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ORIGINAL PAPER

Identification and characterization of a thermostable bifunctional enzyme with phosphomannose isomerase and sugar-1-phosphate nucleotidylyltransferase activities from a hyperthermophilic archaeon, *Pyrococcus horikoshii* OT3

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Abstract Mannosylglycerate is known as a compatible solute, and plays important roles for salinity adaptation and high temperature stability of microorganisms. In the gene cluster for the mannosylglycerate biosynthetic pathway predicted from the genomic data of *Pyrococcus horikoshii* OT3, the PH0925 protein was found as a putative bifunctional enzyme with phosphomannose isomerase (PMI) and mannose-1-phosphate guanylyltransferase (Man-1-P GTase) activities, which can synthesize GDP-mannose when accompanied by a phosphomannomutase/phosphoglucomutase (PMM/PGM) enzyme (PH0923). The recombinant PH0925 protein, expressed in *E. coli,* exhibited both expected PMI and Man-1-P GTase activities, as well as absolute thermostability; 95 °C was the optimum reaction temperature. According to the guanylyltransferase activity (GTase) of the PH0925 protein, it was found that the protein can catalyze glucose-1-phosphate (Glc-1-P) and glucosamine-1-phosphate (GlcN-1-P) in addition to Man-1-P. The analyses of C-terminus-truncated forms of the PH0925 protein indicated that sugar-1-phosphate nucleotidylyltransferase (Sugar-1-P NTase) activity was located in the region from the N-terminus to the 345th residue, and that the C-terminal 114 residue region of the PH0925 protein inhibited the Man-1-P GTase activity. Conversely, the PMI activity was abolished by deletion of the C-terminal 14 residues. This is the first report of a thermostable enzyme with both PMI and multiple Sugar-1-P NTase activities.

Keywords Hyperthermophilic archaea · Sugar-1-phosphate nucleotidylyltransferase · Genomic information · Phosphohexose isomerase · Thermostable enzyme

Abbreviations

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ORF	Open reading frame
MOPS	3-Morpholinopropanesulfonic acid
PMI	Phosphomannose isomerase
Man-1-P GTase	Mannose-1-phosphate guanylyltransferase
GDP-Man	GDP-mannose
Sugar-1-P NTase	Sugar-1-phosphate nucleotidylyltransferase
GlcN-1-P	Glucosamine-1-phosphate

Introduction

Most microorganisms accumulate compatible solutes, low molecular weight organic metabolites, for resistance and adjustment to changes in external osmotic pressures or increase in temperatures in the immediate environment. In an anaerobic hyperthermophilic archaeon, *Pyrococcus*

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horikoshii was isolated from an Okinawa trough in 1992 (González et al. 1998) and for which the entire genomic sequence was published in 1998 (Kawarabayasi et al. 1998), mannosylglycerate (MG) was found to be the main compatible solute that accumulated in response to increased external osmotic pressure (Empadinhas et al. 2001). This archaeon synthesizes MG only via a two-step pathway involving formation of a phosphorylated mannosyl-3-phosphoglycerate with mannosyl-3-phosphoglycerate synthase (MPGS) (Empadinhas et al. 2001).

Based on genomic data, only archaeal species within the genera *Pyrococcus* and *Thermococcus* among all thermophilic microorganisms that accumulate MG, possess a 4-gene cluster for its biosynthesis (Empadinhas and da Costa 2011). The outline of this pathway is shown in the supplemental Fig. 1. The gene cluster in *P. horikoshii* contains four genes encoding phosphomannomutase/phosphoglucomutase (PMM/PGM: PH0923), phosphomannose isomerase/mannose-1-phosphate guanylyltransferase (PMI/Man-1-P GTase: PH0925), mannosyl-3-phosphoglycerate phosphatase (MPGP: PH0926), and MPGS (PH0927). The actual activities on the gene products of PH0926 and PH0927 have already been reported (Empadinhas et al. 2001), and the activity of the PH0923 protein was confirmed by work from our group (Akutsu et al. 2005). However, the actual activities and properties of the PH0925 protein remain undocumented.

The PH0925 gene was expressed in *E. coli,* and purified recombinant protein was used for biochemical analyses to characterize the actual enzymatic activities. In this work, it was shown that the recombinant PH0925 protein actually exhibited PMI and Man-1-P GTase activities as well as extreme thermostability. In addition to the expected Man-1-P GTase activity, the PH0925 protein accepted glucose-1-phosphate (Glc-1-P) and glcosamine-1-phosphate (GlcN-1-P) as substrates for its sugar-1-phosphate nucleotidylyltransferase (Sugar-1-P NTase) activity. Analyses of a series of truncated forms of the PH0925 protein indicated that PMI and Sugar-1-P NTase activities were, respectively, catalyzed by the N- and C-terminal region of the protein.

Materials and methods

Bacterial strains and materials

Pyrococcus horikoshii OT3 (JCM 9974), an anaerobic hyperthermophilic archaeon, was obtained from the Japan Collection of Microorganisms (JCM). Culture of this archaeon and preparation of its genomic DNA were carried out as previously described (Kawarabayasi et al. 1998). *Escherichia coli* strain DH5α, used for plasmid cloning, was obtained from Takara Bio Inc. (Ohtsu, Shiga, Japan), and strain BL21-Codon Plus(DE3)-RIL, used for expression of recombinant protein, was obtained from Stratagene (La Jolla, CA, USA). KOD-plus DNA polymerase used for PCR amplification was purchased from Toyobo Co., Ltd. (Osaka, Japan). The restriction enzymes and T4 DNA ligase used for gene manipulation were obtained from New England Bio Labs, Inc. (Beverly, MO, USA). Phosphoglucose isomerase and glucose-6-phosphate dehydrogenase from *Geobacillus stearothermophilus* were obtained from

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PH0925  MKALILAGGKGTRLWPLSREAMPKQFIKVFSDRSLFQKTVERALIFSKPKEIFVVTNKEYRFRVLDDLNELGLKVPEENILLEPVGKNTLPAIYWGLKVI 100
        *   ** ** * *******   ****     *  *** *  * * *        * *** ** *   *    *      ***** * ** **
Pa      MIPVILSGGSGSRLWPLSRKQYPKQFLALTGDDTLFQQTIKR-LAFDGMQAPLLVCNKEHRFIVQEQLEAQNLAS--QAILLEPFGRNTAPAVAIAAMKL  97

PH0925  NDNYGDSVVAVLPSDHAIEVNESYMEAFKKAEKLAEK-YLVTFGIKPTKPHTGYGYIKPGEKIEVEGKVLGYLVDEFKEKPDLETARKYVENG-YYWNSG 198
             *     ** ** **       *   *   ***   * ***    * ******          *    *  * ****   **  *  * ******
Pa      VAEGRDELLLILPADHVIEDQRAFQQALALATNAAEKGEMVLFGIPASRPETGYGYIRASADAQLPEGVS--RVQSFVEKPDEARAREFVAAGGYYWNSG 195

PH0925  MFMFKVSVFMEEARKHSPDVVK----AFE----EGKSI---EEIYELAPEISVDYGIMEKTNKAAVVPLNTYWNDLGSFDAVYEALEKDENGNAVHVTGF 287
        ** *  *   **  **  *       * *     *         *  *  * **  ****  * ****   *** **         ** ***   **
Pa      MFLFRASRYLEELKKHDADIYDTCLLALERSQHDGDLVNIDAATFECCPDNSIDYAVMEKTSRACVVPLSAGWNDVGSWSSIWDVHAKDANGN---VT-- 290

PH0925  KAKYINVDSRNNLVLT-ERLTATVGVEDLVIIDTGDALLVAKRGETQKVKEVYKKLKEENDERAIVHRTAYRPWGSYTVLEEGERYKIKRLTVLPGKRLS 386
        *      ** * **     *    * ** *   * **   *     * ** * * *          *   *******     * *   *  ** ** ***
Pa      KGDVLVHDSHNCLVHGNGKLVSVIGLEDIVVVETKDAMMIAHKDRVQDVKHVVKDLDAQGRSETQNHCEVYRPWGSYDSVDMGGRFQVKHITVKPGARLS 390

PH0925  LQIHYHRSEHWVVVRGTAKVKVGDKEFILRPGESTFIPAGVPHRLENPGKVILEVIETQIGEYLGEDDIVRLQDDYGR--------------- 464
        ** * ** *** ** *** *   ** * *    ** **    *** ****  ** ** * * ******* ** * ***
Pa      LQMHHHRAEHWIVVSGTAQVTCDDKTFLLTENQSTYIPIASVHRLANPGKIPLEIIEVQSGSYLGEDDIERLEDVYGRTAEPALQVVAGSR 481
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Fig. 1 Comparison of amino acid sequences between the PH0925 protein and the bacterial bifunctional enzymes with PMI and Man-1-P GTase activities. *Pa* indicates a bifunctional enzyme with PMI and Man-1-P GTase activities from *Pseudomonas aeruginosa* (GenBank™ accession number: NP_252241). PH0925 indicates the PH0925 protein used in this work. *Each letters with asterisk* an amino acid residue conserved in these two sequences

Seikagaku Corporation (Tokyo, Japan). Lysozyme from chicken egg white was obtained from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). Deoxyribonuclease I was purchased from Takara Bio Inc. Lysozyme from chicken egg white was obtained from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). The plasmid vector pET28a was purchased from Novagen (Merck, Darmstadt, Germany). NTP, dNTP, GDP-mannose, GDP-glucose, all sugar-1-phosphates used in this study, mannose-6-phosphate (Man-6-P), and nicotinamide adenine dinucleotide phosphate oxidized form (NADP) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA).

Primers used in this work

Each primer sequence and all primer combinations used for PCR amplifications are, respectively, listed in the supplemental Tables 1 and 2.

Construction of expression vectors

To enable the expression of wild-type PH0925 protein with a histidine tag at the N-terminus, two primers (dPH0925F and dPH0925R in supplemental Table 1) were, respectively, designed from the 5′ and 3′ terminal sequences of the PH0925 gene; dPH0925F contained an *Nde* I site and a start codon (respectively, indicated by single underline and italics in supplemental Table 1); dPH0925R contained a *Bam*H I site and a stop codon (respectively, indicated by double underline and in bold in supplemental Table 1). Ten nanograms of *P. horikoshii* OT3 genomic DNA and 10 pmol of each primer were used for PCR amplification in 50 μL of standard PCR reaction solution containing 50 mM Tris–HCl (pH 7.5), 30 mM NaCl, and 2 mM of each dNTP. 30 cycles with a temperature profile of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C were performed with 2.5 units of KOD-plus DNA polymerase. The PCR products were digested with *Nde* I and *Bam*H I and ligated with vector pET28a digested with the same restriction enzymes. After confirmation of their nucleotide sequences, the plasmid possessing the full-length PH0925 coding region with a histidine tag at the N-terminus was designated as pETPH0925H.

To construct expression plasmids encoding a series of C-terminus- or N-terminus-truncated forms of the PH0925 protein, combinations of the dPH0925F and dC_R series primers (for C-terminal truncations) or dN_F series primers and the dPH0925R primer (for N-terminal truncations) were, respectively, used to PCR amplify truncated PH0925 genes. Each amplified PCR fragment was digested with *Nde* I and *Bam*H I, and ligated with the pET28a plasmid digested with the same restriction enzymes. The nucleotide sequence of each expression vector encoding the truncated PH0925 gene was confirmed; plasmid designations and predicted coding information are listed in supplemental Table 2.

Expression and purification of the recombinant proteins

Each confirmed expression vector was introduced into the *E. coli* strain BL21-Codon Plus(DE3)-RIL cells. The transformed *E. coli* was grown in 2 L of LB medium containing 75 μg/mL kanamycin and 50 μg/mL chloramphenicol at 25 °C until the absorbance at 600 nm reached 0.6. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and aeration was continued for 15 h at 25 °C to induce expression of recombinant proteins. After induction, *E. coli* cells were collected by centrifugation at 5000×*g* for 15 min, washed with 300 mL of 50 mM MOPS (pH 7.6), and suspended in 180 mL of 50 mM MOPS (pH 7.6). Lysozyme and deoxyribonuclease I were, respectively, added to a final concentration of 0.2 mg/g of wet-cell pellet and 10 μg/mL, to each cell suspension, then each cell suspension was treated for 30 min on ice. The treated cells were ruptured by sonication with a Vibra cell (SONICS, Newtown, CT, USA), using 30 cycles of 10-s pulses followed by a 50-s rests on ice. Each lysate was centrifuged at 20,000×*g* for 30 min at 4 °C, and each supernatant was collected as a soluble fraction. Each soluble fraction was treated at 80 °C for 30 min and then centrifuged at 20,000×*g* for 30 min at 4 °C. The recombinant proteins were purified by the nickel-loaded HiTrap Chelating HP column (GE healthcare, Piscataway, NJ, USA) according to the manufacturer's instruction. Proteins contained in 3 μL of elution solution were separated by 0.1 % SDS, 10 % polyacrylamide gel electrophoresis and detected by staining with Coomassie Brilliant Blue R-250. The recovered proteins were dialyzed against 50 mM MOPS (pH 7.6). The purified proteins were stored at 4 °C. Concentration of purified proteins was determined by the BCA protein assay reagent kits (Pierce Biotechnology, Inc., Rockford, IL, USA).

Assay of phosphomannose isomerase (PMI) activity

As the PMI activity assay, the reverse direction of the reaction, producing Frc-6-P from mannose-6-phosphate (Man-6-P), was measured. The reaction was performed in a 2 mL standard reaction mixture containing 50 mM MOPS (pH 7.6), 2 mM $MgSO_4$, 1 mM $NADP^+$, 2 U phosphoglucose isomerase from *G. stearothermophilus*, 2 U glucose-6-phosphate dehydrogenase from *G. stearothermophilus* and 2.0 μg of purified recombinant protein. After pre-incubation of the reaction mixture at 65 °C for 3 min, the reaction was started by the addition of Man-6-P to a final

concentration of 0.4 mM, and allowed to continue at 65 °C for one min. Absorbance increase was monitored at 340 nm using a spectrophotometer (V-550, JASCO).

Assay of sugar-1-phosphate nucleotidylyltransferase (Sugar-1-P NTase) activity

The Sugar-1-P NTase activity of the wild-type and truncated forms of the PH0925 protein was analyzed by the method described previously (Zhang et al. 2005) with some modification. The standard assay for the forward reaction producing NDP-sugar from NTP and Sugar-1-P was performed in a 10-μL reaction mixture containing 50 mM MOPS (pH 7.6), 2 mM $MgSO_4$, 0.05 mM one of NTP, 1.0 mM one of Sugar-1-P, and 1 ng of purified recombinant protein. After 1 min of pre-incubation at 85 °C, each reaction was started by the addition of the recombinant protein and progressed at 85 °C for 5 min. 100 μL of 500 mM KH_2PO_4 was added to the reaction mixture immediately after finishing reaction. A 50-μL aliquot of each solution was analyzed on a Hitachi LaChrom Elite HPLC system with a 0.46 × 25 cm column of Wakosil 5C18-200 (Wako, Tokyo, Japan). The flow rate of 500 mM KH_2PO_4 was maintained at 1 mL/min. The product of the reaction, NDP-sugar, was monitored by absorbance at 257 nm, and the amount of product was calculated based on the area under the peaks. The NDP-sugar molecule including in peak was confirmed by coincidence of the elution position with that for the standard sugar-nucleotide molecules.

Analyses of enzymatic properties

The effect of metal ions on PMI and Sugar-1-P NTase activities was analyzed separately. For PMI activity, the standard 2-mL reaction mixture containing either 0.1 mM EDTA alone or 0.1 mM EDTA plus 2 mM of Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, or Ni^{2+} ion was used. For Sugar-1-P NTase activity, the standard 10-μL reaction mixture containing either 0.1 mM EDTA alone or 0.1 mM EDTA plus 2 mM of Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+} ion was utilized.

Results and discussion

Construction of the expression vector and expression of the recombinant PH0925 protein

A 1395-bp PH0925 ORF was predicted to encode a homologue of the well-characterized bacterial bifunctional enzymes with PMI and Man-1-P GTase activities. As shown in Fig. 1, the PH0925 protein showed approximately 44 % identity to the similar enzyme from *Pseudomonus aeruginosa* (Shinabarger et al. 1991; May et al. 1994). The PH0925 gene was therefore predicted as a protein with both PMI and Man-1-P GTase activities in a gene cluster for MG biosynthetic pathway. There was no previous report for archaeal similar bifunctional protein with high thermostability. By construction of the expression vector of the PH0925 gene product containing a histidine tag at the N-terminus, the soluble and thermostable form of the recombinant wild-type PH0925 protein was successfully expressed.

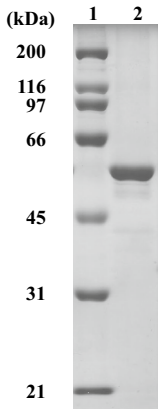

Fig. 2 SDS-PAGE analysis of purified wild-type PH0925 protein. The recombinant protein expressed by and isolated from *E. coli* harboring the expression plasmid pETPH0925H was analyzed on a 10 % polyacrylamide gel containing 0.1 % SDS. *Lane 1* molecular mass protein standards, *Lane 2* purified PH0925 protein. The recombinant protein was purified via nickel-affinity column chromatography on a HiTrap Chelating HP column and visualized by Coomassie Brilliant Blue R-250 staining

In previous analyses of the enzyme from *P. aeruginosa*, PMI activity was found to be located in the C-terminal region based on removal of the C-terminus by chymotryptic digestion (May et al. 1994). However, the exact region essential for each PMI and Man-1-P GTase activity has not been determined. Therefore, we designed a series of N- and C-terminus-truncated forms of the PH0925 protein (supplemental Tables 1 and 2) and constructed expression vectors were expressed in *E. coli* strain BL21-Codon Plus(DE3)-RIL cells. All 6 N-terminus-truncated forms of the PH0925 protein were expressed as insoluble form. Therefore, the only C-terminus-truncated forms of the PH0925 protein were used for their activity analyses. These observations indicated that the N-terminal region of the PH0925 protein, unlike the C-terminal region, played an important role in the solubility of the entire PH0925 protein and in the acquisition of the correct structure of the entire PH0925 protein.

The expressed proteins were purified by affinity chromatography with the nickel-loaded HiTrap chelating HP column. As shown in Fig. 2, SDS-PAGE analysis indicated

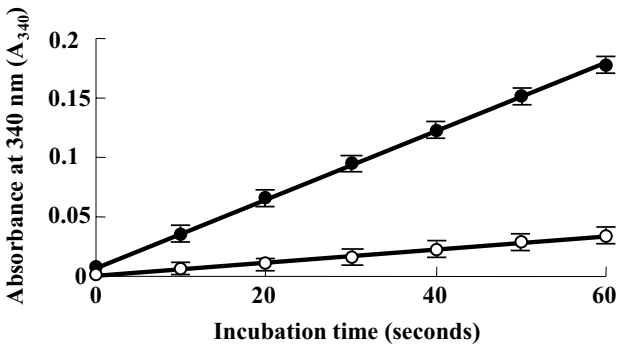

Fig. 3 Absorbance at 340 nm during PMI reaction. A standard PMI activity assay was performed with standard reaction conditions, and absorbance at 340 nm was measured after incubation periods indicated. The *closed* and *open circles* indicate absorbance for the reaction in the presence or absence, respectively, of the PH0925 protein

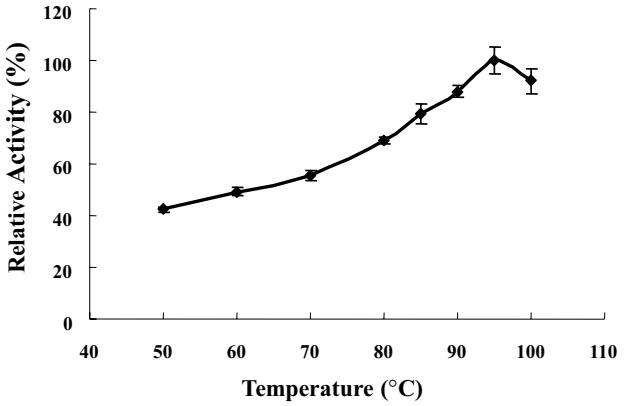

Fig. 4 Relative activities of the PH0925 protein at varying temperatures. Man-1-P GTase activity was measured with temperatures between 50 and 100 °C. Relative activity is expressed as a percentage of the maximum activity at 95 °C

that the purified wild-type PH0925 protein migrated as a single band with a molecular mass of approximately 52.9 kDa, consistent with the molecular mass predicted from the nucleotide sequences.

PMI activity of the PH0925 protein

The reverse reaction of the PH0925 PMI activity, producing Frc-6-P from Man-6-P, was monitored by increasing amount of NADPH. As NADPH was produced from Frc-6-P, product of the PH0925 PMI activity, with two coupling enzymes, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, the amount of NADPH indicated that of Frc-6-P produced. Therefore, NADPH production was monitored by absorbance at 340 nm for measuring the reverse reaction of the PMI activity of the PH0925 protein. As shown in Fig. 3, increased absorbance at 340 nm was evident and time dependent at 60 °C only when the PH0925 protein was added into the reaction solution, revealing that the PH0925 protein expressed in *E. coli* possessed actual PMI activity.

To determine the kinetic constants, standard reaction conditions were used; additionally, the concentrations of the coupling enzymes, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, were five- to tenfold higher than the levels required for catalyzing all products. For the reverse reaction (production of Frc-6-P from Man-6-P) of the PH0925 PMI activity measured at 65 °C, the apparent K_m for Man-6-P and V_{max} values were 98.6 ± 9.7 μM and 28.8 ± 1.8 μmol min^{-1} mg^{-1} protein, respectively.

Analyses of substrate specificity indicated that the PH0925 protein was capable of catalyzing Man-6-P and none of other sugar-6-phosphate molecules. This property of the PH0925 PMI activity was similar to the bacterial bifunctional enzymes with PMI and Man-1-P GTase activities (Shinabarger et al. 1991).

In Archaea, a single-functional PMI enzyme was identified from genomic data of *A. pernix* (Hansen et al. 2004). The single-functional PMI enzyme exhibited conversion ability between Glc-6-P and Frc-6-P as well as between Man-6-P and Frc-6-P. This single-functional PMI/PGI gene from *A. pernix* encodes a 331-residue enzyme (Hansen et al. 2004). However, the PH0925 protein did not show remarkable sequence similarity to the *A. pernix* single-functional PMI/PGI protein. These observations indicated that these two types of phospho-sugar isomerases evolved independently.

Man-1-P GTase activity of the PH0925 protein

The forward reaction of Man-1-P GTase activity, producing GDP-mannose (GDP-Man) from GTP and Man-1-P, was measured by amount of GDP-Man produced with HPLC system as described in Materials and Methods section. GDP-Man was detected after incubation at 85 °C only in the presence of the PH0925 protein (data not shown), revealing that the PH0925 protein actually possessed Man-1-P GTase activity at 85 °C.

For the forward reaction of Man-1-P GTase activity at 85 °C, the apparent K_m values of the PH0925 protein with GTP and Man-1-P were 2.6 ± 1.1 and 16.1 ± 4.1 μM, respectively. The V_{max} for the forward reaction of Man-1-P GTase activity with the PH0925 protein was 11.7 ± 2.4 μmol min^{-1} mg^{-1} protein.

Since the PH0925 protein was isolated from a hyperthermophilic archaeon, *P. horikoshii* OT3 (González et al. 1998), the temperature dependence of the Man-1-P GTase activity was analyzed from 50 to 100 °C. As shown in Fig. 4, the PH0925 protein exhibited relatively high activity

Table 1 Substrate specificity of the forward reaction of the Sugar-1-P NTase activity of the PH0925 protein

Nucleotide substrate	Sugar-1-phosphate substrate	Specific activity (μmol min^{-1} mg^{-1} protein)
GTP	α-D-Mannose-1-phosphate	10.2 ± 0.4
ATP		0.8 ± 0.0
CTP		0.8 ± 0.0
UTP		0.8 ± 0.0
dGTP		8.2 ± 0.5
dATP/dCTP/dTTP		ND
GTP	α-D-Glucose-1-phosphate	5.2 ± 0.4
dGTP		0.2 ± 0.1
ATP/CTP/UTP/dATP/dCTP/dTTP		ND
GTP	α-D-Glucosamine-1-phosphate	7.3 ± 0.7
dGTP		0.5 ± 0.1
ATP/CTP/UTP/dATP/dCTP/dTTP		ND
GTP/dGTP	Fructose-1-phosphate	ND
	α-D-Galactose-1-phosphate	ND
	N-Acetyl-D-glucosamine-1-phosphate	ND

ND non-detectable

between 80 and 100 °C with a maximum activity at 95 °C. This finding indicated that the optimum temperature for the PH0925 Man-1-P GTase activity was very close to the optimal growth temperature of the original host microorganism, *P. horikoshii* OT3. This property might be favorable for industrial applications.

Substrate specificity of the Sugar-1-P NTase activity of the PH0925 protein

As described above, the PH0925 protein actually exhibited Man-1-P GTase activity. In archaeal enzymes involved in carbohydrate metabolism, it was found that some proteins, e.g. the ST0452 protein in *S. tokodaii* (Zhang et al. 2005), exhibited acceptability of multiple substrates for their enzymatic activities. Therefore, the substrate specificity of the forward reaction of the PH0925 protein was examined under standard reaction conditions.

Initially, the specificity for various nucleoside triphosphates was examined. When Man-1-P and GTP were utilized as substrates, the highest activity was detected. As shown in Table 1, the PH0925 protein exhibited approximately 80 % of the maximum activity when Man-1-P and dGTP were used as substrates, but only 8 % of the maximum activity when Man-1-P and ATP, CTP or UTP were used as substrates. As high activity was detected with a combination of GTP or dGTP and Man-1-P, the effects of various sugar-1-P molecules on the PH0925 Sugar-1-P NTase activity was then analyzed using GTP and dGTP.

As shown in Table 1, approximately 51 or 72 % of the maximum PH0925 Sugar-1-P NTase activity were, respectively, detected when Glc-1-P or GlcN-1-P was utilized as substrate with GTP. Only 2.0 or 4.9 % of the maximum PH0925 Sugar-1-P NTase activity were, respectively, detected when same phospho-sugars were used as substrate with dGTP. Combination of other Sugar-1-P molecules, including fructose-1-phosphate (Frc-1-P), galactose-1-phosphate (Gal-1-P) and *N*-acetyl-D-glucosamine-1-phosphate (GlcNAc-1-P), and other NTP or dNTP were not catalyzed by the PH0925 Sugar-1-P NTase activity.

On the similar enzyme from *P. aeruginosa*, substrate specificity of the reverse direction of the Sugar-1-P NTase activity was analyzed and the result indicated that *P. aeruginosa* enzyme possessed only Man-1-P GTase activity (Shinabarger et al. 1991). For other bacterial similar bifunctional enzymes characterized, there has been no report on their acceptability of other Sugar-1-P molecules than Man-1-P as substrate. Additionally, Glc-1-P GTase activity has previously been reported only from pig liver (Ning and Elbein 2000). Conversely, the PH0925 Sugar-1-P NTase activity could utilize Man-1-P, Glc-1-P or GlcN-1-P as substrates. Therefore, the Sugar-1-P NTase activity of the PH0925 protein was distinctly different from those of bacterial similar bifunctional enzymes. The PH0925 protein is the first and sole archaeal protein from a thermophilic archaeon with multiple Sugar-1-P NTase activities including the Glc-1-P GTase activity. These observations proposed that the activity might have some role for production of glucosylglycerate, a compatible solute to function as an osmoprotectant and thermoprotectant (Costa et al. 2007; Empadinhas and da Costa 2011). This multiple substrate acceptability of the PH0925 protein also might be

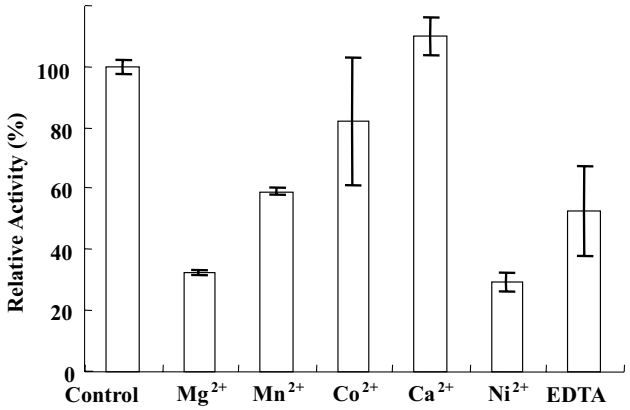

Fig. 5 PMI activity of the recombinant PH0925 protein in the presence of different metal ions. PMI activity in standard reaction condition was measured. The concentration of each metal ion used was 2 mM. The assay conditions were as described under "Materials and methods". The relative activity is shown as a percentage of the activity detected without any metal ions (control). All experiments were repeated three times

convenient for investigation of substrate recognition mechanism of the PH0925 protein.

Detailed kinetic properties of the reactions producing other compounds; ADP-mannose, CDP-mannnose, UDP-mannose, dGDP-mannose, GDP-Glc, dGDP-Glc, GDP-Glucosamine, dGDP-glucosamine, were not determined in this work.

Effect of metal ions on each enzymatic activity of the PH0925 protein

Many enzymes responsible for nucleotide metabolism require metal ions for their activity. Therefore, we examined the effects of metal ions on the PMI and Sugar-1-P NTase activities of the PH0925 protein. The effects of each metal ion were measured in standard reaction solutions in the presence of 0.1 mM EDTA alone or in the presence of both 0.1 mM EDTA and 2 mM of each different divalent metal ion.

For the PMI activity, as shown in Fig. 5, the maximum activity was detected under condition of addition of 2 mM Ca^{2+}. Slightly lower PMI activity was detected even when no metal ion was added. Co^{2+} or Mn^{2+} ions, respectively, decreased to 80 or 60 % of the maximum activity. Approximately 50 % of the maximum activity was detected when 0.1 mM EDTA was added into the reaction solution. Mg^{2+} or Ni^{2+} ions inhibited the PH0925 PMI activity to approximately 30 % of the maximum activity. In contrast, a bacterial bifunctional enzyme with PMI and Man-1-P GTase activities from *P. aeruginosa* indicated that Co^{2+} was the most effective metal cation for its PMI activity, and other 5 kinds of metal ions exhibited only less than 50 % of its

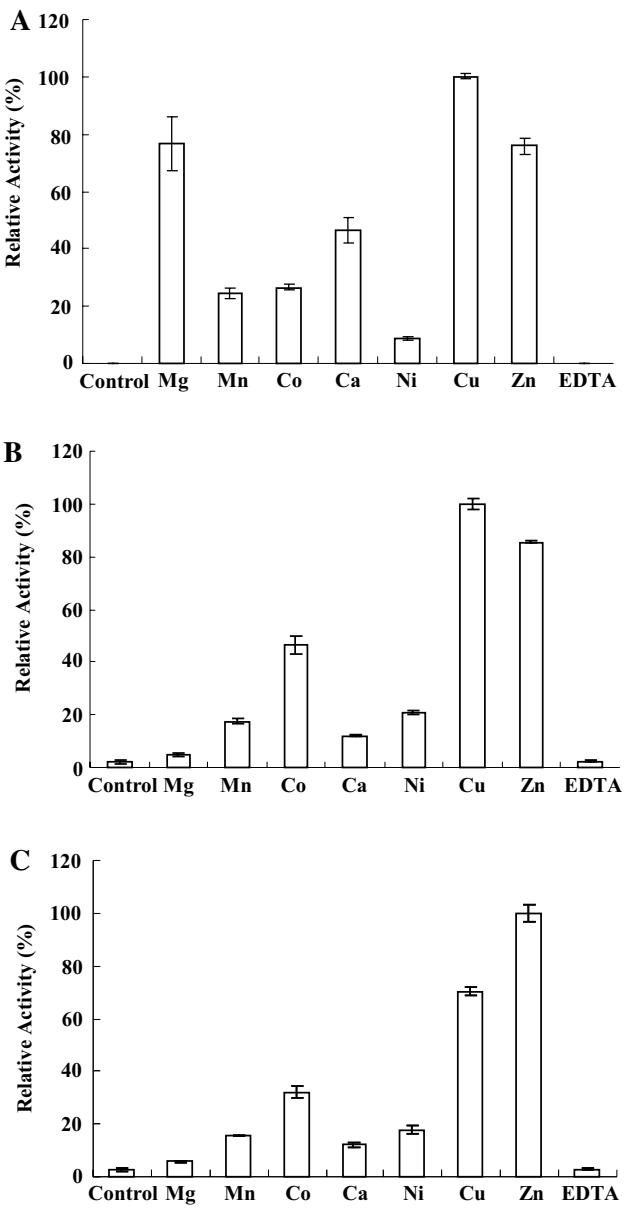

Fig. 6 Sugar-1-P NTase activity of the recombinant PH0925 protein in the presence of different metal ions. The Sugar-1-P NTase activity for three substrates, Man-1-P (A), Glc-1-P (B) and GlcN-1-P (C) were measured under standard reaction conditions. The concentration of each metal ion used was 2 mM. The assay conditions were as described under "Materials and methods". Relative activity is shown as a percentage of the maximum activity obtained for each Sugar-1-P substrate. All experiments were repeated three times

maximum activity detected with Co^{2+} (Shinabarger et al. 1991). The similar effect of metal ions was observed on the bifunctional enzyme with PMI and Man-1-P GTase activities from *Hericobactor pylori* (Wu et al. 2002). The PH0925 protein showed different patterns of effectiveness of the metal ions from characterized bacterial similar enzymes. To fully understand the difference between

Table 2 Specific and relative activities of Man-1-P GTase activity of the PH0925 protein

	Length of truncation from C-terminus (residues)	Specific activity (μmol^{-1} min^{-1} mg^{-1} protein)	Relative activity (%)
PH0925[a]	0	0.20 ± 0.002	100
DN450	14	0.25 ± 0.02	125
DN350	114	0.71 ± 0.05	355
DN345	119	0.16 ± 0.01	80
DN328	136	0.03 ± 0.0002	1.5
DN321	143	0.002 ± 0.0003	1
DN310	154	0.002 ± 0.0001	1
DN271	193	0.0003 ± 0.0001	0.1

Relative activity is indicated by % of the activity observed on the PH0925 protein

Activity was measured three times

[a] The length of the wild-type PH0925 is 464 residues

Table 3 Kinetic properties of Man-1-P GTase activity of the wild-type and the truncated forms of the PH0925 protein

Proteins	Substrates	V_{max} (μmol min^{-1} mg^{-1} protein)	K_m(μM)
PH0925	Man-1-P	3.4 ± 0.7	16.1 ± 4.1
	GTP	11.7 ± 2.4	2.6 ± 1.1
DN345	Man-1-P	2.3 ± 0.1	8.8 ± 0.2
	GTP	5.0 ± 0.3	20.7 ± 2.4
DN350	Man-1-P	7.8 ± 0.2	2.1 ± 0.2
	GTP	4.4 ± 2.0	3.9 ± 0.7
DN450	Man-1-P	1.5 ± 0.1	7.0 ± 0.5
	GTP	9.2 ± 0.8	16.9 ± 1.9

Activity was measured three times

the bacterial and archaeal PMI activities of these similar bifunctional enzymes, more detailed analyses, including determination of 3-D structures of co-crystals with metal ions and introduction of site-directed mutation at the reaction center, are required.

For the Sugar-1-P NTase activity of the PH0925 protein, no Sugar-1-P NTase activity was detected, when divalent cations were not added into or removed by EDTA from the reaction solution (Fig. 6). Figure 6 indicates that the PH0925 Suger-1-P NTase activity was activated by several divalent cations. The order of effectiveness of metal ions for the PH0925 Sugar-1-P NTase activity was found to be different according to the Sugar-1-P molecules used as substrate. When Man-1-P was used as substrate, the order of effectiveness was $Cu^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+}$. When Glc-1-P was used, the order was $Cu^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. For GlcN-1-P NTase activity, effectiveness order was $Zn^{2+} > Cu^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. On the other hand, it was found on the bacterial bifunctional enzymes with PMI and Man-1-P GTase activities that only Mg^{2+} or Mn^{2+} ions were essential for their activity (Shinabarger et al. 1991; Wu et al. 2002). To understand the difference observed on these Man-1-P GTase activities, more detailed analyses should be required.

Analysis of the truncated form of the PH0925 protein

Among all N-terminus- and C-terminus-truncated forms of the PH0925 protein, only C-terminus-truncated forms of the PH0925 protein were used for activity analyses, because all 6 N-terminus-truncated forms of the PH0925 protein constructed were expressed in *E. coli* as insoluble form.

For PMI activity, no activity was detected with any C-terminus-truncated form of the PH0925 protein, indicating that the C-terminal 16-residue region was essential for the PH0925 PMI activity (data not shown).

Conversely, for the Man-1-P GTase activity, from N-terminus to 345th residues or longer form of the PH0925 protein exhibited the remarkable activity (Table 2). Longer C-terminus-truncated forms of the PH0925 protein did not show reliable Man-1-P GTase activity. It revealed that the region comprising the 1st to the 345th residue of the PH0925 protein was essential for PH0925 Man-1-P GTase activity. Moreover, as shown in Table 2, the DN450 and DN350 proteins, respectively, exhibited 1.25- and 3.55-fold higher Man-1-P GTase activity than that of the wild-type PH0925 protein. These findings indicated that a C-terminal 114-residue region had inhibitory effect on the Man-1-P GTase activity that was found to be located at the N-terminus of the PH0925 protein.

To understand effect of the C-terminus-truncation on the PH0925 Man-1-P GTase activity, we determined the kinetic constants. As shown in Table 3, for the DN350 protein, the V_{max} and K_m values with Man-1-P was approximately 2.3-fold larger and 7.7-fold smaller than those for the wild-type

PH0925 protein, respectively. Thus, these differences were thought to be the reasons for the enhanced relative activity of the DN350 protein. For DN450 protein, both V_{max} and K_m values with Man-1-P were smaller than those for the wild-type PH0925 protein. These differences were thought to be the reason for slightly enhanced Man-1-P GTase activity of the DN450 protein.

As the exact mechanism of the inhibition caused by this C-terminal region was not clear, more detailed analyses are required. In future experiments, more detailed analyses including determination of the 3-D structure and construction of point-mutant forms of the PH0925 protein may reveal domains essential for each activity of the PH0925 protein.

In this work, the effects of C-terminus truncation of the PH0925 protein were measured only for Man-1-P GTase activity. In future experiments, enhancement effects with different substrates, Glc-1-P or GlcN-1-P, should be analyzed. These results will provide more information about the mechanism of enhancement caused by deletion of the 114-residue C-terminal region. Notably, this is the first report of a thermostable, bifunctional enzyme with PMI and multiple Sugar-1-P NTase activities. More detailed analyses of this protein will provide important information for both basic and applied sciences.

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