Biochemical Characterization of Pantoate Kinase, a Novel Enzyme Necessary for Coenzyme A Biosynthesis in the Archaea

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Although bacteria and eukaryotes share a pathway for coenzyme A (CoA) biosynthesis, we previously clarified that most archaea utilize a distinct pathway for the conversion of pantoate to 4′-phosphopantothenate. Whereas bacteria/eukaryotes use pantothenate synthetase and pantokate kinase (PanK), the hyperthermophilic archaeon Thermococcus kodakarensis utilizes two novel enzymes: pantoate kinase (PoK) and phosphopantothenate synthetase (PPS). Here, we report a detailed biochemical examination of PoK from T. kodakarensis. Kinetic analyses revealed that the PoK reaction displayed Michaelis-Menten kinetics toward ATP, whereas substrate inhibition was observed with pantoate. PoK activity was not affected by the addition of CoA/acyetyl-CoA. Interestingly, PoK displayed broad nucleotide specificity and utilized ATP, GTP, UTP, and CTP with comparable $k_{cat}/K_m$ values. Sequence alignment of 27 PoK homologs revealed seven conserved residues with reactive side chains, and variant proteins were constructed for each residue. Activity was not detected when mutations were introduced to Ser104, Glu134, and Asp143, suggesting that these residues play vital roles in PoK catalysis. Kinetic analysis of the other variant proteins, with mutations S28A, H131A, R155A, and T186A, indicated that all four residues are involved in pantoate recognition and that Arg155 and Thr186 play important roles in PoK catalysis. Gel filtration analyses of the variant proteins indicated that Thr186 is also involved in dimer assembly. A sequence comparison between PoK and other members of the GHMP kinase family suggests that Ser104 and Glu134 are involved in binding with phosphate and Mg$^{2+}$, respectively, while Asp143 is the base responsible for proton abstraction from the pantoate hydroxy group.

Coenzyme A (CoA) is an important coenzyme that is found in all three domains of life. CoA forms high-energy thioester bonds with various carboxylic acids and plays a central role in carbon and energy metabolism. Its derivatives, such as acetyl-CoA, succinyl-CoA, malonyl-CoA, and hydroxymethylglutaryl-CoA, are key metabolites in a wide range of pathways which include sugar breakdown and β-oxidation, as well as the biosynthesis of fatty acids and isoprenoid compounds.

In bacteria and eukaryotes, the mechanism of CoA biosynthesis has been examined in detail, and it is now known that they share common reactions (14, 27). CoA is synthesized from pantothenate via five enzymatic reactions: pantoate kinase (PanK), 4′-phosphopantothenoylcytoste synthetase (PPCS), 4′-phosphopantothenoylcyclste decarboxylase (PPDC), 4′-phosphopantothenoyl adenyltransferase (PPAT), and dephospho-CoA kinase (PPCK). In addition, microorganisms and plants can synthesize pantothenate from $l$-valine and the phosphorylation reaction has been examined and shown to exhibit PanK activity (28). The PanK from P. torridus has been examined and shown to exhibit PanK activity (28). The gene that encodes PS in these organisms has yet to be identified.

Based on its primary structure, PoK is structurally related to members of the GHMP kinase superfamily (33). This family con-

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prizes a vast number of proteins that include the galactokinases, homoserine kinases, mevalonate kinases, and phosphomevalonate kinases (3). Many members of the GHMP family are involved in intermediary metabolism and utilize ATP to phosphorylate their specific substrates. These kinases harbor a common loop sequence rich in glycine/serine residues (4), which, in the case of human mevalonate kinase (MvK), has been biochemically shown to be involved in binding with Mg$$^{2+}$$ and ATP. The specific residue involved in this binding (Ser146) is highly conserved among the members of the GHMP family (4). Glu193 from this enzyme has also been shown to be involved in ATP binding (18).

Furthermore, mutagenesis studies on Asp204 have indicated that this residue functions as the catalytic base to enhance the nucleophilicity of the hydroxy group to attack the ATP γ-phosphate (18). Both of these residues are conserved in a wide range of GHMP family proteins. In the archaea, several proteins belonging to the GHMP kinase family have been biochemically and/or structurally examined, including the galactokinase (9, 30) from Pyrococcus furiosus (PF-GalK) and the mevalonate kinase (MvK) (32), homoserine kinase (Mj-HSK) (12), and shikimate kinase (5) from Methanocaldococcus jannaschii. Structural analyses of PF-GalK, Mj-MvK, and Mj-HSK confirm the importance of the residues described above. Ser107, Glu139, and Asp151 of PF-GalK, Ser112, Glu144, and Asp155 of Mj-MvK, and Ser98, Glu130, and Asn141 of Mj-HSK correspond to Ser146, Glu144, and Asp155 of Mj-MvK, and Ser98, Glu130, and Asn141, respectively, of human MvK. In the case of Mj-HSK, no strong base can be identified near the phosphoryl acceptor hydroxy group, and Asn141 has been proposed to interact with the hydroxy group of homoserine (12).

As the PoK/PPS pathway can be considered to be the predominant route for CoA biosynthesis in the archaea, in this study, we have performed the first detailed biochemical analysis of PoK. Here, we focused on the reaction kinetics, substrate specificity, and moreover, identification of the amino acid residues involved in the catalysis of this novel enzyme, which can be considered a new archaeal member of the GHMP kinase superfamily.

MATERIALS AND METHODS

Strains, media, and culture conditions. Cultivation of T. kodakarensis KOD1 (2, 16) and its derivative strains was performed under anaerobic conditions at 85°C in a nutrient-rich medium (ASW-YT) or a synthetic medium (ASW-AA). ASW-YT medium consists of 0.8× artificial seawater (ASW), 5.0 g liter$$^{-1}$$ yeast extract, 5.0 g liter$$^{-1}$$ tryptone, and 0.8 mg liter$$^{-1}$$ resazurin. Prior to inoculation, 5.0 g liter$$^{-1}$$ sodium pyruvate (ASW-YT-Pyr medium) or 2.0 g liter$$^{-1}$$ elemental sulfur (ASW-YT-SO$$^2$$ medium) and Na,S were added to the medium until it became colorless. ASW-AA medium consisted of 0.8× ASW, a mixture of 20 amino acids, modified Wolfe’s trace minerals, a vitamin mixture, and 2.0 g liter$$^{-1}$$ elemental sulfur (19, 25). In the case of plate culture used to isolate transformants, elemental sulfur and Na,S 9H$$^2$$O were replaced with 2 ml of a polysulfide solution (10 g Na,S 9H$$^2$$O and 3 g sulfur flowers in 15 ml H$$^2$$O) per liter and 10 g liter$$^{-1}$$ Gelrite was added to solidify the medium. Specific modifications of the medium to isolate transformants are described in the respective sections below. Escherichia coli DH5$$^x$$ used for plasmid construction was cultivated at 37°C in Luria-Bertani (LB) medium containing ampicillin (100 mg liter$$^{-1}$$). Unless mentioned otherwise, all chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Production and purification of the wild-type pantoate kinase. The TK2141 gene encoding pantoate kinase (PoK), with a His tag on its N terminus, was overexpressed in T. kodakarensis. The TK2141 overexpression strain (ETK2141) (33) was cultivated in ASW-YT-Pyr for 12 h at 85°C. Cells were harvested, resuspended in 20 mM potassium phosphate buffer (pH 7.4) containing 0.5 M KCl and 40 mM imidazole, and disrupted by sonication. After centrifugation (20,000 × g, 15 min), the supernatant was applied to His GraviTrap (GE Healthcare Biosciences, Piscataway, NJ), and the His-tagged proteins were eluted with 20 mM potassium phosphate buffer (pH 7.4), 0.5 M KCl, and 0.5 M imidazole. After the buffer was exchanged for 50 mM Tris-HCl (pH 8.0) using a PD-10 column (GE Healthcare Biosciences), the sample was applied to anion-exchange chromatography (HiTrap Q HP; GE Healthcare Biosciences), and proteins were eluted with a linear gradient of NaCl (0 to 1.0 M) in 50 mM Tris-HCl (pH 8.0) at a flow rate of 2.5 ml min$$^{-1}$$.

The protein concentration was determined with the Bio-Rad protein assay system (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard.

Examinations of pantoate kinase activity. PoK activity was measured by quantifying the ADP generated from the PoK reaction with the pyruvate kinase/lactate dehydrogenase (PK/LDH) reaction. The PoK reaction was carried out either prior to or simultaneously with the PK/LDH reaction. In the former case, unless mentioned otherwise, the PoK reaction mixture contained 6 mM L-pantoate, 4 mM ATP (Oriental Yeast, Tokyo, Japan), 5.7 μg ml$$^{-1}$$ recombinant PoK protein, 10 mM MgCl$$^2$$, and 50 mM Tris-HCl (pH 7.5). L-Pantoate was prepared by hydrolyzing L-pantolactone (Sigma-Aldrich, St. Louis, MO) in 0.4 M KOH for 1 h at 95°C. After the reaction mixture was preincubated without ATP for 2 min at the desired temperature, the reaction was initiated by the addition of ATP. The reaction was stopped by cooling the mixture on ice for 30 min, followed by the removal of PoK by ultrafiltration with an Amicon ultra 0.5-ml 10K centrifugal filter (Millipore, Billerica, MA). An aliquot was applied to the PK/LDH reaction mixture, which contained 5 mM phosphoenolpyruvate (PEP), 0.2 mM NADH (Oriental Yeast), 7.4 U ml$$^{-1}$$/9.3 U ml$$^{-1}$$ PK/LDH enzymes from rabbit muscle (Sigma-Aldrich), 10 mM MgCl$$^2$$, and 50 mM Tris-HCl (pH 7.5), and incubated at 42°C. The PK/LDH reaction mixture was preincubated for 2 min at 42°C, and the reaction was started by the addition of the aliquot of the PoK reaction. The
decrease in absorption at 340 nm was measured using a spectrophotometer at 42°C. The decrease in NADH after the addition of an aliquot of the PoK reaction mixture without pantoate was subtracted from each assay result. Standard activity measurements were performed by carrying out the PoK and PK/LDH reactions simultaneously at 42°C. The reaction mixture contained 6 mM pantoate, 4 mM ATP, 5 mM PEP, 0.2 mM NADH, 5.7 μg ml⁻¹ of recombinant PoK, 14.8 U ml⁻¹/18.6 U ml⁻¹ of PK/LDH enzymes, 10 mM MgCl₂, and 50 mM Tris-HCl (pH 7.5). The mixture without ATP was preincubated for 2 min at 42°C, and ATP was added to start the reaction. The rate of the decrease in absorption at 340 nm was measured consecutively. Kinetic parameters for pantoate and ATP were determined using the standard method at 42°C with various concentrations of pantoate (with 4 mM ATP) and ATP (with 6 mM pantoate). For analysis of the variant proteins, the reaction mixtures were slightly modified and are described in the respective sections.

**Thermostability and effects of pH and temperature.** For examining thermostability, purified PoK protein (0.57 mg ml⁻¹) in 50 mM Tris-HCl (pH 8.0) was incubated for various periods of time at 60, 70, 80, or 90°C. After the protein solutions were cooled on ice for 30 min, PoK activity was measured with the standard method. In order to examine the effects of pH, the PoK reaction was first performed at various pHs, followed by the PK/LDH reaction. The PoK reaction mixtures contained one of the following buffers at a final concentration of 50 mM: 2-morpholinoethanesulfonic acid, monohydrate (MES) (pH 5.5 to 7.0), piperezine-1,4-bis(2-ethanesulfonic acid) (Pipes) (pH 6.5 to 7.5), HEPES (pH 7.0 to 8.0), Tris (Tris-HCl) (pH 7.5 to 8.0), N,N-bis(2-hydroxyethyl)glycine (bicine) (pH 8.0 to 9.0), and 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) (pH 9.0 to 10). The pH values of all buffers except Tris-HCl were adjusted with NaOH. The reactions were performed at 75°C for 1, 3, and 5 min, and the rate of ADP formation was used to calculate the activity. In order to examine the effects of temperature, the PoK reaction was carried out at various temperatures for 1, 3, and 5 min, followed by the PK/LDH reaction at 42°C. The data obtained were used to make an Arrhenius plot. The rate constant k (s⁻¹) was calculated for each temperature, based on the equation V = k [ES] ≈ k [E]₀ assuming that the observed velocities were almost equal to V_max. Here, V (μmol s⁻¹), [ES] (μmol), and [E]₀ (μmol) represent the observed initial velocity, the concentration of enzyme-substrate complexes, and the initial concentration of enzyme, respectively.

**Effects of K⁺, CoA, acetyl-CoA, and l-cysteine.** PoK activity in the presence of various concentrations of K⁺, CoA, acetyl-CoA, or l-cysteine was examined using the standard method at 42°C. We confirmed that the addition of these compounds had no effect on the PK/LDH reaction. When we examined the effects of KCl, pantoate was prepared by hydrolyzing pantolactone in 0.4 M NaOH instead of KOH.

**Substrate specificity of pantoate kinase for phosphate donors.** In order to examine the nucleotide specificity of PoK, various concentrations of nucleoside triphosphate (NTP; ATP, GTP, UTP, or CTP) (Sigma-Aldrich) were added to the PoK reaction mixture and incubated at 42 or 75°C. ATP, GTP, and UTP were trisodium salts, whereas CTP was a disodium salt. In order to examine the effects of temperature, the PoK reaction was carried out at various temperatures for 1, 3, and 5 min, followed by the PK/LDH reaction. The rate of the decrease in absorption at 340 nm was measured consecutively. Kinetic parameters for pantoate and ATP were determined using the standard method at 42°C with various concentrations of pantoate (with 4 mM ATP) and ATP (with 6 mM pantoate). For analysis of the variant proteins, the reaction mixtures were slightly modified and are described in the respective sections.

**CD spectroscopy.** Circular dichroism (CD) spectroscopy for the wild-type and all variant proteins was carried out at 42°C using a Jasco J-820 spectropolarimeter. The far-UV spectra of the proteins were measured from 200 to 260 nm in 50 mM Tris-HCl (pH 8.0). The instrument settings were as follows: protein concentration, 0.2 mg ml⁻¹; speed, 10 nm s⁻¹; scans, average of 100; and path length, 1 mm.

**RESULTS**

**Production and purification of recombinant pantoate kinase.** Pantoate kinase (PoK, encoded by TK2141) was expressed as an insoluble protein in *E. coli* (33), and therefore, it was expressed in its native host, *T. kodakarenensis* ETK2141. As a result, recombinant PoK harboring a His₆ tag sequence at the N terminus was purified to apparent homogeneity by nickel chelate affinity chromatography and anion-exchange chromatography. A single band corresponding to the molecular weight of PoK was observed after SDS-PAGE. The protein exhibited PoK activity and was used for further biochemical analyses.

**Basic enzymatic properties of PoK.** At 75°C, PoK showed high activity toward pantoate as a phosphate donor. The Michaelis-Menten constant for pantoate was 19 μM. The kinetic parameters for pantoate and ATP were determined using the standard method at 42°C with various concentrations of pantoate (with 4 mM ATP) and ATP (with 6 mM pantoate). For analysis of the variant proteins, the reaction mixtures were slightly modified and are described in the respective sections.

**Expression and purification of the PoK variant proteins.** The PoK overexpression cassette ([pET−TK2141::His₆]) was amplified from the PoK overexpression strain (33) with the primers 2141- EcoRI-NotI (see Table S1 in the supplemental material). After digestion of the amplified DNA fragment and pLC64 (24) with EcoRI and NotI, the cassette was inserted into pLC64 in the same manner as the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase insertion reported by Santangelo et al. (24). The plasmid constructed, pLC64/pok, was introduced into the *T. kodakarenensis* ΔpoK strain as follows. *T. kodakarenensis* ΔpoK (ΔpypF ΔtrpE ΔpoK), which shows tryptophan and uracil auxotrophy, was cultivated in ASW-YT-Pyr for 12 h at 85°C. Cells were harvested and resuspended in 200 μl of 0.8× ASW, followed by incubation on ice for 30 min. After treatment with 3.0 μg of pLC64 and further incubation on ice for 1 h, cells were cultivated in ASW-AA medium without tryptophan (ASW-AA−W) for 24 h at 85°C. Cells were harvested, diluted with 0.8× ASW, and spread onto solid ASW-AA−W. After cultivation for 4 days at 85°C, transformants displaying tryptophan prototrophy were isolated and cultivated in ASW-AA−W. The presence of the plasmids was confirmed by PCR using 8 primer sets (pLC64/F1−R4, F5−R8, F9−R12, F13−R16, F17−R20, F21−R24, F25−R28, and F29−R2; see Table S1 in the supplemental material) that anneal to pLC64. Inverse PCR was performed to construct the plasmids for overexpression of PoK variant proteins. Seven primer sets for introduction of point mutations (Ser28F/-R, Ser104F/-R, His131F/-R, Glu134F/-R, Asp143F/-R, Arg155F/-R, and Thr186F/-R; see Table S1) were used to amplify the entire pLC64/pok. After self-ligation and sequence confirmation, plasmids were introduced into the *T. kodakarenensis* ΔpoK strain. After cultivation of these strains in ASW-AA−W with elemental sulfur and 1 mM CoA for 24 h at 85°C, the strains were cultivated in ASW-YT-Pyr with 0.5 mM CoA for 15 h at 85°C or 2 days at 70°C (for the T186A variant). Cells were harvested by centrifugation, and the variant proteins were purified by methods identical to that used for wild-type PoK. When we purified the T186A variant, the protein was further applied to gel filtration chromatography using Superdex 200 (GE Healthcare Biosciences) at a flow rate of 1.0 ml min⁻¹ after exchanging the buffer for 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl using an Amicon ultra 0.5-ml 10K centrifugal filter. To examine the molecular mass of wild-type and variant PoK proteins, gel filtration was carried out with a Superdex 200 column with a mobile phase of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at a flow rate of 0.8 ml min⁻¹. The size markers were conalbumin (75 kDa), albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

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**Basic enzymatic properties of PoK.** At 75°C, PoK showed high...
activity at neutral pH with a maximum at pH 7.5 in 50 mM PIPES or 50 mM Tris-HCl (data not shown). In terms of temperature, PoK showed the highest activity at 80°C (Fig. 2A). An Arrhenius plot of the data (Fig. 2B) displayed linearity between 55°C and 80°C, indicating that the active site of PoK maintains its structure within this temperature range. The activation energy of the reaction was calculated to be 27.5 kJ mol$^{-1}$. In terms of thermostability, PoK exhibited half-lives of 65 h and 11 h at 80 and 90°C, respectively. Decreases in activity could not be detected at 60 and 70°C for at least 48 h. PoK activity was moderately stimulated in the presence of potassium cations, with an optimal concentration of 10 mM. The addition of CoA sodium salt, acetyl-CoA, and L-cysteine did not affect PoK activity at 42°C. We previously reported that a 0.1 mM concentration of CoA resulted in a slight decrease (20 to 25%) in PoK activity (33), but we found here that this decrease in activity was not due to CoA itself but to the lithium cations in the CoA lithium salt reagent.

**Kinetic examinations of the pantoate kinase reaction.** We carried out kinetic studies on the pantoate kinase reaction. Activity measurements were performed at 42°C in the presence of PK/LDH. The apparent kinetic constants for ATP were determined in the presence of 6 mM pantoate. As shown in Fig. 3A, the reaction followed Michaelis-Menten kinetics. The apparent kinetic constants for pantoate were determined in the presence of 4 mM ATP. We previously concluded that the kinetics of PoK toward pantoate followed Michaelis-Menten kinetics (33), but examinations at higher substrate concentrations revealed that the initial velocity decreases at concentrations higher than 6 mM pantoate (Fig. 3B). An equation taking into account substrate inhibition via binding at a second inhibitory binding site for pantoate fit well to the observed data. This model, toward the substrate homoserine, has previously been proposed for homoserine kinase from *Escherichia coli* (26). The apparent kinetic constants toward ATP that were obtained (apparent maximum initial velocity $V_{\text{max(app)}} = 2.72 \pm 0.03 \mu\text{mol min}^{-1} \text{mg}^{-1}$ [mean \pm standard deviation], $K_{m(app)} = 0.32 \pm 0.01 \text{mM}$, and $k_{\text{cat(app)}} = 1.48 \pm 0.02 \text{s}^{-1}$) were very similar to those we reported previously (33). In the case of the other substrate, pantoate, the kinetic parameters obtained were $V_{\text{max(app)}} = 5.17 \pm 0.23 \mu\text{mol min}^{-1} \text{mg}^{-1}$, $K_{m(app)} = 2.92 \pm 0.23 \text{mM}$, $K_{i(app)} = 9.75 \pm 0.72 \text{mM}$, and $k_{\text{cat(app)}} = 2.82 \pm 0.13 \text{s}^{-1}$.

**Nucleotide specificity of PoK.** In order to examine the nucleotide specificity of PoK, the PoK reactions were performed in the presence of 1, 3, or 5 mM ATP, GTP, UTP, or CTP at 75°C. To our surprise, PoK displayed the highest activity with UTP, whereas the activities with GTP and CTP were about half of that with ATP.
TABLE 1 Apparent kinetic constants of wild-type pantoate kinase toward various NTPs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>V_{max(app)} (μmol min⁻¹ mg⁻¹)</th>
<th>K_{m(app)} (mM)</th>
<th>k_{cat(app)} (s⁻¹)</th>
<th>k_{cat(app)}/K_{m(app)} (s⁻¹ mM⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>1.82 ± 0.05</td>
<td>0.45 ± 0.06</td>
<td>0.99 ± 0.03</td>
<td>2.2</td>
</tr>
<tr>
<td>GTP</td>
<td>0.58 ± 0.02</td>
<td>0.43 ± 0.07</td>
<td>0.32 ± 0.01</td>
<td>0.74</td>
</tr>
<tr>
<td>UTP</td>
<td>2.03 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>1.11 ± 0.03</td>
<td>6.5</td>
</tr>
<tr>
<td>CTP</td>
<td>0.87 ± 0.02</td>
<td>0.34 ± 0.04</td>
<td>0.47 ± 0.01</td>
<td>1.4</td>
</tr>
</tbody>
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*PoK activity with various concentrations of NTP was measured in the presence of 6 mM pantoate. Values are means ± standard deviations or ratios.

(data not shown). We performed kinetic examinations of the PoK reaction with each NTP at 42°C. As shown in Fig. 3C, all reactions followed Michaelis-Menten kinetics, and the parameters obtained are shown in Table 1. In this analysis, the PoK reaction was carried out prior to the PK/LDH reaction. A slightly lower apparent V_{max} value toward ATP was obtained compared to that from the simultaneous assay method, but the apparent K_{m} value was similar. The results indicate that PoK displays broad substrate specificity toward NTPs with comparable k_{cat}/K_{m} values. In addition, we examined whether PoK could utilize pyrophosphate or triphosphate by coupling the reaction with the PPS reaction. While the formation of 4'-phosphopantothenate was clearly detected in the presence of ATP, we could not detect product formation with either pyrophosphate or triphosphate in the PoK reaction, indicating that PoK does not utilize pyrophosphate or triphosphate.

Conserved amino acid residues in archaeal PoK. PoK is a novel enzyme that does not display similarity to PanK, and thus, its structure-function relationship is of interest. In order to identify the amino acid residues involved in catalysis and/or substrate recognition, we searched for highly conserved residues by aligning the amino acid sequences of 27 PoK protein homologs from diverse archaeal species. We found that the PoK homologs are present in the recently sequenced genomes of *Thaumarchaeota Cenarchaeum symbiosum* (8) and *Nitrosopumilus maritimus* (31). The alignment with nine representative sequences is shown in Fig. 4. As described above, PoK is structurally related to members of the GHMP kinases. The phosphate-binding loop, which includes this conserved region, plays an important role in Mg²⁺ coordination. Asp residue that acts as an internal nucleophile, which is highly conserved among the archaeal PoK sequences.

Ser28, His131, and Arg155, seem to be conserved only in PoK. The other three residues, Thr186, play a role in phosphate binding. The other three residues, Ser28, His131, and Arg155, seem to be conserved only in PoK.

Wild-type and variant PoK proteins with single amino acid substitutions in the seven conserved residues were produced in *T. kodakarensis*. Genes were inserted into pLC64, a plasmid that displays autonomous replication in both *E. coli* and *T. kodakarensis* (24). The plasmid was introduced into a ΔpoK strain in order to avoid interaction of the variant protein with wild-type PoK encoded in the native locus. We confirmed that the plasmids were stably maintained in *T. kodakarensis* under the culture conditions applied for gene expression. After cultivation, cells were disrupted and the proteins were purified by methods identical to those applied for wild-type PoK, with the exception of the T186A protein. The expression levels of this protein, as well as those of the R155A variant, to a lesser extent, were exceptionally low, and an additional gel-filtration step was necessary to purify the protein. Cells were inoculated into 5-fold larger volumes of medium and grown at a suboptimal temperature of 70°C. The results of SDS-PAGE analysis of the purified proteins are shown in Fig. 5A. In order to compare the secondary structures of PoK variant proteins with those of wild-type PoK, the CD spectrum of each protein was measured. As shown in Fig. 5B, all variant proteins showed spectra that were almost identical to that of the wild-type enzyme, suggesting that residue exchange did not disturb the overall monomeric structure of the protein. In order to compare the quaternary structures of the variant proteins with that of wild-type PoK, the purified proteins were applied to gel filtration. Six of the seven variant proteins, with the exception of the T186A variant, eluted as a peak corresponding to a dimer. We observed multiple peaks in the chromatogram of the T186A variant, suggesting that the Thr186 residue is important in the dimer assembly of PoK. The results indicate that with the exception of the T186A protein, we can interpret the changes in PoK activity observed in the variant proteins as direct consequences brought about by residue exchange.

Activity measurements of the variant proteins. The activities of the wild-type and seven variant PoK proteins were examined in the presence of 6 mM pantoate and 4 mM ATP at 42°C. As shown in Fig. 6A, decreases in activity were detected for all variant proteins, and activity could not be detected in five proteins. In order to examine whether the loss of activity was due to a decrease in the affinity toward substrate, we measured activity in the presence of excessively high substrate concentrations: 60 mM pantoate and 10 mM ATP. As shown in Fig. 6B, high activities were detected for the S28A and H131A proteins, and we could also detect activity in the R155A and T186A proteins. On the other hand, we could not detect activity in the S104A, E134A, and D143A proteins even under these conditions, indicating that these three residues are vital for PoK catalysis. Ser104, Glu134, and Asp143 are the three residues that were also conserved in all GHMP kinases.

The S28A, H131A, R155A, and T186A variant proteins displayed sufficient levels of activity for kinetic examination (Fig. 7). The substrate concentrations applied for each variant protein are indicated in the legend of Fig. 7. When kinetic studies on the S28A variant were performed, the kinetics of the S28A protein toward ATP were similar to those of the wild-type protein (Fig. 7A), whereas the affinity for pantoate decreased (Fig. 7B). In addition, a sigmoidal curve was obtained and the substrate inhibition observed in the wild-type protein was not observed. The H131A protein also showed kinetics similar to those of the wild-type PoK in terms of ATP, and the affinity for pantoate displayed a significant decrease, as in the case of the S28A protein (Fig. 7C and D). In the case of the R155A protein, decreases in the affinities for both ATP and pantoate were observed (Fig. 7E and F). As for the T186A protein, which displayed a defect in dimer assembly, the apparent K_{m} value for ATP was comparable with that of the wild-type protein, whereas the affinity for pantoate decreased dramatically (Fig. 7C and D).
A sequence alignment of archaeal PoK proteins and galactokinase from Pyrococcus furiosus (PoFK-GalK, AAL80569, PF0445) (sequence accession numbers are from NCBI; locus tags are also shown), mevalonate kinase from Methanocaldococcus jannaschii (PoFK-MvK, AAB99088, MJ1087), and homoserine kinase from M. jannaschii (PoFK-HskK, AAB99107, MJ1104). The amino acid sequences of PoK homologs from 27 archaeal sequences were aligned with the ClustalW program provided by the DNA Data Bank of Japan (DDBJ). Nine representative sequences from Thermoplasma acidophilum (PoFK-Tko, BAD86330, TK2141), Halobacterium sp. NRC-1 (PoFK-Hba, AAG18848, VNG0251C), Archaeoglobus fulgidus (PoFK-Afu, AAB89596, AF1645), M. jannaschii (PoFK-Mja, AAB98974, MJ0968), Methanopyrus kandleri (PoFK-Mka, AAM01947, MK0733), Pyrobaculum aerophilum (PoFK-Pae, AAL64892, PAE3407), Aeropyrum pernix (PoFK-Ape, BAA80948, APE1939), Sulfolobus solfataricus (PoFK-Sso, AAK42544, SSO2397), and Cenarchaeum symbiosum (PoFK-Csy, ABK78124, AKN0302), are shown. Highly conserved residues among the 27 PoK sequences, which are present in at least 24 sequences, are indicated in red. The residues completely conserved in all 27 PoK sequences and Pf-GalK, Mj-MvK, and Mj-HsK are indicated with asterisks. Other PoK protein sequences used for the alignment but not shown here are from Haloarcula marismortui (PoFK-Hma, AAV46584, rrnAC1672), Haloquadratum walsbyi (CAJ52140, HQ1271A), Methanococcoides burtonii (PoFK-Mcb, ABE52109, Mbur1339), Methanococcus maripaludis (PoFK-Mmp, CAF29954, MMP0398), Methanosarcina acetivorans (PoFK-Mac, AAM04698, MA1279), Methanosarcina barkeri (PoFK-Mbb, AAZ72136, MbarA3255), Methanosarcina mazei (PoFK-Mmm, AAM31978, MM2282), Methanosphaera stadtmanae (PoFK-Msa, ABK53437, Msp1187), Methanospirillum hungatei (PoFK-Msh, ABD40583, Mhun0831), Methanothermobacter thermautotrophicus (PoFK-Mta, AAB85083, MTH772), Natronomonas pharaonis (PoFK-Nmp, CAI48513, NP0844A), Nitrosopumilus maritimus (PoFK-Nmm, ABX13614, Nmar1718), Pyrococcus abyssi (PoFK-Pab, CAB49305, PAB2145), Pyrococcus furiosus (PoFK-Pfu, AAL80569, PF0445), Pyrococcus horikoshii (PoFK-Phe, BAA80948, APE1939), Sulfolobus solfataricus (PoFK-Sso, AAK42544, SSO2397), Sulfolobus tokodaii (PoFK-Stt, BAB65525, ST0530).
7G and H). Furthermore, the initial velocity of the T186A protein was low even in the presence of high concentrations of substrate. The results indicate that Thr186 plays multiple roles involved in protein assembly, pantoate binding, and PoK catalysis. Although we could not propose equations that fit the [pantoate]-V plots shown in Fig. 7, the [ATP]-V plots for all variant proteins followed Michaelis-Menten kinetics. The apparent kinetic constants obtained for the S28A, H131A, R155A, and T186A variants for ATP, along with those of the wild-type PoK, are shown in Table 2.

**DISCUSSION**

In this study, we carried out a detailed biochemical examination of pantoate kinase (PoK), a novel enzyme involved in archaeal CoA biosynthesis (33). The enzyme displayed several notable features, including broad nucleotide specificity, substrate inhibition observed with pantoate, and a lack of inhibition in the presence of CoA/acyetyl-CoA.

We found that the apparent $k_{cat}/K_m$ values of PoK for all of the nucleotides examined were comparable, indicating that any of the nucleotides could be used as a phosphate donor, depending on the intracellular concentration. This was not due to the fact that our measurements were performed at relatively low temperatures of 42°C, as we also observed the same broad nucleotide specificity at 75°C (data not shown). In contrast, the PanK from *E. coli* displays a strict preference toward ATP. Interestingly, the PanK from *P. torridus*, which is an exceptional member of the archaea that does not rely on the PoK/PPS system, also shows broad nucleotide specificity (28). The lack of nucleotide specificity toward ATP and the lack of inhibition by CoA may be related to one another, as the inhibition of PanK by CoA is due to the binding of CoA competing with ATP binding through the ADP moieties found in both molecules (34).

Regardless of the different routes utilized to convert pantoate to 4'-phosphopantothenate, the biosynthesis of CoA can be regarded as a costly process. In order to synthesize one molecule of CoA, one molecule each of L-valine, β-alanine, and L-cysteine are consumed, along with 5 molecules of ATP and an NADPH. Therefore, it can be expected that a regulatory mechanism exists to prevent the excess synthesis of CoA. In bacteria and eukaryotes, the regulatory mechanisms of CoA biosynthesis have been studied in detail. In *E. coli*, the activities of PanK and PPAT are controlled by feedback inhibition by CoA and its thioesters (15, 22, 29). Multiple PanK isozymes are found in mammalian cells, and several have been found to be inhibited by CoA, acetyl-CoA, malonyl-CoA, and/or palmitoyl-CoA (11, 20, 21). However, CoA and acetyl-CoA did not have any effect on PoK activity. Other enzymes in the upstream portion of the pathway, such as KPHMT, KPR, or the other archaeon-specific enzyme PPS, may be the target of feedback inhibition. In terms of competitive inhibition, PPS would be the more likely candidate, as ATP (which contains an ADP moiety) is a substrate. We did observe substrate inhibition for PoK in the case of pantoate. This substrate inhibition mechanism may function in order to prevent excessive PoK reactions when pantoate has accumulated in the cell. Similar features have been reported in PS from *Arabidopsis thaliana* and homoserine kinase from *E. coli*, which are inhibited by the substrates pantoate and homoserine, respectively (10, 26). However, the moderate decrease in activity at relatively high concentrations of pantoate does not strongly support a major regulatory role of this substrate in-
hibition in CoA biosynthesis. Another alternative that should be explored is regulation at the transcription/translation level.

We have identified seven amino acid residues that are highly conserved in PoK homologs. Among these, our results indicate that Ser104, Glu134, and Asp143 are vital for PoK activity. Sequence alignment indicates that the three residues correspond to residues that play important roles in GHMP family enzymes. Ser104 of PoK corresponds to Ser107 of Pf-GalK, Ser112 of Mj-MvK, and Ser98 of Mj-HSK. All are included in a phosphate-binding loop [PXGL(G/S)SSA] that is highly conserved in

FIG 7  Kinetic analyses of variant PoK enzymes. S28A protein toward ATP (A) and pantoate (B), H131A protein toward ATP (C) and pantoate (D), R155A protein toward ATP (E) and pantoate (F), and T186A protein toward ATP (G) and pantoate (H). Concentrations of pantoate were 50 mM in panel C and 150 mM in panels A, E, and G. Concentrations of ATP were 4 mM in panels B, D, and H and 12 mM in panel F.
GHMP kinases (4, 5) and involved in binding to Mg\(^{2+}\) and the phosphate groups of ATP. Glu134 of PoK corresponds to Glu139 of Pf-GalK, Glu144 of Mj-MvK, and Glu130 of Mj-HSK, which bind to Mg\(^{2+}\). Furthermore, Asp143 of PoK corresponds to Asp151 of Pf-GalK, Asp155 of Mj-MvK, and Asn141 of Mj-HSK, which are responsible for the proton abstraction from the hydroxy group that eventually attacks the \(\gamma\)-phosphate of ATP. The loss of activity in these variant proteins indicates that the mechanisms utilized by PoK for phosphorylation are similar to those found in previously identified GHMP kinases. It should be noted that, although the levels are lower than our detection limits, S104A, E134A, and D143A proteins may still exhibit trace levels of activity, though the levels are lower than our detection limits, S104A, E134A, and D143A proteins may still exhibit trace levels of activity. PoK activity with various concentrations of ATP was measured in the presence of 6 mM (wild type), 50 mM (H131A variant), or 150 mM (S28A, R155A, and T186A variants) pantoate. Values are means \(\pm\) standard deviations.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(V_{\text{max(app)}}) ((\mu\text{mol min}^{-1}\ \text{mg}^{-1}))</th>
<th>(K_{\text{m(app)}}) (mM)</th>
<th>(k_{\text{cat(app)}}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td>2.72 (\pm) 0.03</td>
<td>0.32 (\pm) 0.01</td>
<td>1.48 (\pm) 0.02</td>
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<tr>
<td>S28A variant</td>
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<td>0.22 (\pm) 0.02</td>
<td>1.81 (\pm) 0.02</td>
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<tr>
<td>H131A variant</td>
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<td>0.13 (\pm) 0.01</td>
<td>1.45 (\pm) 0.01</td>
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<tr>
<td>R155A variant</td>
<td>1.09 (\pm) 0.04</td>
<td>2.82 (\pm) 0.31</td>
<td>0.59 (\pm) 0.02</td>
</tr>
<tr>
<td>T186A variant</td>
<td>0.38 (\pm) 0.01</td>
<td>0.27 (\pm) 0.03</td>
<td>2.1 (\pm) 0.01</td>
</tr>
</tbody>
</table>

\(a\) PoK activity with various concentrations of ATP was measured in the presence of 6 mM (wild type), 50 mM (H131A variant), or 150 mM (S28A, R155A, and T186A variants) pantoate. Values are means \(\pm\) standard deviations.

REFERENCES


