KNDWTYPKWAKQ, and FIAGGG) are the proposed regions of glucose, polyP, and adenosine binding in the polyP/ATP-GK (9), respectively.

for the *M. tuberculosis* polyP/ATP-GK (3, 9). The internal region of the *M. phosphovorus* polyP-GK between Phosphate-1 and Connect-1 was longer by two amino acids than that of the polyP/ATP-GK. A putative glucose-binding region proposed in the polyP/ATP-GK (9) was conserved in polyP-GK but not well conserved in ATP-GK. An adenosine-binding region (9) was slightly changed in the polyP-GK (Fig. 2). However, the location of the adenosine-binding region is highly speculative in the polyP-GK. The amino acid sequences which may specifically enable the polyP-GK to utilize polyP as the sole phosphoryl donor remain to be identified. Interestingly, ATP-GK contains a larger insertion between Phosphate-2 and Connect-2 motifs than those seen with polyP and polyP/ATP-GKs (Fig. 2).

A long-standing question was whether a strictly polyP-dependent glucokinase exists in contemporary organisms. The present results convincingly showed the existence of polyP-GK, which has been considered a hypothetical evolutionary origin of glucokinases. Very interestingly, *M. phosphovorus* strain NM-1 was found to release P_i, concomitantly with the degradation of intracellular polyP, when it took up glucose and accumulated glycogen under anaerobic conditions (8). This evidence, together with the existence of polyP-GK, strongly suggests that polyP can be directly used to phosphorylate glucose in *M. phosphovorus*. After the glucose-P_i is converted to glycogen, the resultant P_i might be released. The polyP-GK may be responsible for the release of P_i of this bacterium. We could not detect a polyP-dependent phosphofructokinase activity in *M. phosphovorus* strain NM-1. Only ATP phosphofructokinase was present in this organism (data not shown). These results suggest that polyP is not a sole phosphoryl donor for driving the whole glycolytic pathway in *M. phosphovorus* strain NM-1. When the *M. phosphovorus* cells are grown aerobically in the glucose medium with excess P_i, the cellular levels of polyP (mainly 100 to 200 P_i residues) reach over 5 mmol/g of cells in P_i equivalents (8), which far exceeds those seen with ATP (typically 7 μmol/g of *Escherichia coli* cells). However, it is unclear whether ATP is directly used for the biosynthesis of polyP, because significant polyphosphate kinase activity was not detected with *M. phosphovorus* strain NM-1. The cellular environment of this unique bacterium may represent a hypothetical ancient world in which polyP could be preferentially utilized in metabolic roles.

Nucleotide sequence accession number. The nucleotide sequence of the *ppgk* gene has been deposited in DDBJ with the *accession number* AB075018.

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