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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].

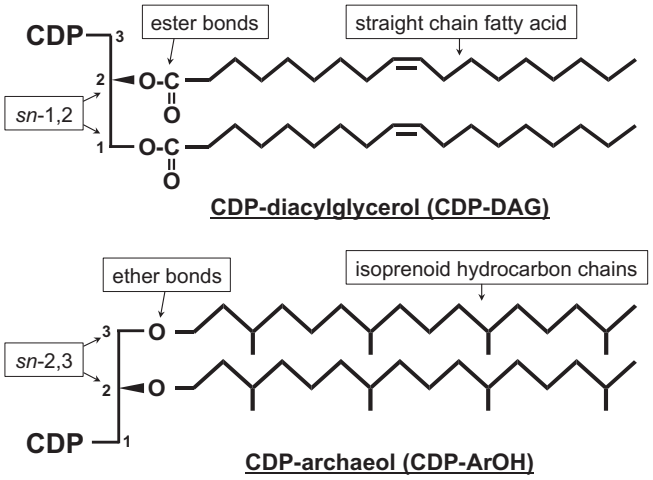

Fig. 1. Structures of lipid substrates, CDP-diacylglycerol and CDP-archaeol for PIP synthase and AIP synthase. Three differences (*sn*-1,2 and *sn*-2,3, ester bonds and ether bonds, straight fatty acyl chains and highly methyl-branched saturated isopranyl chains) are shown.

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).

3. Results

3.1. Distribution of PIPS and AIPS in Bacteria and Archaea

Table 1 shows the distribution of PIPS and AIPS in Bacteria and Archaea. PIPS and AIPS activity was confirmed in all recombinant cells transformed with the respective gene constructs for the four bacterial species and the two archaeal species. Inositol was not incorporated. The enzymes from the bacterial and archaeal species in Table 1 were annotated as PIS, etc., by the National Center for Biotechnology Information, but were exact matches for PIPS and AIPS. In the case of the two eukaryotic species, *S. cerevisiae* and *H. sapiens,* PIS activity was observed; however, inositol 1-phosphate was not incorporated.

3.2. Lipid substrate specificities

There are three fundamental differences between CDP-DAG and CDP-ArOH (Fig. 1). The first is the stereochemical structure of di-*O*-radylglycerol moiety (*sn*-1,2 and *sn*-2,3). The second is the nature of the linkage between the glycerol and hydrocarbon chains (ester and ether). The third is the nature of hydrocarbon chains themselves (straight fatty acyl chains and highly methyl-branched saturated isopranyl chains). CDP-ArOH was used as the substrate for PIPS in Bacteria, and CDP-DAG was used as the substrate for AIPS in Archaea (Table 1). CDP-ArOH was used as the substrate for PIS in eukaryotes, but its activity was very weak (Table 1). The reaction products of CDP-DAG and CDP-ArOH with the membrane fraction of *E. coli* pET21a-Coryne-PIPS (GI:41325888, PIP synthase of *C. glutamicum*) and *E. coli* pET21a-Aero-AIPS (GI:118431493, AIP synthase of *A. pernix)* were tested using TLC (Fig. 2). Regardless of the enzyme used, when CDP-DAG was used as the substrate, PIP and small quantities of PI were produced, and when CDP-ArOH was used as the substrate, AIP and small quantities of AI were produced. When *E. coli* pET21a-Aero-AIPS was used as the enzyme, the proportion of minor reaction products, PI and AI (besides the main products PIP and AIP), was greater than that of *E. coli* pET21a-Coryne-PIPS.

3.3. Phylogenic analysis of PIPS homologs

The phylogenetic tree based on the amino acid sequence of PIPS, AIPS, and PIS (Fig. 3) shows that these three enzymes are closely related despite different substrate specificities. The amino acid sequence of PIPS and AIPS was highly homologous and differed from that of PIS in that they had a common site with four arginine residues downstream of the CDP-alcohol phosphatidyltransferase motif [2]. This is believed to be the recognition site for the inositol phosphate group substrates.

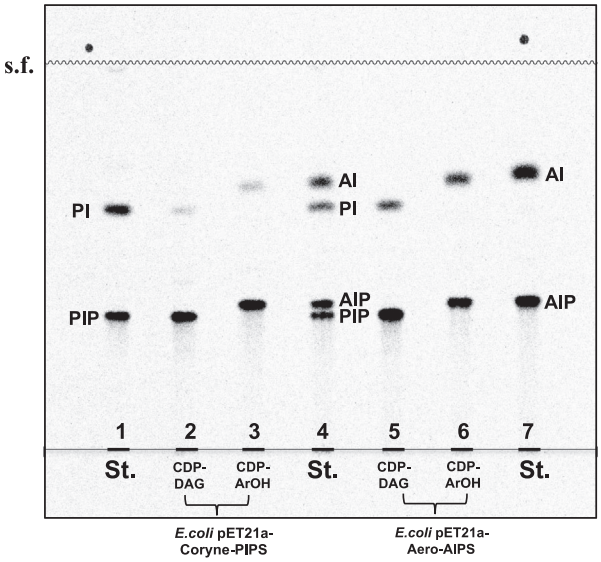

Fig. 2. Identification of the reaction products of PIP synthase and AIP synthase. Thin-layer chromatogram of ^{14}C-labeled products of the PIP(AIP) synthase reaction is shown. The source of the enzyme was the membrane fraction of *E. coli* pET21a-Coryne-PIPS (GI:41325888, PIP synthase of *Corynebacterium glutamicum*) (lanes 2 and 3) or *E. coli* pET21a-Aero-AIPS (GI:118431493, AIP synthase of *Aeropyrum pernix*) (lanes 5 and 6). Lipid substrate in the enzyme reaction was CDP-DAG (lanes 2 and 5) or CDP-ArOH (lanes 3 and 6). Products were extracted and developed by TLC. Radioactive spots were detected by autoradiography. s.f., solvent front; St., standard ^{14}C-labeled PI and PIP (lane 1), ^{14}C-labeled AI, PI, AIP, and PIP (lane 4), and ^{14}C-labeled AI and AIP (lane 7). AI, archaetidylinositol; AIP, archaetidylinositol phosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate.

4. Discussion

Both PIPS and AIPS activity were confirmed in all recombinant cells transformed with the respective gene constructs for the four bacterial and two archaeal species. Inositol was not incorporated. The TLC method identified the enzymatic minor reaction products from PIPS and AIPS, and confirmed that these were PI and AI,

Table 1
Specific activity of PIP synthase, AIP synthase, PI synthase and AI synthase in various organisms.

Source of enzyme[a] (GI No.)	PIP synthase	AIP synthase	PI synthase	AI synthase
	(nmol/h/mg)		(pmol/h/mg)	
Bacteria				
Mycobacterium bovis[b] (GI:31793797)	138 ± 2	5 ± 2	–	
Streptomyces avermitilis (GI:29610491)	76 ± 3	20 ± 1	–	
Propionibacterium acnes (GI:50840158)	88 ± 2	14 ± 0	–	
Corynebacterium glutamicum (GI:41325888)	175 ± 6	174 ± 8	–	
Rhodococcus equi (GI:312139484)	124 ± 1	33 ± 1	–	
Archaea				
Sulfolobus solfataricus (GI:13813721)	179 ± 6	107 ± 6		–
Aeropyrum prenix (GI:118431493)	179 ± 1	112 ± 5		–
Eukarya				
Saccharomyces cerevisiae (GI:6325370)	–		107 ± 15	7 ± 0
Homo sapiens (GI:119600384)	–		34 ± 2	2 ± 0

–, No activity.
Results are expressed as means ± SE for two experiments.
[a] Each recombinant was assembled by chemical synthesis and expressed in *Escherichia coli* except that of *Mycobacterium bovis*.
[b] The gene-encoding PIPS from *M. bovis* was cloned and expressed in *E. coli* [2].

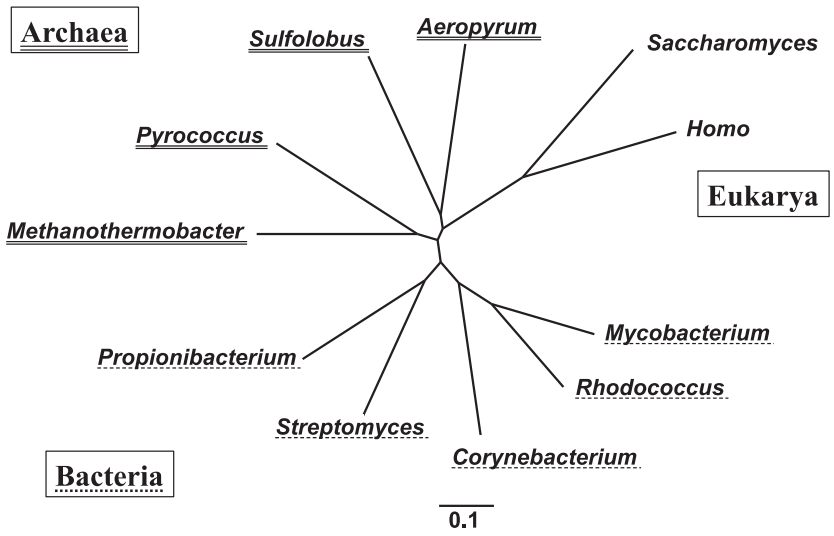

Fig. 3. Phylogenic analysis of PIP synthase, AIP synthase, and PI synthase. Amino acid sequences were aligned using ClustalW (http://clustalw.ddbj.nig.ac.jp/) and the default parameters. The tree was built using FigTree v1.4.0 software.

respectively. It is believed that these were generated from the phosphatase activity of the host cell *E. coli* and were synthesized from PIP and AIP. Alkaline phosphatase of *E. coli* hydrolyzes a wide variety of compounds containing monosubstituted phosphate [18,19]. The assay using *E. coli* pET21a-Aero-PIPS was performed at 60 °C, pH 8.0. Alkaline phosphatase of *E. coli* is very active at this high temperature [20], which could be the reason for the greater proportion of minor reaction products, PI and AI. No tests were performed for PIP and AIP dephosphorylation enzyme activity in the strains used. Based on the fact that PI and AI exist within the cell membrane, however, it is believed that a pathway synthesizing PI or AI through the dephosphorylation of the intermediates, PIP and AIP, is universal in Bacteria and Archaea, which contain inositol phospholipid, and that there is no biosynthetic pathway that can use free inositol in Eukarya.

The findings from recombinant cells transformed with the PIS gene in eukaryotic species *S. cerevisiae* and *H. sapiens*, however, confirmed that both species had PIS activity, but did not incorporate inositol 1-phosphate. The results indicate that in these two eukaryotic species, unlike in Bacteria, there is no pathway to convert PIP to PI. The only pathway converts free inositol and CDP-DAG directly into PI.

These findings confirm that bacterial PIPS can use CDP-ArOH as a substrate to synthesize AIP, and that archaeal AIPS uses CDP-DAG as a substrate to produce PIP. The AIPS activity was higher with CPD-DAG as the substrate instead of the canonical CDP-ArOH substrate. Because the amino acid sequences of the two enzymes are also highly homologous, PIPS and AIPS could be essentially regarded as the same enzyme (CDP-activated core lipid:inositol 1-phosphate-phosphatidyltransferase). It is possible that either the PIPS gene or the AIPS gene was horizontally transferred from one domain (Bacteria or Archaea) to the other domain (Archaea or Bacteria).

Fig. 3 shows a phylogenetic tree based on the amino acid sequences of the inositol phospholipid enzymes (PIPS, AIPS, and PIS) from the three species confirmed previously [1,2] and the four bacterial, two archaeal, and two eukaryotic species examined in the present study. This tree shows that they are closely related. Daiyasu et al. also showed that AIPS, archaetidylglycerol phosphate synthase, PIPS, phosphatidylglycerol phosphate synthase, and PIS are all homologous, based on the genomic information [21].

The ultimate products of Bacteria, Archaea, and Eukarya are PI and AI. The three-dimensional structure of the inositol-containing polar head groups of these products is exactly the same as 1D-*myo*-inositol 1-phosphate [22–25]. It is possible that the inositol phospholipid biosynthesis pathway in Eukarya was originally the same as that in Bacteria and used inositol phosphate as a substrate. This ultimately leads to an interesting question as to why the phosphate group was removed and why the free inositol was used as a substrate in Eukarya.

PIP is not formed in the process of PI synthesis in humans because inositol reacts directly with CDP-DAG. PIP synthase in the pathway is a promising target for the development of new anti-harmful actinomycetes drugs [16]. Furthermore, because the distribution of PI is limited to a few eubacteria, synthesis of agents specific to actinomycetes with no effects on indigenous bacterial flora in humans is expected.

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