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# Ubiquitous distribution of phosphatidylinositol phosphate synthase and archaetidylinositol phosphate synthase in Bacteria and Archaea, which contain inositol phospholipid

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ABSTRACT

In Eukarya, phosphatidylinositol (PI) is biosynthesized from CDP-diacylglycerol (CDP-DAG) and inositol. In Archaea and Bacteria, on the other hand, we found a novel inositol phospholipid biosynthetic pathway. The precursors, inositol 1-phosphate, CDP-archaeol (CDP-ArOH), and CDP-DAG, form archaetidylinositol phosphate (AIP) and phosphatidylinositol phosphate (PIP) as intermediates. These intermediates are dephosphorylated to synthesize archaetidylinositol (AI) and PI. To date, the activities of the key enzymes (AIP synthase, PIP synthase) have been confirmed in only three genera (two archaeal genera, *Methanothermobacter* and *Pyrococcus*, and one bacterial genus, *Mycobacterium*). In the present study, we demonstrated that this novel biosynthetic pathway is universal in both Archaea and Bacteria, which contain inositol phospholipid, and elucidate the specificity of PIP synthase and AIP synthase for lipid substrates. PIP and AIP synthase activity were confirmed in all recombinant cells transformed with the respective gene constructs for four bacterial species (*Streptomyces avermitilis*, *Propionibacterium acnes*, *Corynebacterium glutamicum*, and *Rhodococcus equi*) and two archaeal species (*Aeropyrum pernix* and *Sulfolobus solfataricus*). Inositol was not incorporated. CDP-ArOH was used as the substrate for PIP synthase in Bacteria, and CDP-DAG was used as the substrate for AIP synthase in Archaea, despite their fundamentally different structures. PI synthase activity was observed in two eukaryotic species, *Saccharomyces cerevisiae* and *Homo sapiens*; however, inositol 1-phosphate was not incorporated. In Eukarya, the only pathway converts free inositol and CDP-DAG directly into PI. Phylogenic analysis of PIP synthase, AIP synthase, and PI synthase revealed that they are closely related enzymes.



## 1. Introduction

Our study describes a novel biosynthetic pathway of inositol phospholipids in methanogenic Archaea [1] that is similar to the pathway in mycobacteria [2]. The mechanism of phosphatidylinositol (PI) synthesis in mycobacteria has been revised [2]. In eukaryotes, PI is biosynthesized from CDP-diacylglycerol (CDP-DAG) and inositol (Reaction 1) [3–6].

$$\text{CDP-DAG} + \text{inositol} \rightarrow \text{PI} + \text{CMP} \quad (1)$$

In methanoarchaeon and mycobacteria, however, the precursors are inositol 1-phosphate, CDP-archaeol (CDP-ArOH), and CDP-DAG, which form archaetidylinositol phosphate (AIP) and phosphatidylinositol phosphate (PIP) as intermediates (Reaction 2). These are dephosphorylated to synthesize archaetidylinositol (AI) and PI (Reaction 3) [1,2].

$$\text{CDP-ArOH(CDP-DAG)} + \text{inositol 1-phosphate} \rightarrow \text{AIP(PIP)} + \text{CMP} \quad (2)$$

$$\text{AIP(PIP)} \rightarrow \text{AI(PI)} + \text{Pi} \quad (3)$$

Inositol phospholipids are ubiquitously distributed in Eukarya, in most of Archaea [7], and in the class *Actinobacteria* among Bacteria [8–12]. The enzymes associated with inositol phospholipid biosynthesis of Bacteria and Archaea are PIP synthase (PIPS) and AIP synthase (AIPS). To date, the activities of these enzymes have only been confirmed in three genera. AIPS activity has been observed in the genera *Methanothermobacter* and *Pyrococcus* in

*Abbreviations:* CDP-DAG, CDP-diacylglycerol; CDP-ArOH, CDP-archaeol; PI, phosphatidylinositol; PIS, phosphatidylinositol synthase; PIP, phosphatidylinositol phosphate; PIPS, phosphatidylinositol phosphate synthase; AI, archaetidylinositol; AIS, archaetidylinositol synthase; AIP, archaetidylinositol phosphate; AIPS, archaetidylinositol phosphate synthase; TLC, thin layer chromatography.

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Archaea [1], and PIPS activity has only been confirmed in the genus *Mycobacterium* in Bacteria [2]. The structures of the two lipid substrates for these enzymes, CDP-DAG and CDP-ArOH, are different, because Archaea has characteristic structural lipids compared with that of Bacteria and Eukarya [13]. We aimed to demonstrate that this novel biosynthetic pathway is universal in both Archaea and Bacteria, which contain inositol phospholipid, and elucidate the specificity of PIPS and AIPS for lipid substrates. We confirmed PIPS and AIPS activity among different species by testing four bacterial species (*Streptomyces avermitilis*, *Propionibacterium acnes*, *Corynebacterium glutamicum*, and *Rhodococcus equi*), and two archaeal species (*Aeropyrum pernix* and *Sulfolobus solfataricus*). Thus far, studies of the presence or absence of inositol incorporation in mycobacteria have been performed using bacterial homogenates; a method, however, that can lead to incorrect interpretations of the result due to the PI/inositol exchange reaction [14] and inositol kinase activity [2]. In the present experiment, we used recombinant cells containing genes for artificially synthesized proteins homologous to PIPS or AIPS to test for the activity of the enzymes involved in the biosynthesis of inositol phospholipids (i.e., PIPS, AIPS, and PI synthase [PIS]).

## 2. Materials and methods

### 2.1. Materials

[$^{14}$C(U)]Glucose 6-phosphate (3.7 MBq/ml) was obtained from Moravek Biochemicals, Inc. (Brea, CA). [2-$^{3}$H]*myo*-Inositol (37 MBq/ml) was obtained from Perkin Elmer, Inc. (Waltham, MA). [$^{14}$C]Inositol 1-phosphate, which was not commercially available, was prepared as previously described [1]. 1,2-Dioleoyl-*sn*-glycero-3-phosphate was obtained from Avanti Polar Lipids Inc. (Alabaster, AL).

### 2.2. Chemical synthesis of CDP-DAG and CDP-ArOH

CDP-1,2-di-*O*-oleoyl-*sn*-glycerol (CDP-DAG; Fig. 1) was chemically synthesized with cytidine 5′-monophosphomorpholidate from 1,2-dioleoyl-*sn*-glycero-3-phosphate, as described previously [15]. Similarly, CDP-2,3-di-*O*-phytanyl-*sn*-glycerol (CDP-ArOH; Fig. 1) was chemically synthesized from archaetidic acid, as previously described [15].

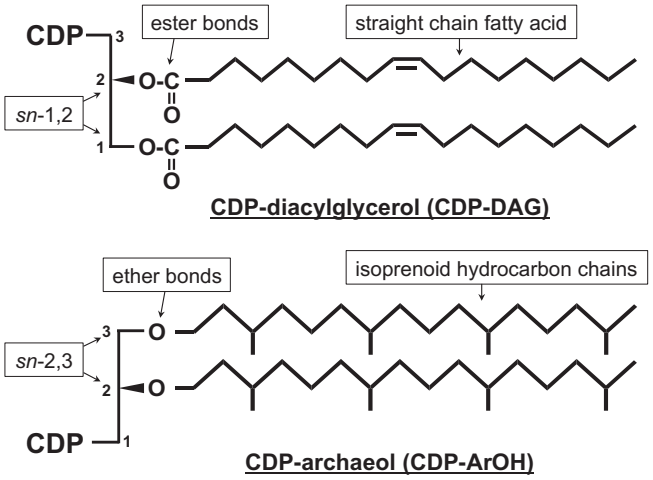


**Fig. 1.** Structures of lipid substrates, CDP-diacylglycerol and CDP-archaeol for PIP synthase and AIP synthase. Three differences (*sn*-1,2 and *sn*-2,3, ester bonds and ether bonds, straight fatty acyl chains and highly methyl-branched saturated isopranyl chains) are shown.

### 2.3. Enzymatic preparation of [$^{14}$C]labeled standard PI, PIP, AI, and AIP

[$^{14}$C]PIP and [$^{14}$C]PI were enzymatically prepared using the purified cell wall fraction of *Mycobacterium smegmatis* as the enzyme, as described previously [2] except that the incubation time was prolonged 2 h. [$^{14}$C]AIP and [$^{14}$C]AI were enzymatically prepared using the membrane fraction of *Methanothermobacter thermautotrophicus* as the enzyme, as described previously [1], except that the incubation time was prolonged 1 h.

### 2.4. Construction of the expression plasmids for the PIPS, AIPS, and PIS genes of various organisms

An artificial gene construct for a protein homologous to PIPS of *Mycobacterium tuberculosis* and AIPS of *M. thermautotrophicus* was introduced into *Escherichia coli* by transformation using a plasmid with a pET21a(+) vector insert (GenScript, Piscataway, NJ) for each of the four bacterial species (*S. avermitilis, P. acnes, C. glutamicum*, and *R. equi*) and the two archaeal species (*S. solfataricus* and *A. pernix*). Similarly, a gene construct for PIS was introduced into *E. coli* for the two eukaryotic species (*Saccharomyces cerevisiae* and *Homo sapiens*). The recombinant cells expressing these various enzymes were suspended in buffer A (0.1 M Bicine buffer [pH 8.0] containing 10 mM 2-mercaptoethanol) and disrupted by sonication using a SONIFIER 250 (1 cm probe; Branson, Danbury, CT) for 10 min (5 × 60 s pulses with 60-s cooling intervals between pulses). Cell fragments and intact cells were separated using low-speed centrifugation (10,000*g*, 10 min). The remaining homogenate was centrifuged (100,000*g*, 2 h), and the membrane fraction was fractionated, suspended in buffer A, and used as the enzyme [16].

### 2.5. Measurement of PIPS and AIPS activity

The complete assay mixture (final volume, 0.2 ml) contained 0.1 mM [$^{14}$C]inositol 1-phosphate (20 nmol, 2947 Bq), 40 nmol CDP-DAG or CDP-ArOH, 50 mM Bicine buffer (pH 8.0), 10 mM $MgCl_2$, 5 μg protein of *E. coli* transformant cells. The reaction mixture was incubated at 37 °C, 10 min for Bacteria and *H. sapiens*; at 30 °C, 10 min for *S. cerevisiae*; or at 60 °C, 10 min for Archaea. After incubation, the mixture of the reactants and the product was partitioned into aqueous and organic components with $CHCl_3$, methanol, and 1 M $MgCl_2$. The radioactivity in the chloroform-soluble organic materials was counted [2].

### 2.6. Measurement of PIS and AI synthase activity

The complete assay mixture (final volume, 0.2 ml) contained 0.5 mM [$^{3}$H]inositol (100 nmol, 74 KBq), 40 nmol CDP-DAG, 50 mM Tris–HCl buffer (pH 8.0), 2 mM $MnCl_2$, 2.4 mM Triton X-100 (0.15%), and the membrane fraction (200 μg protein) of the *E. coli* transformant cells (*E. coli* pET21a-PIS). The reaction mixture was incubated at 37 °C, 20 min for Bacteria and *H. sapiens*; at 30 °C, 20 min for *S. cerevisiae*; or at 60 °C, 20 min for Archaea. After incubation, the radioactivity in the organic component of the reaction mixture was counted [3,17].

### 2.7. Thin layer chromatography

Thin layer chromatography (TLC) of lipids was performed on a Silica Gel 60 plate (Merck, Tokyo, Japan) with the following solvent: chloroform, methanol, acetic acid, and water (80:30:20:10). Radioactive spots on the TLC plate were recorded using a Fujifilm FLA-5000 fluor-image analyzer with an imaging plate (Fujifilm type BAS-MS for $^{14}$C material, Fujifilm, Japan).