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Purification and Properties of *sn*-Glycerol-1-Phosphate Dehydrogenase from *Methanobacterium thermoautotrophicum*: Characterization of the Biosynthetic Enzyme for the Enantiomeric Glycerophosphate Backbone of Ether Polar Lipids of Archaea

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The enzyme which seems to be responsible for the formation of the enantiomeric configuration of the glycerophosphate backbone (*sn*-glycerol-1-phosphate) of archaeal ether lipids was purified from a methanogenic archaeon, *Methanobacterium thermoautotrophicum*, and characterized. The enzyme, *sn*-glycerol-1-phosphate: NAD(P)$^+$ oxidoreductase (*sn*-glycerol-1-phosphate dehydrogenase), was purified 7,600-fold from a cell free extract by ammonium sulfate fractionation and seven steps of chromatography. The final preparation exhibited a specific activity of 617 μmol/min/mg (V_{max}) and gave a single band corresponding to 38 kDa on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The native enzyme showed an apparent molecular mass of 302 kDa on gel-filtration chromatography, indicating it is present as a homooctamer. Maximum activity was observed at 75°C at near neutral pH. The activity was stimulated by potassium ions. The K_m for dihydroxyacetone phosphate was 7.5 times smaller than that for *sn*-glycerol-1-phosphate, suggesting that the formation of *sn*-glycerol-1-phosphate is the natural direction in the cell. Under the assay conditions used, no product inhibition was observed. The N-terminal amino acid sequence was determined.

Key words: archaea, ether lipid, glycerophosphate dehydrogenase, *Methanobacterium thermoautotrophicum*, lipid biosynthesis.

Archaea (archaebacteria) is a new domain defined on the basis of the 16S rRNA sequence homology (*1*), which has been introduced into the classification of living organisms as a third one, *i.e.* in addition to *Bacteria* (eubacteria) and *Eucarya* (eukaryotes). It includes anaerobic methanogens, extreme halophiles, and aerobic or anaerobic thermophiles. The designation of *Archaea* as a distinct domain is supported by several unique biochemical features, such as ribosomal RNA sequences (*2*), RNA polymerase subunits (*3*), cell wall structures (*4*), unique cofactors (*5*), and membrane lipid structures (*6*). One of the distinctive molecular features common to all members of *Archaea* that distinguishes *Archaea* from *Bacteria* and *Eucarya*, and unites their disparate phenotypes is the nature of their membrane polar lipids. The polar lipids of the members of *Archaea* consist of di- and tetraethers of glycerol with isoprenoid alcohols bound at the *sn*-2 and *sn*-3 positions of the glycerol moiety. That is, the archaeal polar lipids have the enantiomeric configuration of a glycerophosphate backbone [*sn*-glycerol-1-phosphate (G-1-P)] that is the mirror image structure of the bacterial or eucaryal counterpart [*sn*-glycerol-3-phosphate (G-3-P)] (*6, 7*). This means that the absolute stereochemistry of the glycerol moiety in all archaeal polar lipids is opposite to that of glycerol ester lipids in bacteria and eucarya. No exception to this difference has been found so far.

Methanobacterium thermoautotrophicum is an autotrophic archaeon which synthesizes sugars from CO_2 *via* dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde phosphate (GAP) (*8, 9*). Zhang *et al.* have shown that G-1-P is the direct precursor for ether lipid synthesis in this microorganism (*10, 11*). Other compounds such as DHAP, glycerol, and G-3-P do not serve as substrates for the enzyme reaction. The source of the hydrocarbon chain is geranylgeranyl pyrophosphate. These results indicate that the enzyme responsible for G-1-P formation must be a key enzyme in the biosynthesis of the enantiomeric polar lipid structure in this organism.

We found the enzyme activity that formed G-1-P from DHAP for the first time in a cell-free extract of this archaeon, and the enzyme was identified as *sn*-glycerol-1-phosphate:NAD(P)$^+$ oxidoreductase (*sn*-glycerol-1-phosphate dehydrogenase) after partial purification (*12*). The enzyme catalyzes a single step reaction located at the point where lipid biosynthesis branches off from the gluconeogenesis pathway.

In order to compare the properties of G-1-P dehydrogenase with those of bacterial G-3-P dehydrogenase (the corresponding enzyme responsible for formation of the

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Abbreviations: DHAP, dihydroxyacetone phosphate; G-1-P, *sn*-glycerol-1-phosphate; G-3-P, *sn*-Glycerol-3-phosphate; GAP, D-glyceraldehyde-3-phosphate.

G-3-P backbone of bacterial diacyl phospholipids [EC 1.1.1.94]), the enzyme was purified to homogeneity and characterized.

MATERIALS AND METHODS

Growth of Organisms—*M. thermoautotrophicum* ΔH (DSM 1053) was grown in a 15-liter fermenter and harvested as described previously (*13*). The cells were stored at −20°C until use. *Halobacterium cutirubrum* (DSM 669) was grown at 37°C with shaking at 100 rpm in a 3-liter glass bottle containing 1 liter of medium No. 97 described in the DSM catalog, 1987.

Enzyme Assays—The activity of G-1-P dehydrogenase was assayed in both directions spectrophotometrically at 340 nm. One unit corresponds to 1 μmol NAD(P) or NAD(P)H formed per min on DHAP reduction or G-1-P oxidation, respectively. Except when otherwise stated, the activity in the direction of DHAP reduction was measured at 65°C in 1.5 ml cuvettes containing 1.2 ml of the following assay mixture: triethanolamine buffer, pH 7.4, 50 mM; KCl, 70 mM; DHAP, 2.1 mM; NADH, 0.32 mM; and 0.1 ml of enzyme preparation (standard assay mixture). NADPH (0.32 mM) was used instead of NADH for some kinetic experiments. The reaction was initiated by the addition of 0.015–0.05 unit of the enzyme preparation. In the direction of G-1-P oxidation, the reduction of NAD or NADP was measured at 65°C. The assay mixture (1.2 ml) contained the following: triethanolamine buffer, pH 7.4, 50 mM; KCl, 70 mM; G-1-P, 8.3 mM; NAD or NADP, 5.0 mM; and 0.1 ml of enzyme fraction. The reaction was initiated by the addition of 0.015–0.05 unit of the enzyme. Blank values were recorded in the absence of a substrate.

Enzyme Purification—All manipulations were performed at 0–4°C. The activity was monitored routinely in the direction of DHAP reduction. Four types of buffer were used; Buffer A, 50 mM K^+-phosphate (pH 7.4); Buffer B, 100 mM K^+-phosphate (pH 7.4); Buffer C, 20 mM K^+-phosphate (pH 7.4); and Buffer D, 20 mM triethanolamine and 30 mM K^+-phosphate (pH 8.3). A Pharmacia Gradi-Frac System or Hitachi 655 A-11 Liquid Chromatograph was used for liquid chromatography.

A cell free extract was prepared in buffer A from 55 g wet weight of *M. thermoautotrophicum* cells as described previously (*12*).

Step 1. Ammonium sulfate fractionation: Solid ammonium sulfate was added to the extract to 40% saturation. After gentle stirring for 2 h, the supernatant obtained on 20-min centrifugation at 11,000×*g* was adjusted to 60% saturation by adding solid ammonium sulfate. After stirring for 2 h and centrifugation, the pellet was resuspended in buffer B. This fraction was dialyzed against buffer B overnight with one change of the dialyzing buffer.

Step 2. Butyl-Toyopearl chromatography: Solid ammonium sulfate and buffer B were added to the dialyzed fraction to 1.5 M concentration. The fraction was applied to a Butyl-Toyopearl column (2.2×20 cm, Tosoh, Tokyo) pre-equilibrated with 1.5 M ammonium sulfate in buffer B. The column was eluted with a linear gradient of 1.5 to 0 M ammonium sulfate in buffer B. The elution profiles of protein throughout the chromatography steps were monitored by measuring A_{280}. The fractions containing the enzyme activity (0.42–0.24 M) were pooled and dialyzed against buffer C overnight with a change of the dialyzing buffer.

Step 3. Hydroxyapatite chromatography: A column (2.5×15 cm) packed with hydroxyapatite (Wako Chemicals) was pre-equilibrated with buffer C. The active fraction obtained from step 2 was applied to the column and eluted with a linear gradient of 20 to 150 mM K^+-phosphate (pH 7.4). The fractions with the enzyme activity (50–80 mM) were pooled and dialyzed against buffer D overnight with one change of the dialyzing buffer.

Step 4. DEAE-Toyopearl chromatography: The active fraction from step 3 was applied to a DEAE-Toyopearl column pre-equilibrated with buffer D and then eluted with a linear gradient of 0 to 0.6 M KCl in buffer D. The fractions containing high enzyme activity (0.27–0.33 M) were pooled and dialyzed against buffer A overnight with one change of the dialyzing buffer.

Step 5. Sephacryl S-300 chromatography: The active fraction from step 4 was applied to a Sephacryl S-300 gel-filtration column (2.6×60 cm) and then eluted with buffer A. The fractions containing enzyme activity (corresponding to around 300 kDa) were pooled.

Step 6. Cosmogel DEAE chromatography: This chromatography was performed the same as for step 4 except that a column (0.8×7.5 cm) of Cosmogel DEAE (Nacalai Tesque, Kyoto) was used and the elution gradient comprised 0 to 1.0 M KCl. The fractions containing the enzyme activity (0.45–0.55 M) were pooled and dialyzed against buffer C overnight with one change of the dialyzing buffer.

Step 7. Second hydroxyapatite chromatography: This chromatography was performed the same as for step 3 except that a Tosoh TSK-gel HA-1000 column (0.75×7.5 cm) was used. The fractions exhibiting the high enzyme activity (45–50 mM) were pooled.

Step 8. Cosmosil 5 Diol-300 chromatography: Cosmosil 5 Diol-300 gel filtration column (Nacalai Tesque) chromatography was performed for the final step with buffer A. Several peaks were observed, and one of them, which was clearly separated from the others, contained the enzyme activity. The fractions corresponding this peak were collected and pooled as the purified enzyme preparation, which was stored at −20°C until use.

Analytical Procedures—Protein concentrations were measured by the bicinchoninic acid method (*14*). Denaturing SDS-PAGE was performed on a commercially available polyacrylamide gel PAGEL series (Atto Manufacturing, Tokyo). Silver staining was carried out with Nacalai staining kits as described by the manufacturer. The molecular mass of the denatured enzyme was determined from the retardation coefficient obtained by Ferguson plotting (*15*) of the data obtained on SDS-PAGE using PAGEL series with 5, 7.5, 10, and 12.5% gel concentrations. The N-terminal amino acid sequence of the purified enzyme was determined with an Applied Biosystems 477 A protein/peptide sequencer using the protocol of the manufacturer.

Materials—G-1-P was prepared from lipids of *H. cutirubrum*, which are exclusively composed of *sn*-2,3-di-*O*-phytanyl glycerol core (archaeol) (*16*). The total lipid extract of *H. cutirubrum* was subjected to strong acid methanolysis (*17*), and then archaeol was purified from the chloroform-soluble hydrolysate by thin-layer chromatography with a developing solvent system of light petroleum/

diethyl ether/acetic acid (50 : 50 : 1). The archaeol was converted to archaetidic acid (archaeal diether analog of phosphatidic acid) with dimethylphosphoryl chloride and trimethylsilyl bromide as described by Zhang and Poulter (*11*). Archaetidic acid was purified by thin-layer chromatography with a developing solvent system of chloroform/methanol/acetic acid (200 : 50 : 10). G-1-P was derived from archaetidic acid by dealkylation with BCl_3 (*18*). G-3-P was purchased from Boehringer Mannheim. GAP and molecular mass standards were purchased from Sigma. DHAP, NADH, NADPH, NAD, NADP, DL-α-glycerophosphate, *sn*-glycerol-2-phosphate, and trimethylsilyl bromide were the products of Nacalai Tesque. Dimethylphosphoryl chloride was obtained from Aldrich. All other reagents were of reagent grade.

RESULTS

Purification—G-1-P dehydrogenase of *M. thermoautotrophicum* was purified approximately 7,600-fold, in a final yield of 25%, to apparent homogeneity. The eight-step procedure is outlined in Table I. During this purification, the presence of phosphate ions at a concentration of more than 20 mM in the buffers proved to be necessary for stabilization of the enzyme. SDS-PAGE of the final enzyme preparation gave a single band (Fig. 1). The molecular mass was estimated to be 38 kDa by Ferguson plotting using myosin (205 kDa), β-galactosidase (116 kDa), phospholyase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa) as standards. The molecular mass of the native enzyme was determined to be 302 kDa by gel-filtration chromatography at purification step 8, in comparison with molecular mass standards of thyroglobulin (733 kDa), ferritin (421 kDa), catalase (232 kDa), β-amylase (200 kDa), aldolase (177 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). It is most likely that the native enzyme is present as a homo-octamer. The purified enzyme was used for further experiments.

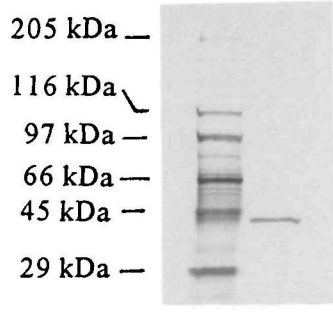

Fig. 1. **Analysis of the purified G-1-P dehydrogenase by SDS/PAGE.** The purified enzyme and molecular markers were electrophoresed on a 5-20% gradient polyacrylamide gel and then stained with silver staining reagent. A: Molecular mass standards: myosin (205 kDa), β-galactosidase (116 kDa), phospholyase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). B: Purified G-1-P dehydrogenase.

Substrate Specificity and the Reaction Product—The purified enzyme was active toward both DHAP and G-1-P. No activity was observed toward G-3-P, *sn*-glycerol-2-phosphate, GAP, glycerol, glyceraldehyde, or dihydroxyacetone. α-Glycerophosphate was identified as the sole product of DHAP reduction on GC-MS analysis of the reaction mixture after trimethylsilylation, and the enzyme was determined to be G-1-P by using stereospecific G-3-P dehydrogenase as described previously (*12*).

Requirements and Properties—Except when otherwise stated, the properties were determined from initial velocity measurements in the direction of DHAP reduction with the standard assay mixture at 65°C. The enzyme exhibited a broad optimum pH (pH 6.6-7.4) around neutral pH. The activity decreased gradually at pH over 7.4 or below 6.6.

The enzyme activity was greatly stimulated in the presence of K^+ (Fig. 2). Maximum activity was observed at

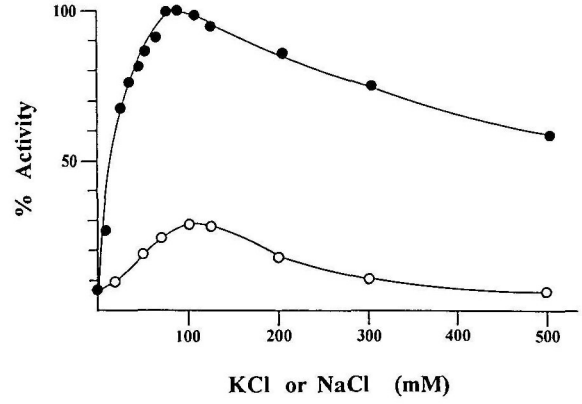

Fig. 2. **Effects of KCl and NaCl on enzyme activity.** The enzyme activity was measured in the direction of DHAP reduction in the standard assay mixture containing KCl or NaCl instead of KCl at the concentrations indicated. The activities are expressed as percentages relative to the highest value obtained (60-70 mM KCl). (●) KCl, (○) NaCl.

TABLE I. **Purification of G-1-P dehydrogenase from *M. thermoautotrophicum* cells.** The activity was measured in the direction of G-1-P synthesis with the standard assay mixture.

	Activity (units)	Protein (mg)	Specific activity (μmol/min/mg)	Purification (fold)	Recovery (%)
Crude extract	103.9	2,522	0.041	1	100
40-60% $(NH_4)_2SO_4$	102.3	1,364	0.075	1.8	99
Chromatography					
Buthyl-Toyopearl	94.1	179	0.525	12.8	91
Hydroxyapatite	90.0	41.1	2.19	53.4	87
DEAE-Toyopearl	57.1	24.4	2.34	57.1	55
Sephacryl S-300	53.0	9.49	5.59	136	51
Cosmogel DEAE	43.2	2.90	14.9	363	42
Hydroxyapatite	32.0	0.225	142	3,463	31
Cosmosil 5-Diol	25.5	0.081	314	7,659	25

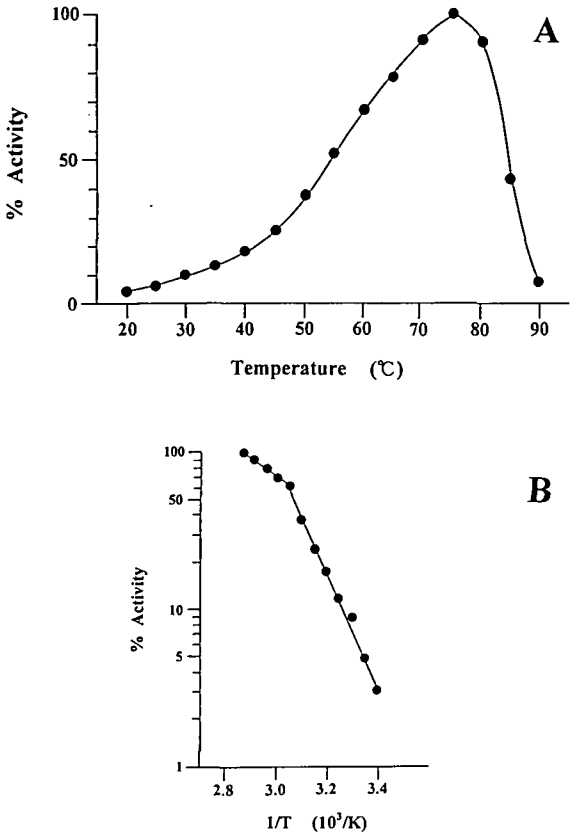

Fig. 3. **Effect of temperature on enzyme activity.** The enzyme activity was measured in the direction of DHAP reduction in the standard assay mixture. A: The activity is expressed as a percentage relative to the highest value obtained (75°C). B: Arrhenius plot.

60-80 mM. The stimulatory effect of Na^+ was one-third of that of K^+. Under the conditions where K^+ was present at a saturating level for the reaction, Na^+ was essentially ineffective. Divalent metal cations such as Co^{2+}, Cu^{2+}, and Ni^{2+} at 1 mM inhibited 33-64% of the G-1-P dehydrogenase activity. Dithiothreitol was inhibitory (45% inhibition at 1 mM), but mercaptoethanol was essentially non-effective.

The purified enzyme showed apparent maximum activity at 75°C (Fig. 3A). The temperature-activity profile, an Arrhenius plot (Fig. 3B), showed a transition point of a linear relationship at 51°C. This indicates that a temperature-induced conformational change occurs near 51°C. Because DHAP and NADH were rapidly decomposed over 70°C (data not shown), the usual assay was carried out at 65°C, which is the optimum temperature for growth of the organism. The rapid decrease in the apparent activity over 75°C may be partially explained by the breakdown of the substrate and coenzymes at high temperature.

Kinetic Parameters—Kinetic constants were determined in the directions of both DHAP reduction and G-1-P oxidation. The apparent K_m values and V_{max} obtained are shown in Table II. The K_m for DHAP (2.17 mM) was 7.5 times smaller than that for G-1-P (16.3 mM) when NAD(H) was used as a coenzyme. NADP(H) was also active as well as NAD(H). In this case the K_m for DHAP (0.58 mM) was 8.3 times smaller than that for G-1-P (4.8 mM). In the direction of DHAP reduction, half-maximal velocities were obtained with 129 μM NADH and 25 μM NADPH. The V_{max}/K_m for NADH and NADPH were 4.73 and 12.12, respectively.

TABLE II. **Kinetic properties of G-1-P dehydrogenase of *M. thermoautotrophicum*.**

	K_m (mM)	V_{max} (μmol/min/mg)	
DHAP reduction			
NADH	0.129	610	(DHAP 4.0 mM)
NADPH	0.025	303	(DHAP 2.1 mM)
DHAP(NADH)	2.17	617	(NADH 0.32 mM)
DHAP(NADPH)	0.58	323	(NADPH 0.32 mM)
G-1-P oxidation			
NAD	0.127	39.8	(G-1-P 49 mM)
NADP	0.270	23.9	(G-1-P 49 mM)
G-1-P(NAD)	16.3	39.4	(NAD 5 mM)
G-1-P(NADP)	4.80	14.6	(NADP 5 mM)

Racemic α-glycerophosphate in the assay mixture did not inhibit the enzyme activity up to 500 μM, indicating that both G-1-P and G-3-P were not inhibitory for G-1-P formation.

N-Terminal Sequence—The N-terminal sequence could be determined up to 23 amino acids. The sequence was MDPRKIQLPREIYTGPGVIEDTG.

DISCUSSION

The details of the properties of G-1-P dehydrogenase presented in this paper suggest that the enzyme is likely to be responsible for the formation of the enantiomeric glycerophosphate backbone structure of polar lipids in *M. thermoautotrophicum*, as discussed in the previous paper (*12*). The enzyme was active only toward DHAP and G-1-P. The fact that the K_m for DHAP was 7.5 or 8.3 times smaller than that for G-1-P indicates that the formation of G-1-P is the natural direction in the cell. NADPH is likely to be a real coenzyme in the cell because the K_m for NADPH was 5.2 times smaller than that for NADH, and the V_{max}/K_m for NADPH (12.12) was higher than that for NADH (4.73), although the V_{max} of DHAP reduction with NADH was higher than that with NADPH.

In *E. coli*, the G-3-P dehydrogenase involved in lipid biosynthesis is regulated by allosteric inhibition by the product, G-3-P (*19-21*). This inhibition is important for keeping the intracellular pool of G-3-P small and for regulating lipid biosynthesis when glycerol or G-3-P is supplied exogeneously to heterotrophic *E. coli*. The G-1-P dehydrogenase of *M. thermoautotrophicum* was not affected by the product, G-1-P, nor by G-3-P, probably because autotrophic methanogens do not utilize glycerol or glycerophosphate, and the level of the lipid biosynthetic precursor must not change significantly. In fact, *M. thermoautotrophicum* cells did not contain glycerol kinase (*12*), which is considered to be the first enzyme for glycerol dissimilation. It does not seem likely that the enzyme is regulated by the K^+ concentration in the cell because the intracellular KCl concentration was estimated to be 700-1,000 mM (*22, 23*), which is much higher compared to the regulation level observed.

We have already found the activity of G-1-P dehydrogenase in cell-free extracts of an extreme halophilic archaeon, *H. cutirubrum*, and a hyperthermophilic archaeon, *Pyrococcus* (unpublished results). These observations

may suggest that G-1-P formation is a common pathway operating for polar lipid biosynthesis in archaea.

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