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ORIGINAL PAPER

A detailed biochemical characterization of phosphopantothenate synthetase, a novel enzyme involved in coenzyme A biosynthesis in the Archaea

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Abstract We have previously reported that the majority of the archaea utilize a novel pathway for coenzyme A biosynthesis (CoA). Bacteria/eukaryotes commonly use pantothenate synthetase and pantothenate kinase to convert pantoate to 4′-phosphopantothenate. However, in the hyperthermophilic archaeon *Thermococcus kodakarensis*, two novel enzymes specific to the archaea, pantoate kinase and phosphopantothenate synthetase, are responsible for this conversion. Here, we examined the enzymatic properties of the archaeal phosphopantothenate synthetase, which catalyzes the ATP-dependent condensation of 4-phosphopantoate and β-alanine. The activation energy of the phosphopantothenate synthetase reaction was 82.3 kJ mol^{-1}. In terms of substrate specificity toward nucleoside triphosphates, the enzyme displayed a strict preference for ATP. Among several amine substrates, activity was detected with β-alanine, but not with γ-aminobutyrate, glycine nor aspartate. The phosphopantothenate synthetase reaction followed Michaelis–Menten kinetics toward β-alanine, whereas substrate inhibition was observed with 4-phosphopantoate and ATP. Feedback inhibition by CoA/acetyl-CoA and product inhibition by 4′-phosphopantothenate were not observed. By contrast, the other archaeal enzyme pantoate kinase displayed product inhibition by 4-phosphopantoate in a non-competitive manner. Based on our results, we discuss the regulation of CoA biosynthesis in the archaea.

Keywords Coenzyme A · Phosphopantothenate synthetase · Pantoate kinase · *Thermococcus* · Archaea

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Introduction

Coenzyme A (CoA) is a ubiquitous coenzyme utilized in all three domains of life (Genschel 2004; Leonardi et al. 2005b; Spry et al. 2008). It plays important roles in various metabolic pathways including the tricarboxylic acid cycle, the β-oxidation pathway for fatty acid degradation, and the mevalonate pathway for isoprenoid biosynthesis.

The pathways for CoA biosynthesis have been studied in a variety of bacteria and eukaryotes, and have been found to utilize a common enzymatic pathway (Leonardi et al. 2005b; Spry et al. 2008). In plants, yeast and bacteria, the precursor compound is 2-oxoisovalerate, which can be

generated from pyruvate or converted from valine by aminotransferase. This reaction is followed by the addition of a hydroxymethyl group (ketopantoate hydroxymethyltransferase), reduction by ketopantoate reductase, and condensation with β-alanine by pantothenate synthetase (PS) to generate pantothenate. Animals, which lack these enzymes, must obtain exogenous pantothenate to synthesize CoA. Pantothenate is further converted by 5 enzymes catalyzing phosphorylation (pantothenate kinase, PanK), condensation with cysteine (4′-phosphopantothenoylcysteine synthetase), decarboxylation (4′-phosphopantothenoylcysteine decarboxylase), adenylylation (4′-phosphopantetheine adenylyltransferase), and phosphorylation (dephospho-CoA kinase). The eight reactions utilize 2-oxoisovalerate, a hydroxymethyl group from 5,10-methylenetetrahydrofolate, β-alanine, cysteine and an ATP as structural precursors, along with 2 phosphate groups provided by 2 molecules of ATP. The pathway also consumes an NAD(P)H and 2 molecules of ATP/CTP for reduction and condensation, respectively.

As CoA biosynthesis requires relatively high levels of both carbon and energy, the pathway has been shown to be strictly regulated in many bacteria and eukaryotes. In *Escherichia coli*, PanK is strongly inhibited by CoA and is a key regulator of the pathway (Rock et al. 2003; Vallari et al. 1987; Yun et al. 2000). CoA acts as a competitive inhibitor with the substrate ATP (Yun et al. 2000), and an approximately 90 % decrease in activity is observed in the presence of 80 μM CoA and 1 mM ATP (Rock et al. 2003). Despite to a lesser extent, PanK is also inhibited by acetyl-CoA, succinyl-CoA and other acyl-CoA derivatives (Vallari et al. 1987). 4′-Phosphopantetheine adenylyltransferase has also been shown to be inhibited by CoA, but the effects are moderate when compared to those observed with PanK (Miller et al. 2007). Quantitative analysis of intermediate metabolites confirms that PanK represents the primary regulating step. *E. coli* cells grown on glucose produce 15-fold higher levels of pantothenate than the amounts actually utilized for CoA synthesis (Rock et al. 2003). Accumulation and release of 4′-phosphopantetheine are also observed in *E. coli*, suggesting that 4′-phosphopantetheine adenylyltransferase also plays a role in regulating CoA biosynthesis. There are exceptional cases in which PanK is not inhibited by CoA and its thioesters, such as the enzyme from *Staphylococcus aureus* (Leonardi et al. 2005a). CoA biosynthesis in this organism does not seem to be regulated and intracellular CoA levels have been reported to be relatively high.

In humans and mice, at least four isoforms of PanK have been identified (Leonardi et al. 2010; Zhang et al. 2005). All contain a common catalytic core with or without amino- or carboxy-terminal extensions. The isoforms display differences in their subcellular localization, tissue distribution, and their response to CoA. The isoform PanK1α from mice is strongly inhibited by acetyl-CoA and malonyl-CoA (Rock et al. 2002). PanK3 is strongly inhibited by acetyl-CoA and palmitoyl-CoA (Zhang et al. 2005). The isoform PanK1β is also inhibited by acetyl-CoA, but with lower sensitivity compared to PanK3/PanK1α, and is only weakly regulated by malonyl-CoA and not at all by palmitoyl-CoA (Rock et al. 2002).

In the archaea, genes that resemble either bacterial or eukaryotic genes related to CoA biosynthesis can be found for 4′-phosphopantothenoylcysteine synthetase, 4′-phosphopantothenoylcysteine decarboxylase and 4′-phosphopantetheine adenylyltransferase (Genschel 2004). In many bacteria, the 4′-phosphopantothenoylcysteine synthetase and 4′-phosphopantothenoylcysteine decarboxylase reactions are catalyzed by single, bifunctional proteins referred to as the Dfp proteins. In *Methanocaldococcus jannaschii*, a putative Dfp protein has been examined in detail (Kupke and Schwarz 2006). Domains corresponding to 4′-phosphopantothenoylcysteine synthetase and 4′-phosphopantothenoylcysteine decarboxylase have been individually characterized and each was found to display their expected activity. The 4′-phosphopantetheine adenylyltransferase from *Pyrococcus abyssi* has also been biochemically characterized, and activity has been detected (Armengaud et al. 2003; Nalezkova et al. 2005). The presence of feedback inhibition by CoA toward these enzymes has not been examined. Very few, if any, archaeal genomes harbor homologs for PS and PanK, and studies to identify the proteins/genes involved in the conversion from pantoate to 4′-phosphopantothenate have been carried out (Genschel 2004; Ronconi et al. 2008; Takagi et al. 2010). From a comparative genomics approach, followed by determination of activity and genetic evaluation in the hyperthermophilic archaeon *Thermococcus kodakarensis*, we found that this conversion is carried out by two enzymes, pantoate kinase (PoK) and phosphopantothenate synthetase (PPS), that catalyze novel reactions (Fig. 1) (Yokooji et al. 2009). In this PoK/PPS pathway, pantoate is first phosphorylated by PoK to generate 4-phosphopantoate, followed by condensation with β-alanine catalyzed by PPS. In the PS/PanK pathway of bacteria/eukaryotes, the condensation of pantoate with β-alanine occurs first, followed by the phosphorylation of pantothenate. Homologous genes of PoK and PPS from *T. kodakarensis* (PoK: TK2141, PPS: TK1686) are conserved in almost all archaeal genomes with the exception of those of the Thermoplasmatales and *Nanoarchaeum equitans*, suggesting that the PoK/PPS pathway is the major pathway for synthesis of 4′-phosphopantothenate in the archaea (Yokooji et al. 2009). Consistent with the lack of PoK/PPS in the Thermoplasmatales, a novel PanK has been identified in *Picrophilus torridus* (Takagi et al. 2010). Interestingly, the *P. torridus* PanK does not undergo feedback inhibition by CoA.

We have recently reported the enzymatic properties of the PoK from *T. kodakarensis* (Tomita et al. 2012). Here,

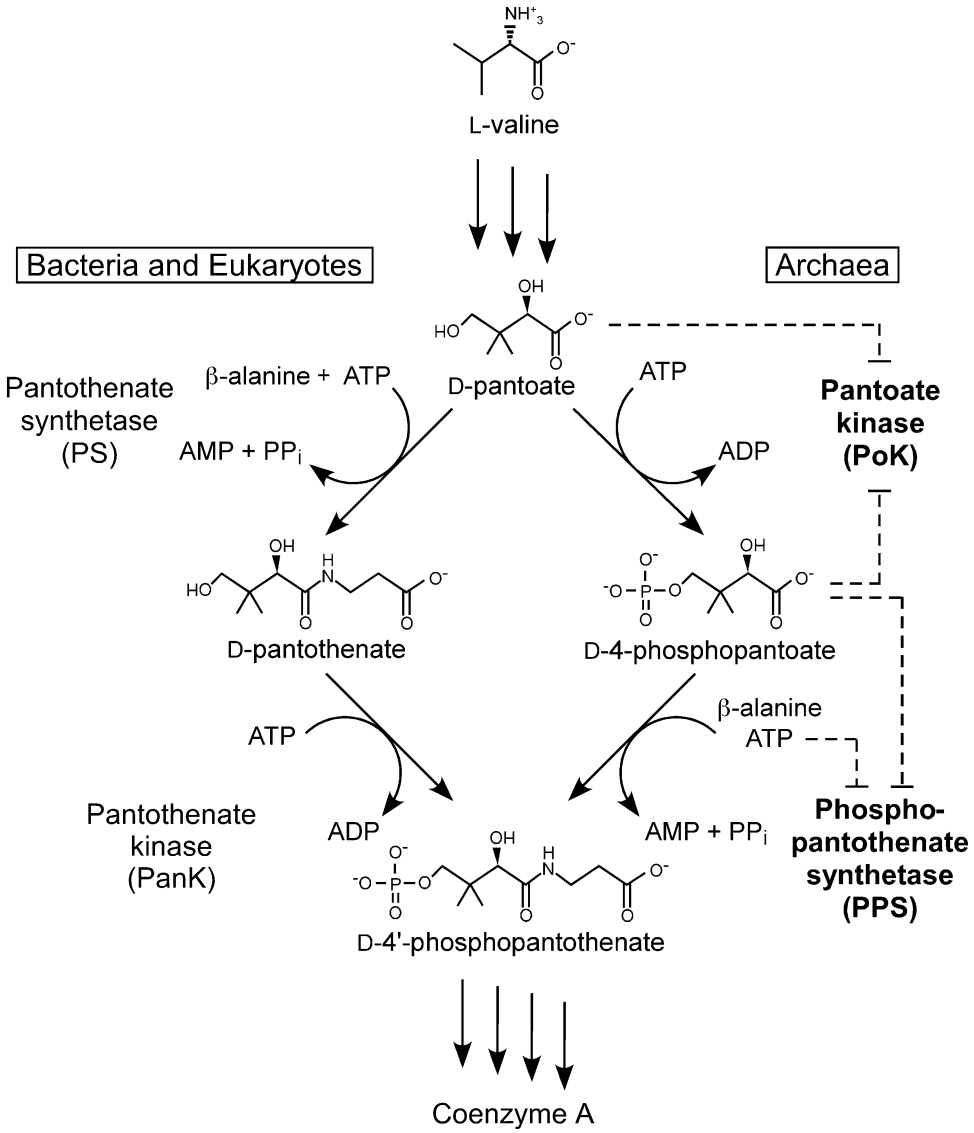

Fig. 1 Two pathways from pantoate to 4′-phospho-pantothenate in CoA biosynthesis. Bacteria and eukaryotes utilize the PS/PanK pathway (*left*), whereas most archaea utilize the PoK/PPS pathway (*right*). The *dotted lines* indicate the substrate and product inhibition observed in this study and in another recent study (Tomita et al. 2012)

we report the first biochemical characterization of the archaea-specific PPS. The study focuses on the substrate specificity and kinetics of the reaction catalyzed by PPS, and whether PPS activity is inhibited by the intermediates in the CoA biosynthetic pathway. Product inhibition in the PoK reaction was also examined.

Materials and methods

Strains and culture conditions

T. kodakarensis KOD1 (Atomi et al. 2004; Morikawa et al. 1994) and its derivative strains were cultivated under anaerobic conditions at 85 °C in a nutrient-rich medium (ASW-YT). ASW-YT medium consists of 0.8× artificial seawater (ASW), 5.0 g l^{-1} yeast extract, 5.0 g l^{-1} tryptone, and 0.8 mg l^{-1} resazurin. Sodium pyruvate (5.0 g l^{-1}, ASW-YT-Pyr medium) or elemental sulfur (2.0 g l^{-1}, ASW-YT-S^0 medium) was supplemented prior to inoculation. Na_2S was added to the medium until it became colorless. *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Agilent, Santa Clara, CA, USA), used for heterologous gene expression, was cultivated at 37 °C in Luria–Bertani (LB) medium containing 100 mg l^{-1} ampicillin. Unless mentioned otherwise, all chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Production and purification of PoK, PPS from *T. kodakarensis*

The TK2141 gene encoding PoK, with a His_6-Tag on its N-terminus, was overexpressed in *T. kodakarensis*. The TK2141 overexpression strain (*T. kodakarensis* ETK2141)

(Yokooji et al., 2009) was cultivated at 85 °C in ASW-YT-Pyr for 12 h. 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. All columns used for protein purification were the products of GE Healthcare Biosciences (Piscataway, NJ, USA). After sonication, the extract was centrifuged (20,000×*g*, 15 min, 4 °C), and the supernatant was applied to His GraviTrap, followed by elution with 20 mM potassium phosphate (pH 7.4), 0.5 M KCl, and 0.5 M imidazole. The buffer was exchanged with 50 mM Tris–HCl (pH 8.0) using a PD-10 column, and the sample was applied to anion-exchange chromatography (HiTrap Q HP). Proteins were eluted with a linear gradient of NaCl (0–1.0 M) in 50 mM Tris–HCl (pH 8.0) at a flow rate of 2.5 ml min^{-1}.

The TK1686 gene encoding PPS was overexpressed in *E. coli* (Yokooji et al. 2009). Cells were cultivated at 37 °C in LB medium until the optical density at 660 nm reached ~0.5. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM to induce expression, and cells were cultivated for a further 4 h. Cells were harvested, resuspended in 50 mM Tris–HCl buffer (pH 8.0), and disrupted by sonication. After centrifugation (20,000×*g*, 15 min, 4 °C), the soluble cell extract was incubated at 70 °C for 15 min. After removing thermolabile proteins deriving from the host by centrifugation (20,000×*g*, 15 min, 4 °C), the supernatant was applied to anion-exchange chromatography (HiTrap Q HP), and proteins were eluted with a linear gradient of NaCl (0–1.0 M) in 50 mM Tris–HCl (pH 8.0) at a flow rate of 2.5 ml min^{-1}. After concentrating the sample and exchanging the buffer to 50 mM Tris–HCl (pH 8.0) and 150 mM NaCl using Amicon Ultra-4 10K (Millipore, Billerica, MA, USA), the sample was applied to a Superdex 200 HR 10/30 gel filtration column with a mobile phase of 50 mM Tris–HCl (pH 8.0) and 150 mM NaCl at a flow rate of 0.5 ml min^{-1}. Protein concentration was determined with the Protein Assay System (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard.

Chemical synthesis of 4-phosphopantoic acid

4-Phosphopantoate, the substrate of the PPS reaction, was chemically synthesized and used for experiments for substrate specificity toward nucleoside phosphates, product inhibition of PoK and kinetic studies with ATP. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AVANCEIII 600 spectrometer (600 MHz for ^{1}H) (Bruker Biospin, Yokohama, Japan). Chemical shifts are reported in parts per million relative to the external standards [sodium 3-(trimethylsilyl)propanesulfonate for ^{1}H and ^{13}C; 85 % H_3PO_4 for ^{31}P]. High resolution mass spectrometry (HRMS) data were recorded on a JEOL JMS-700 spectrometer (JEOL, Tokyo, Japan). Optical rotation was measured on a HORIBA SEPA-500 polarimeter (HORIBA, Kyoto, Japan). Reverse-phase medium-pressure column chromatography was carried out using a Büchi Sepacore chromatography system (Büchi, Switzerland) with a COSMOSIL $5C_{18}$-PAQ column (20 mm ID × 250 mm, Nacalai Tesque). Hydrogen gas was bubbled into a mixture of (*R*)-2-benzyloxy-4-[(dibenzyloxyphosphoryl)oxy]-3,3-dimethylbutanoic acid (1.42 g, 2.85 mmol), methanol (20 ml), water (20 ml) and palladium black (1.00 g) for 3.5 h at room temperature (Fig. 2). (*R*)-2-Benzyloxy-4-[(dibenzyloxyphosphoryl)oxy]-3,3-dimethylbutanoic acid was prepared from D-pantolactone (Tokyo Chemical Industry, Tokyo, Japan) as described elsewhere (Mandel et al. 2004). After the reaction, the catalyst was filtered off and the filtrate was concentrated under reduced pressure. The residue was purified by reverse-phase medium-pressure column chromatography (water/methanol = 85:15; flow rate: 5 ml/min) to give 4-phosphopantoic acid (443 mg, 68 %) as a pale yellow oil after lyophilization. ^{1}H NMR (600 MHz, D_2O) δ_H: 0.93 (3H, s), 0.98 (3H, s), 3.69 (1H, dd, $J = 9.6$ and 4.8 Hz), 3.81 (1H, dd, $J = 9.6$ and 4.8 Hz), 4.13 (1H, s). ^{13}C NMR (151 MHz, D_2O) δ_C: 21.7, 23.2, 41.1 (d, $J = 8.1$ Hz), 74.0 (d, $J = 5.6$ Hz), 76.6, 179.4. ^{31}P NMR (243 MHz, D_2O) δ_P: 0.04 (dd, $J = 4.7$ and 4.7 Hz). $[\alpha]_D^{27}$ −6.3° (*c* 0.187, D_2O). HRMS-FAB (*m*/*z*): $[M + H]^+$ calcd for $C_6H_{14}O_7P$, 229.0477; found, 229.0478.

Enzymatic preparation of 4-phosphopantoate

When preparing 4-phosphopantoate enzymatically with PoK, the reaction mixture contained 40 mM D-pantoate, 20 mM ATP (Oriental Yeast, Tokyo, Japan), 30 μg ml^{-1} PoK protein, 10 mM $MgCl_2$, and 10 mM Tris–HCl (pH 8.0). D-Pantoate was prepared by hydrolyzing D-pantolactone (Sigma-Aldrich, St. Louis, MO, USA) in 0.4 M KOH for 1 h at 95 °C. The PoK reaction was performed for 1 h at 75 °C and stopped by cooling the mixture on ice. The PoK protein was removed by ultrafiltration (5,000×*g*, 15 min, 4 °C) with Amicon Ultra-15 10K (Millipore). The 4-phosphopantoate concentration was determined by quantifying the ADP produced in the PoK reaction by

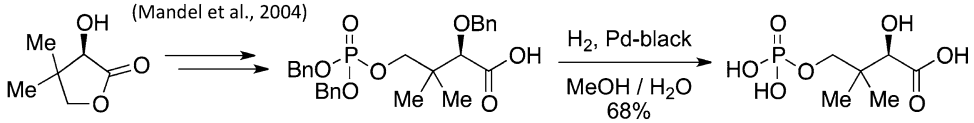

Fig. 2 Chemical synthesis of 4-phosphopantoic acid. Procedures are described in the text

HPLC. When generating 4-phosphopantoate with PoK, the stoichiometric ratio of 4-phosphopantoate:ADP generation was regarded as 1:1. In a typical reaction, approximately 11 mM of 4-phosphopantoate was generated in the PoK reaction. The amount of ATP in this reaction mixture was taken into account when adjusting the ATP concentration in the PPS reaction mixture.

Examination of PPS activity

Phosphopantothenate synthetase activity was measured by measuring the rate of AMP formation by HPLC. Unless mentioned otherwise, the standard PPS reaction mixture contained 5 mM 4-phosphopantoate, 5 mM ATP, 5 mM β-alanine (Sigma-Aldrich), 10 μg ml^{-1} PPS protein, 10 mM $MgCl_2$, and 50 mM MES-NaOH (pH 6.5). The reaction was initiated by the addition of β-alanine and incubated at 85 °C for 3, 5 and 7 min. The reaction was stopped by cooling the mixture on ice. The PPS proteins were removed by ultrafiltration with Amicon Ultra-0.5 10 K (Millipore), and 10 μl aliquots were applied to a COSMOSIL $5C_{18}$-PAQ column. Compounds were separated with 20 mM NaH_2PO_4 in water at a flow rate of 1.0 ml min^{-1} and detected by measuring absorbance at 210 nm. The amount of AMP generated in the reactions after 3, 5 and 7 min was measured and used to calculate the reaction rate. The relationship between the amount of AMP and the peak area was determined beforehand by examining the peak areas of various amounts of commercially available AMP (Oriental Yeast). For every assay, reactions were also carried out without the PPS protein, and AMP formed by thermal degradation of ATP was subtracted from the results. A linear increase in AMP was observed under these conditions, allowing us to calculate the initial velocity of the PPS reaction. Kinetic parameters for β-alanine, 4-phosphopantoate and ATP were determined using the standard method with varying concentrations of β-alanine, 4-phosphopantoate or ATP. When examining the kinetics of one substrate, the concentrations of the other two were set at 5 mM.

Thermostability, and effects of pH, temperature and addition of CoA, acetyl-CoA and 4′-phosphopantothenate

Thermostability of PPS was examined by incubating purified PPS protein (91 μg ml^{-1}) in 50 mM Tris–HCl (pH 8.0) for varying periods of time at 70, 80 or 90 °C, and measuring residual PPS activity with the standard method. To examine the effect of pH, the PPS reaction was carried out at 85 °C at various pH using the following buffers at a concentration of 50 mM: 2-morpholinoethanesulfonic acid, monohydrate (MES) (pH 5.5-6.5), piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) (pH 6.5–7.5), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (pH 7.0–8.0) and *N,N*-Bis(2-hydroxyethyl)glycine (Bicine) (pH 8.0-8.5). Effect of temperature was examined by performing the PPS reaction at varying temperatures under the standard reaction condition. To examine the inhibitory effects of CoA, acetyl-CoA and 4′-phosphopantothenate, these compounds were added to the standard reaction mixture at various concentrations. 4′-Phosphopantothenate was enzymatically produced with the PanK from *E. coli* (Ec-PanK). The Ec-PanK gene was expressed with a His_6-Tag on its N-terminus using the pET21a(+)/*E. coli* BL21(DE3)CodonPlus RIL system (Yokooji et al. 2009). Cultivation and expression conditions were the same as those applied for PPS expression. Harvested cells were suspended in 20 mM potassium phosphate buffer (pH 7.4) containing 0.5 M KCl and 20 mM imidazole, and disrupted by sonication. The extract was centrifuged (20,000×*g*, 15 min, 4 °C), and the supernatant was applied to His GraviTrap, followed by elution with 20 mM potassium phosphate (pH 7.4), 0.5 M KCl, and 0.5 M imidazole. The buffer was exchanged with 50 mM Tris–HCl (pH 8.0) with a PD-10 column. For preparation of 4′-phosphopantothenate, the reaction mixture contained 4 mM pantothenate (Sigma-Aldrich), 5 mM ATP, 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.0) and enzyme. The reaction was carried out for 1 h at 37 °C and stopped by cooling on ice. The PanK protein was removed by ultrafiltration with Amicon Ultra-15 10K (5,000×*g*, 15 min, 4 °C). The 4′-phosphopantothenate concentration was determined by quantifying the pantothenate remaining in the reaction mixture by HPLC. The relationship between the amount of pantothenate and the peak area was determined beforehand by examining the peak areas of various amounts of commercially available pantothenate.

Substrate specificity of PPS toward nucleoside triphosphates and amine compounds

Substrate specificity toward nucleoside triphosphates (NTPs) was measured using the chemically synthesized 4-phosphopantoate as substrate in the presence of 5 mM ATP, GTP, UTP or CTP (Sigma-Aldrich) and measuring the generation of AMP, GMP, UMP and CMP by HPLC, respectively. PPS activity was also measured with β-alanine, γ-aminobutyrate (GABA), glycine, or L-aspartate at a concentration of 5 mM.

Examination of product inhibition for the PoK reaction

PoK activity was measured in the presence of varying concentrations of chemically synthesized 4-phosphopantoate. PoK activity was measured at 42 °C by quantifying the rate of ADP generation with pyruvate kinase/lactate dehydrogenase (PK/LDH). The reaction mixture contained 4 mM

ATP, 5 mM phosphoenolpyruvate, 0.2 mM NADH (Oriental yeast), 6.7 μg ml^{-1} of recombinant PoK, 14.8 U ml^{-1}/18.6 U ml^{-1} of PK/LDH enzymes from rabbit muscle (Sigma-Aldrich), 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5) and various concentrations of pantoate. 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.

Results

Establishing a quantitative assay method to measure PPS activity

In our previous study (Yokooji et al. 2009), we confirmed that the TK1686 protein exhibited PPS activity, but the specific activity of the enzyme was not determined, as we could not provide saturating concentrations of 4-phosphopantoate to the PPS reaction. In this study, by examining varying concentrations of pantoate, ATP and PoK protein, we optimized the conditions for PoK reaction to produce enzymatically the PPS substrate 4-phosphopantoate at concentrations up to 11 mM. The use of this solution allowed us to examine PPS activity with 4-phosphopantoate at concentrations up to 5 mM, which was sufficient to estimate specific activity and allow kinetic examinations (see below). PPS activity was calculated by measuring the rate of AMP generation by HPLC, as quantifying AMP led to much higher sensitivity than detecting the levels of the other reaction product, 4′-phosphopantothenate.

Thermostability, and effects of pH and temperature

Recombinant PPS was produced in *E. coli*, and purified to apparent homogeneity by heat treatment, anion-exchange chromatography and gel filtration chromatography. We examined the effect of pH on the PPS reaction, and observed high activity at neutral pH with a maximum at pH 6.5 in 50 mM MES-NaOH buffer (Fig. 3a). The effect of temperature on PPS was also examined. PPS activity continued to increase with temperature elevation from 65 °C up to 95 °C (Fig. 3b). An Arrhenius plot of the data showed linearity indicating that the active site of PPS maintains its structure within this temperature range (Fig. 3c). The activation energy of the reaction was calculated to be 82.3 kJ mol^{-1}. The thermostability of PPS was examined by incubating the protein at 70, 80 and 90 °C for various periods of time and by measuring residual activity. No decrease in PPS activity was detected at 70 °C for at least 6 h, and the half-lives of the activity at 80 and 90 °C were 13.6 and 8.2 h, respectively (Fig. 3d).

Substrate specificity of PPS

We have previously reported that PPS does not exhibit PS activity, i.e. it does not recognize pantoate as a substrate

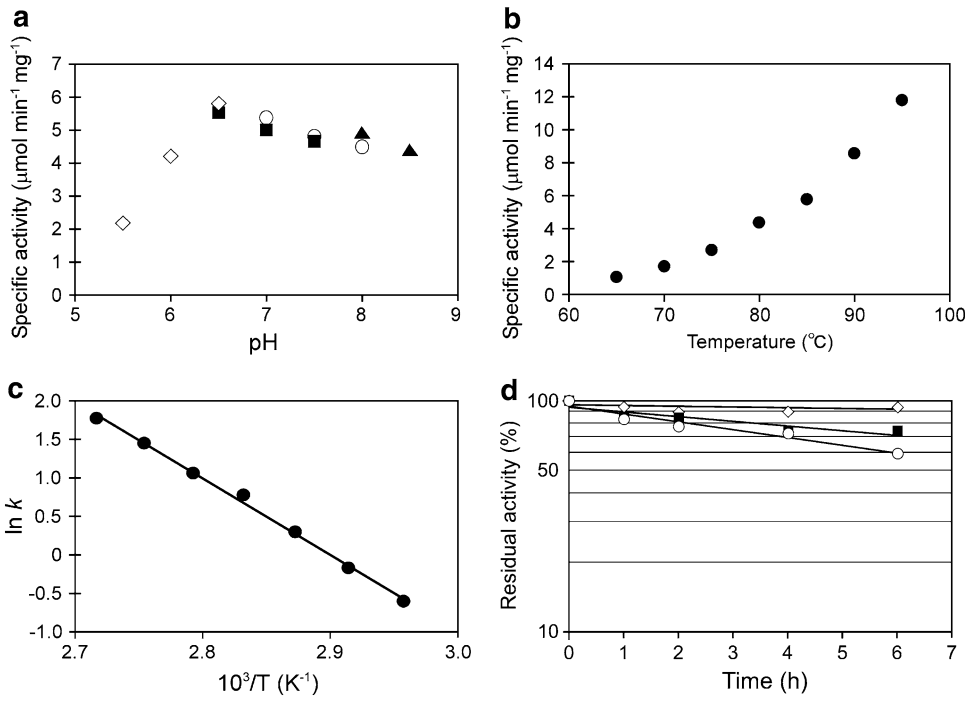

Fig. 3 The effects of pH (**a**) and temperature (**b**) on PPS activity. **a** Buffers were MES (*open diamonds* pH 5.5–6.5), PIPES (*closed squares* pH 6.5–7.5), HEPES (*open circles* pH 7.0–8.0) and Bicine (*closed triangles* pH 8.0–8.5). **c** An Arrhenius plot of the data shown in **b**. **d** Thermostability of the PPS protein. PPS activity was measured after incubation at 70 °C (*open diamonds*), 80 °C (*closed circles*) and 90 °C (*open circles*). All measurements were performed in the presence of 5 mM *β*-alanine, 5 mM 4-phosphopantoate and 5 mM ATP

(Yokooji et al. 2009). Here, we tried to detect PS activity in PPS in the presence of excess concentrations (10 mM) of pantoate, but no activity was observed. We also examined the specificity of the enzyme toward nucleoside triphosphates and amine compounds. PPS reactions were carried out with 5 mM 4-phosphopantoate, 5 mM β-alanine and 5 mM of ATP, GTP, UTP or CTP. In this experiment, we used chemically synthesized 4-phosphopantoate to avoid carrying residual ATP from the PoK reaction into the PPS reaction. The activity level of PPS with ATP was 5.42 μmol min^{-1} mg^{-1}, whereas those with GTP, UTP and CTP were all lower than 0.01 μmol min^{-1} mg^{-1}, indicating a strict preference for ATP. As for the amino compounds, those examined here were β-alanine, γ-aminobutyrate (GABA, one methylene unit longer than β-alanine), glycine (one methylene unit shorter) and L-aspartate (a carboxylated derivative of β-alanine). As a result, PPS activity with 5 mM of 4-phosphopantoate, ATP and β-alanine was 5.42 μmol min^{-1} mg^{-1}. Activity with GABA was as low as 0.05 μmol min^{-1} mg^{-1}, and those for the other compounds were even lower. The results indicate that PPS displays strict specificity for its natural substrates, 4-phosphopantoate, ATP and β-alanine.

Kinetic analysis

Kinetic studies on the PPS reaction were performed by varying the concentration of one substrate with those of the other two substrates being set at constant (5 mM). The kinetics toward β-alanine followed Michaelis–Menten kinetics (Fig. 4a). In the case of 4-phosphopantoate, using the chemically synthesized compound, we observed a decrease in initial velocity at concentrations higher than 2 mM (Fig. 4b), indicating that the PPS reaction displays substrate inhibition toward 4-phosphopantoate. We found that our data fit well to one of the typical substrate inhibition models, expressed as $v = V_{max}[S]/(K_{s1} + [S] + [S]^2/K_{s2})$, where v is reaction velocity, V_{max} is maximum velocity, [S] is substrate concentration, K_{s1} is the dissociation constant between enzyme and the first substrate, and K_{s2} is the dissociation constant between the enzyme–substrate complex and the second, inhibitory substrate. Substrate inhibition was also observed with ATP (Fig. 4c), and the data fit well with the same kinetic model proposed for 4-phosphopantoate. Kinetic parameters for the three substrates are shown in Table 1.

Effects of CoA, acetyl-CoA and 4′-phosphopantothenate on PPS activity

As described above, the biosynthesis of CoA is strictly regulated in bacteria and eukaryotes via feedback inhibition of PanK by CoA or its thioesters (Rock et al. 2002,

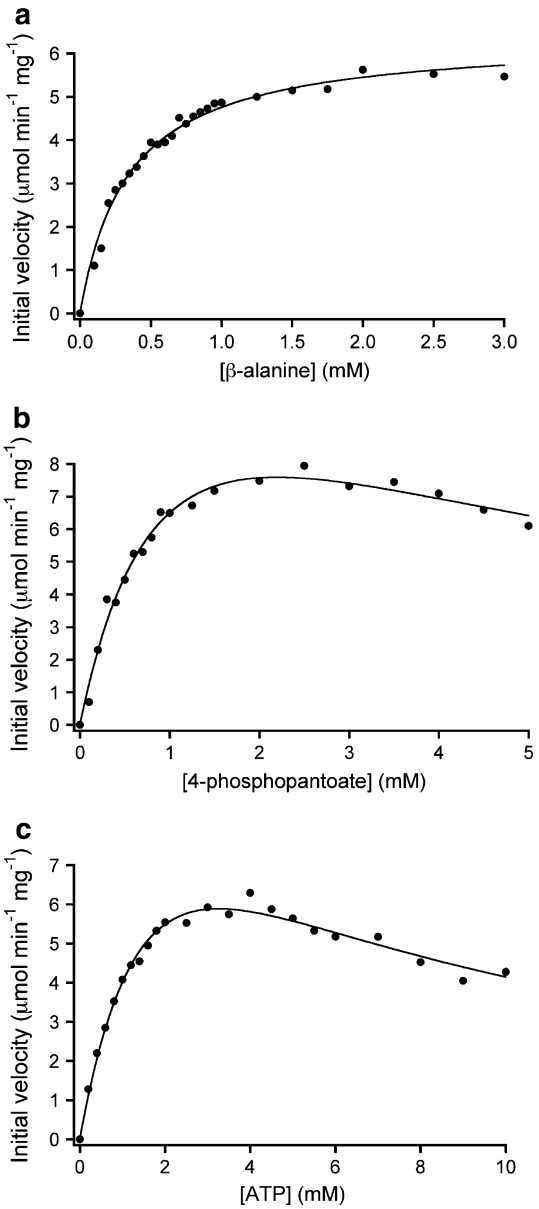

Fig. 4 Kinetic analyses of PPS toward β-alanine (**a**), 4-phosphopantoate (**b**) and ATP (**c**). The measurements were carried out at varying concentrations of one substrate in the presence of 5 mM of the other two substrates

Table 1 Kinetic parameters of the PPS reaction with its substrates

Substrate	K_m or K_{s1} (mM)	K_{s2} (mM)	Apparent V_{max} (μmol min^{-1} mg^{-1})
β-alanine	0.34 ± 0.02	–	6.38 ± 0.12
4-phosphopantoate	1.16 ± 0.23	4.16 ± 1.05	15.6 ± 2.0
ATP	2.44 ± 0.41	4.33 ± 0.79	14.7 ± 1.7

In the case of 4-phosphopantoate and ATP, the following equation was used: $v = V_{max}[S]/(K_{s1} + [S] + [S]^2/K_{s2})$

2003; Vallari et al. 1987; Zhang et al. 2005). We thus examined the possibilities of feedback regulation of PPS. PPS reactions were carried out in the presence of various concentrations of CoA or acetyl-CoA. As a result, no change in PPS activity was observed in the presence of CoA or acetyl-CoA up to 2 mM (data not shown). The results indicate that CoA and acetyl-CoA neither activate nor inhibit PPS. We also examined the effect of 4′-phosphopantothenate (product inhibition) on PPS activity. This experiment was carried out with 4′-phosphopantothenate prepared enzymatically using Ec-PanK. The main components in the PanK mixture after the reaction are the residual substrate ATP and the products ADP and 4′-phosphopantothenate. As the measurement of PPS activity is based on quantifying AMP, the use of the PanK reaction mixture containing ADP does not directly interfere with PPS assay. As a result, the addition of 4′-phosphopantothenate did not affect PPS activity, indicating no product inhibition with PPS.

Effect of 4-phosphopantoate on PoK activity

As the chemically synthesized 4-phosphopantoate was available, we next examined the possibilities of product inhibition of PoK. In the presence of 0.5 mM pantoate, the PoK substrate, we observed a gradual decrease in activity with increasing concentrations of 4-phosphopantoate (Fig. 5a). A decrease in activity of approximately 30 % was observed in the presence of 2 mM 4-phosphopantoate. We then examined whether the inhibition was affected by the concentration of pantoate. Regardless of the pantoate concentration in the range of 0.1–2 mM, the relative decrease in activity brought about by a given concentration of 4-phosphopantoate was constant at a range of 20–30 %, suggesting that the inhibition was not competitive (Fig. 5b).

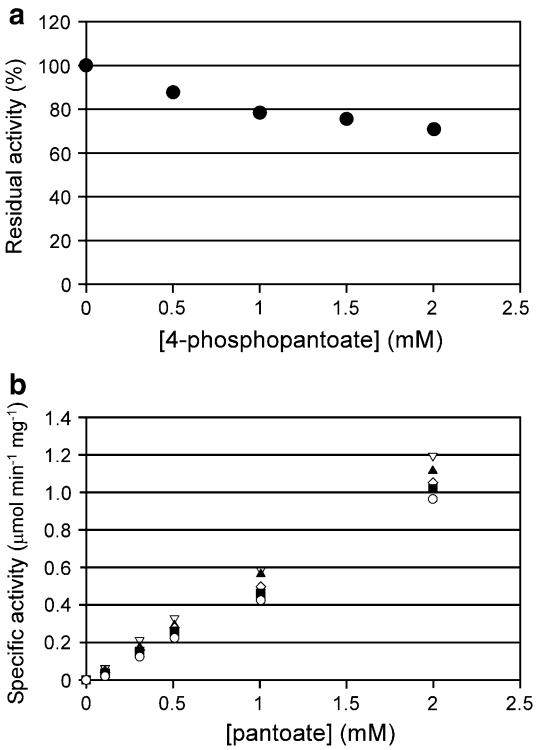

Fig. 5 a PoK activity in the presence of various 4-phosphopantoate concentrations. All reactions were performed with 0.5 mM pantoate and 4 mM ATP. **b** The effects of pantoate concentration on the product inhibition by 4-phosphopantoate. PoK activity was measured with various concentrations of pantoate in the presence of 4 mM ATP and 0 mM (*open triangles*), 0.5 mM (*closed triangles*), 1.0 mM (*open diamonds*), 1.5 mM (*closed squares*) and 2.0 mM (*open circles*) of 4-phosphopantoate

Discussion

Here, we have performed the first biochemical characterization of PPS, an enzyme that catalyzes the condensation reaction of 4-phosphopantoate and *β*-alanine to produce 4′-phosphopantothenate, a reaction specific to the Archaea. As the enzyme is not found in bacteria/eukaryotes and has never been characterized so far, there is no enzyme with which we can directly compare its enzymatic properties. The reaction catalyzed by pantothenate synthetase (PS), found in both bacteria and eukaryotes, can be regarded as the counterpart of the archaeal PPS reaction. PS enzymes from bacteria and eukaryotes have high similarity with each other in their primary structure (Genschel 2004). Crystal structures of the enzymes from *E. coli* (von Delft et al. 2001), *Mycobacterium tuberculosis* (Wang and Eisenberg 2003) and *Staphylococcus aureus* (Satoh et al. 2010) indicate high structural similarity among these proteins. These enzymes are considered as the members of the cytidylyltransferase superfamily and harbor a conserved HXGH motif (von Delft et al. 2001). The His residues in this motif are considered to bind to the α-phosphate of nucleoside triphosphates and stabilize the acyl adenylate intermediates (Weber et al. 1999). Although PPS and PS catalyze similar reactions, the primary structure of PPS has no similarity to those of the PS enzymes and does not contain the HXGH motif. In addition, when we aligned 24 archaeal PPS sequences, we found that over 30 residues are completely conserved, but a histidine residue is not found among these residues. This raises the possibility that the residues/structures and mechanisms involved in substrate recognition and catalysis of archaeal PPS completely differ from those of the bacterial/eukaryotic PS. PPS did not catalyze the PS reaction at all even in the presence of excess concentrations of pantoate, suggesting that binding of 4-phosphopantoate to PPS is highly dependent on recognition of the phosphate group. Differences are also found in the substrate specificities and kinetic behavior of PPS and PS. The reaction catalyzed by the PS from *E. coli*

follows regular Michaelis–Menten kinetics toward all three substrates, pantoate, β-alanine and ATP (Miyatake et al. 1978), whereas substrate inhibition has been observed in the PPS reaction toward 4-phosphopantoate and ATP as shown in Fig. 4b, c, respectively. There has been a report of substrate inhibition by pantoate in the PS from *Arabidopsis thaliana* (Jonczyk and Genschel 2006). Although the *E. coli* PS is an ATP-dependent enzyme, activity levels of approximately 10 % can also be detected with GTP, UTP, CTP and ITP (Miyatake et al. 1978). The PPS seems to have higher selectivity for ATP, as no activity can be detected with other NTPs.

As described above, CoA biosynthesis in most organisms is strictly regulated by means of feedback inhibition of PanK by CoA and its thioesters. Crystal structures of *E. coli* PanK bound to CoA and ATP analogs indicated that the phosphate binding sites overlap, providing an explanation for the competitive inhibition of CoA toward the PanK substrate ATP (Yun et al. 2000). The reaction catalyzed by PoK, the enzyme catalyzing the reaction preceding the PPS reaction, was not affected by the presence of CoA or acetyl-CoA (Tomita et al. 2012). In this study, we have shown that PoK exhibits product inhibition by 4-phosphopantoate. An approximately 30 % decrease in activity was observed in the presence of 2 mM 4-phosphopantoate. The PPS reaction was not affected either by the presence of CoA or acetyl-CoA. In contrast to PoK, product inhibition of PPS was not observed with 4′-phosphopantothenate at concentrations up to 2 mM. On the other hand, the enzyme showed substrate inhibition with 4-phosphopantoate and ATP, but the extent of which was moderate. Although a number of inhibitory mechanisms are possible for both PoK and PPS, we still hesitate in concluding that they actually play a major role in regulating CoA biosynthesis in vivo, because relatively high concentrations of 4-phosphopantoate are required to onset product inhibition (PoK) or substrate inhibition (PPS) to a significant extent. Biochemical studies on other enzymes of the CoA biosynthesis pathway must be carried out to unravel post-translational regulation in the Archaea. Feedback inhibition on enzymes responsible for reactions relatively upstream in the pathway, such as ketopantoate reductase, would be the most efficient. Although archaeal enzymes have not yet been experimentally examined, ketopantoate reductase from *E. coli* (Ciulli et al. 2007; Lobley et al. 2005; Zheng and Blanchard 2000a, b, 2003) and *Saccharomyces cerevisiae* (King et al. 1974; King and Wilken 1972; Wilken et al. 1975) use NAD(P)H as an electron donor. As the NAD(P)H and CoA molecules display structural similarities, both containing an ADP moiety, there is a possibility that CoA may bind to the enzyme in a competitive manner with NAD(P)H. Likewise, enzymes dependent on ATP, such as phosphopantothenoylcysteine synthetase, are also candidates, as the common ADP moiety found in both ATP and CoA may enable competitive inhibition, which is actually the case in bacterial and eukaryotic PanK enzymes.

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