[Original]

Preparation of 1L-*myo*-Inositol 1-Phosphate as a Substrate of Phosphatidylinositol Phosphate Synthase

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Abstract : Novel drugs possessing a mechanism of action specific to pathogenic mycobacteria, including *Mycobacterium tuberculosis*, are needed. In 2010, we discovered that the biosynthetic pathway of phosphatidylinositol, which is a membrane phospholipid, differs between humans and mycobacteria. The key enzyme responsible for this difference is phosphatidylinositol phosphate (PIP) synthase, which is present only in a few bacteria belonging to the phylum Actinobacteria. Discovering compounds that inhibit the activity of this enzyme will lead to the development of new drugs specific to pathogenic mycobacteria. Measuring PIP synthase activity requires the isotope-labeled substrate 1L-*myo*-inositol 1-phosphate (1L-Ino-1P). Because this substrate is not commercially available, we synthesized it from [¹⁴C] glucose 6-phosphate ([¹⁴C] Glc-6P), using a crude enzyme solution isolated from the methanoarchaeon 1L-Ino-1P synthase. The activity of 1L-Ino-1P synthase in the crude enzyme mixture was low, and quantitative analysis of the synthesized 1L-Ino-1P was inaccurate due to impurities present in the crude enzyme mixture. In the present study, we describe a method for synthesizing 1L-Ino-1P using a solution containing recombinant 1L-Ino-1P synthase derived from the hyperthermophilic archaeon *Aeropyrum pernix*. In addition, we elucidate the conditions leading to the almost complete conversion of Glc-6P into 1L-Ino-1P using this enzyme. Quantitation of the synthesized 1L-Ino-1P was performed by colorimetry and gas liquid chromatography. Further, we confirmed that isotope-labeled 1L-Ino-1P, which is difficult to quantitate by gas liquid chromatography, can be accurately quantified by colorimetry. We also confirmed that 1D-inositol 1-phosphate cannot be a substrate for PIP synthase.

Keywords : inositol, phospholipid, phosphatidylinositol phosphate.

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Introduction

Mycobacterium tuberculosis is a pathogen that causes many deaths worldwide [1]. Tuberculosis is treated with several antituberculosis drugs, such as isoniazid, rifampicin, and streptomycin, but increased infection by mycobacteria resistant to multiple antitu-

berculosis drugs has become a problem. Group outbreaks of tuberculosis infection still occur in Japan, although the tuberculosis morbidity rates continue to decrease. The prevalence of infectious diseases caused by nontuberculous mycobacteria is rapidly increasing, but effective treatment for these diseases has not yet been established [2]. Novel drugs with a mechanism

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of action specific to pathogenic mycobacteria, including *M. tuberculosis*, are needed.

In 2010, we discovered that the synthetic pathway of phosphatidylinositol (PI) in membrane phospholipids differs between mycobacteria and humans [3]. In mycobacteria, 1L-*myo*-inositol 1-phosphate (1L-Ino-1P) synthesized from glucose 6-phosphate (Glc-6P) reacts with cytidine diphosphate diacylglycerol (CDP-DAG) to produce an intermediate compound, phosphatidylinositol phosphate (PIP), in which an extra phosphate is attached to PI (Fig. 1). The phosphate is then removed to produce PI. In humans, free inositol, in which the phosphate has been removed from 1L-Ino-

1P, reacts with CDP-DAG, so that PIP is not produced during the PI biosynthetic process. In mycobacteria, the membrane phospholipid component PI, which is synthesized via PIP, is a starting compound for the biosynthesis of lipoarabinomannan, a structural component of the cell wall. In all mycobacteria, including *M. tuberculosis*, lipoarabinomannan plays an important role in enabling the bacteria to elude the host immune system [4–6]. Therefore, discovering compounds that inhibit PIP biosynthesis in *M. tuberculosis* will lead to the development of new drugs that specifically inhibit the growth of pathogenic mycobacteria without affecting humans. Bacteria that belongs to genera other than

Fig. 1. Biosynthetic pathway of phosphatidylinositol in mycobacteria and humans. Reproduced modification from ref. (Morii H *et al* (2013): Studies of inositol 1-phosphate analogues as inhibitors of the phosphatidylinositol phosphate synthase in mycobacteria. *J Biochem* 153: 257–266, Fig. 1. Biosynthetic pathway of phosphatidylinositol in mycobacteria and humans [9]), with permission of the Japanese Biochemistry Society.

Actinomyces do not possess PI, so it should be possible to develop drugs that do not affect endogenous bacteria important to humans, but are able to specifically inhibit *M. tuberculosis* and its related pathogenic bacteria by targeting mycobacteria-specific PIP synthase.

Phosphatidylinositol phosphate (PIP) synthase activity was measured using [^{14}C] 1L-Ino-1P as the substrate. Isotope-labeled 1L-Ino-1P is not commercially available, and must therefore be prepared from [^{14}C] Glc-6P. Until now, [^{14}C] 1L-Ino-1P has been prepared from [^{14}C] Glc-6P using the supernatant of homogenized methanarchaeon, which is a crude mixture of enzymes that includes 1L-Ino-1P synthase [3, 7–9]. Quantification of 1L-Ino-1P prepared from the crude enzyme mixture is performed by colorimetric measurement of inorganic phosphate released after NaIO_4 oxidation [10]. Inorganic phosphate is not released from Glc-6P by NaIO_4 oxidation. In a reaction mixture containing the crude enzyme mixture and 1L-Ino-1P formed from Glc-6P, however, 87% of the inorganic phosphate released after NaIO_4 oxidation is due to the release of inorganic phosphate from the crude enzyme mixture alone. A high inorganic phosphate background would interfere with the measurement of 1L-Ino-1P. Although an accurate concentration of 1L-Ino-1P is not required for detecting only the presence of PIP synthase activity, it is definitely necessary for examining the nature of PIP synthase in detail and for verifying its inhibitor. Therefore, we studied the optimum conditions for preparing 1L-Ino-1P using a solution containing recombinant 1L-Ino-1P synthase derived from *Aeropyrum pernix* and quantitated the production of 1L-Ino-1P by colorimetry and gas liquid chromatography (GLC). We also examined whether commercially available [^3H] 1D-inositol 1-phosphate can be a substrate for PIP synthase.

Materials and Methods

Materials

A solution of thermostable, recombinant inositol 1-phosphate synthase was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). D-Glucose 6-phosphate (Glc-6P) and 1D-*myo*-inositol 1-monophosphate (1D-Ino-1P) were purchased from Sigma-Aldrich (Tokyo, Japan). DL-*myo*-inositol 1-phosphate was purchased from Biochem AG (Bubendorf, Switzerland).

land). CDP-dioleoylglycerol {CDP-DAG (dioleoyl)} was chemically synthesized from 1,2-dioleoyl-*sn*-glycero-3-phosphate (Avanti Polar Lipids Inc., Alabaster, AL, USA), as described previously [11]. Glucitol 6-phosphate was prepared by NaBH_4 reduction of D-Glc-6P [12]. [^{14}C (U)]Glucose 6-phosphate (3.7 MBq/ml) was obtained from Moravsek Biochemicals, Inc. (Brea, CA, USA). [$2\text{-}^3\text{H}$] 1D-*myo*-inositol 1-phosphate (1.85 MBq/ml) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA).

Preparation of 1L-*myo*-inositol 1-phosphate (1L-Ino-1P) using a crude enzyme

1L-*myo*-inositol 1-phosphate (1L-Ino-1P) was prepared from Glc-6P using a crude enzyme as follows. Cells of *Methanothermobacter thermautotrophicus* (DSM 1053) were grown and harvested at log phase, as described previously [11]. The *M. thermautotrophicus* cells were washed twice with 5 mM dithiothreitol (DTT). The pelleted cell paste was stored at -20°C until use. The frozen *M. thermautotrophicus* cells were thawed in 10 ml of 5 mM DTT containing 5 mg deoxyribonuclease I (DNase I) (Sigma-Aldrich) and passed through a French pressure cell operated at $1,400\text{ kg/cm}^2$. This process was repeated three times. Cell debris and unbroken cells were removed by centrifugation ($10,000 \times g$) for 10 min. The resultant homogenate was centrifuged at $100,000 \times g$ for 2 h to separate the supernatant and membrane fractions. The supernatant fraction was used as a crude enzyme containing 1L-Ino-1P synthase. The complete reaction mixture (final volume, 5.0 ml) contained the supernatant fraction of *M. thermautotrophicus* homogenates (59.5 mg protein), 50 mM bicine buffer (pH 8.5), 0.5 mM Glc-6P, 5 mM nicotinamide adenine dinucleotide (NAD), 1 mM DTT, and 1 mM ammonium acetate. The reaction mixture was incubated at 60°C for 2 h. After incubation, it was filtered with an Ultrafilter (USY-1 M=10,000, Advantec) to remove macromolecular compounds. The filtrate contained 1L-Ino-1P, unreacted Glc-6P, and other low molecular-weight compounds in the reaction mixture.

Preparation of 1L-*myo*-inositol 1-phosphate (1L-Ino-1P) using a recombinant enzyme

1L-*myo*-inositol 1-phosphate (1L-Ino-1P) was pre-

pared from Glc-6P using a solution containing recombinant 1L-Ino-1P synthase produced by expressing the relevant gene from *A. pernix*. The complete reaction mixture (final volume, 0.25 ml) contained the recombinant enzyme (0.032 units), 40 mM NaHCO₃ (pH 9.0), 0.5–2.0 mM D-Glc-6P, 1 mM NAD, and 15 mM NH₄Cl. The reaction mixture was incubated at 85°C for 5–90 min.

Measurement of 1L-myo-inositol 1-phosphate (1L-Ino-1P) by colorimetry

Synthesized 1L-Ino-1P was measured essentially based on the measurement of inorganic phosphate (Pi) released by periodate oxidation of *myo*-inositol 1-phosphate, as described by Barnett *et al* [10]. The reaction mixture (0.5 ml) of 1L-Ino-1P prepared from Glc-6P using the enzyme was stopped by the addition of 0.2 ml of 20% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant was incubated at 37°C for 1 h with 0.2 M NaIO₄, after which 1 M Na₂SO₃ was added to destroy excess NaIO₄ and liberated Pi was measured using the method of Bartlett [13]. The net Pi value was obtained by subtracting the blank value of the reaction mixture that received Na₂SO₃ before the addition of NaIO₄.

Measurement of 1L-myo-inositol 1-phosphate (1L-Ino-1P) by gas liquid chromatography (GLC)

After incubation of 1L-Ino-1P prepared from Glc-6P using the enzyme, the reaction mixture was filtered with an Ultrafilter (USY-1 M = 10,000, Advantec) to remove macromolecular compounds without the addition of trichloroacetic acid. Glucitol 6-phosphate was added to the supernatant as an internal GLC standard, followed by evaporation at 40°C in a water bath under nitrogen gas and dryness in a high vacuum. This dried residue was trimethylsilylated in trimethylchlorosilane:hexamethyldisilazane:anhydrous pyridine = 2:2:5 (v/v) at 80°C for 10 min. DL-*myo*-inositol 1-phosphate was similarly trimethylsilylated and used as the GLC standard. Gas liquid chromatography (GLC) was performed using a Shimadzu GC-14B gas-liquid chromatograph equipped with flame ionization detectors. Trimethylsilylated inositol 1-phosphate was chromatographed on a 2-m glass column packed with 3% OV-17 on Uniport HP (80–100 mesh) at 215°C.

Measurement of phosphatidylinositol phosphate (PIP) synthase activity

The complete assay mixture (final volume, 0.2 ml) contained 0.1 mM [¹⁴C] 1L-Ino-1P (20 nmol, 11.8 kBq) or 0.1 mM [³H] 1D-Ino 1P (20 nmol, 18.5 kBq), 40 nmol CDP-DAG, 50 mM bicine buffer (pH 8.0), 10 mM MgCl₂, and 20 µg protein of *E. coli*-pET21a-BCG-PIPS membrane fraction [3]. The sequence of the cloned PIP synthase gene of *Mycobacterium bovis* BCG was the same as that of *M. tuberculosis* (CAE55505.1). The reaction mixture was incubated at 37°C for 30 min. After incubation, the mixture of the reactants and product was partitioned into aqueous and organic components with 2.5 ml CHCl₃, 2.5 ml of 0.1 M HCl in methanol, and 2.05 ml of 1 M MgCl₂. The radioactivity in the chloroform-soluble organic materials was counted using a liquid scintillation spectrometer (Aloka LSC-8000, Japan) with Aquasol-2 (Packard, Meriden, CT) as the scintillation cocktail.

Results and Discussion

1L-*myo*-inositol 1-phosphate (1L-Ino-1P), a PIP synthase substrate, was prepared from Glc-6P using a crude enzyme solution (1L-Ino-1P synthase) contained in the supernatant of homogenized *M. thermotrophicus*. Many components of this crude enzyme solution release inorganic phosphate after NaIO₄ oxidation, which reduces the reliability of quantitative measurement of 1L-Ino-1P. Therefore, we performed partial purification of the crude enzyme solution using ammonium sulfate fractionation, gel filtration, and ultrafiltration. We observed almost no 1L-Ino-1P synthase activity, however, in the partially purified enzyme solution (data not shown).

1L-*myo*-inositol 1-phosphate (1L-Ino-1P) was prepared from Glc-6P using a solution containing recombinant 1L-Ino-1P synthase produced by expressing the relevant gene from *A. pernix*. Inorganic phosphate was not released in this enzyme solution without substrate after NaIO₄ oxidation (data not shown). The activities of the crude and recombinant enzymes are shown in Table 1. Figure 2 shows the time-course of the ratios of the 1L-Ino-1P formed from Glc-6P. The 1L-Ino-1P was measured by colorimetry. Free inorganic phosphate Pi was released after NaIO₄ oxidation of the 1L-Ino-1P produced from 0.5, 1.0, and 2.0 mM Glc-6P using

Table 1. Determination of 1L-inositol 1-phosphate (1L-Ino-1P) after an inositol 1-phosphate synthase (IPS) reaction using crude and recombinant enzyme

Glc-6P	Enzyme (origin of IPS)	Assay Condition	1L-Ino-1P (mM) ^a	
			NaIO ₄ -P _i ^b	GLC ^c
0.5 mM	Crude ^d (<i>M. thermotrophicus</i>)	60°C 2 h	0.266 ± 0.004	–
0.5 mM	Recombinant ^e (<i>A. pernix</i>)	85°C 1 h	0.492 ± 0.018	0.508
2.0 mM	Recombinant ^e (<i>A. pernix</i>)	85°C 1 h	0.784 ± 0.008	0.776

^a: Concentration of 1L-Ino-1P in the enzyme reaction mixture,

^b: 1L-Ino-1P was measured by colorimetric measurement of the inorganic phosphate released after NaIO₄ oxidation, ^c: 1L-Ino-1P was measured by GLC using glucitol 6-phosphate as an internal standard, ^d: crude enzyme, homogenate of *M. thermotrophicus* (59.5 mg protein/5.0 ml well), ^e: recombinant enzyme derived from *A. pernix* (0.032 units/0.25 ml well). Glc-6P: D-glucose 6-phosphate, GLC: gas liquid chromatography

Fig. 2. Time-course of the ratios of 1L-Ino-1P formed from glucose 6-phosphate (Glc-6P). 1L-Ino-1P were prepared from ●: 0.5 mM Glc-6P, ▲: 1.0 mM Glc-6P, ■: 2.0 mM Glc-6P using the recombinant enzyme at 80°C, and then the reaction mixture was incubated with 0.1 mM NaIO₄. The released Pi, which is correlated with the amount of 1L-Ino-1P, was measured by colorimetry. 1L-Ino-1P: 1L-inositol 1-phosphate.

the recombinant enzyme. The released Pi was measured by colorimetry. Almost all the Glc-6P reacted within 30 min when the concentration of Glc-6P was 0.5 mM, and the amount of Pi did not change thereafter. When the same reaction was performed with Glc-6P at concentrations of 1.0 and 2.0 mM, the reaction rates

slowed within approximately 30 min under both concentrations. Moreover, the reactions did not progress further, leaving a large amount of unreacted Glc-6P.

It is difficult to analyze isotope-labeled 1L-Ino-1P by GLC; therefore, a simple quantitative method (colorimetry) must be established to analyze it. The quantitative analysis results for 1L-Ino-1P obtained by colorimetry and GLC are shown in Table 1. Analysis with GLC revealed that the products obtained from the crude enzyme reaction solution had many compound peaks in addition to 1L-Ino-1P and Glc-6P. Because a peak appearing in the same position as the internal standard glucitol 1-phosphate was detected, it was not possible to quantitate 1L-Ino-1P by GLC. When the recombinant enzyme was used, the concentration of 1L-Ino-1P measured by GLC was the same as that measured by colorimetry (Table 1). Therefore, a simple quantitative method can be used to accurately measure the concentration of (isotope-labeled) 1L-Ino-1P.

On the basis of these results, we established the following method for preparing a substrate necessary for measuring PIP synthase activity: addition of the recombinant enzyme (0.032 units/0.25 ml well) derived from *A. pernix* to Glc-6P (to a final concentration of 0.5 mM), followed by heat treatment for 1 h at 85°C. The 1L-Ino-1P prepared using the method described above contained very little Glc-6P. In a previous method for measuring of PIP synthase activity, [¹⁴C] 1L-Ino-1P prepared using a crude enzyme solution was used despite a large amount of unreacted [¹⁴C] Glc-6P in the solution. The concentration of 1L-Ino-1P prepared according to the newly established method was 0.5 mM. When PIP synthase activity is measured, 0.1 mM 1L-Ino-1P is usually used; therefore, the prepared 1L-Ino-1P should be diluted 5-fold. The concentration of 1L-Ino-1P can be accurately determined by colorimetric measurement of the inorganic phosphate released after NaIO₄ oxidation, and thereafter inhibitors of PIP synthase can readily be discovered.

The three-dimensional structure of the inositol-containing polar head region of PI, which is 1D-*myo*-inositol 1-phosphate, is identical in mycobacteria and humans (Fig. 1). A novel pathway for the synthesis of inositol phospholipids using CDP-inositol as the donor of the polar head group in the thermophilic bacterium *Rhodothermus marinus* was recently reported [14].

Fig. 3. Structural relations among 1D-myo-inositol 1-phosphate (1D-Ino-1P), phosphatidylinositol phosphate (PIP) and phosphatidylinositol (PI) in mycobacteria, humans and *R. marinus*. A PI with the same structure as that of *R. marinus* could be created using a phosphatase on the product formed by PIP, provided that PIP synthase can utilize 1D-Ino-1P as a substrate. CDP: cytidine diphosphate, CDP-DAG: CDP-diacylglycerol.

The configuration of CDP-inositol is CDP-1L-myo-inositol [15]. Therefore, the configuration of the inositol-containing polar head region of PI in *R. marinus* is 1L-myo-inositol 1-phosphate, not 1D-myo-inositol 1-phosphate (Fig. 3). A PI with the same structure as that of *R. marinus* could be created using a phosphatase on the product formed by PIP, provided that PIP synthase can utilize 1D-Ino-1P as a substrate (Fig. 3).

The [^{14}C] 1L-Ino-1P prepared using the method developed in this study and commercially available [^3H] 1D-Ino-1P were used as substrates to examine the PIP synthase activity of each. The PIP synthase activity, as measured colorimetrically, was high (76.2 ± 1.2 nmol/h/mg) when using [^{14}C] 1L-Ino-1P as the substrate, and almost nonexistent (< 0.2 nmol/h/mg) when using [^3H] 1D-Ino-1P as the substrate (Table 2). 1D-Ino-1P is

produced when PI is decomposed by phospholipase C in mycobacteria. 1D-Ino-1P, however, is not a substrate for PIP synthase (Fig. 3).

Conflict of Interest

The authors declare no conflict of interest.

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Table 2. Substrate specificity of phosphatidylinositol phosphate (PIP) synthase in mycobacteria

Substrate	PIP synthase specific activity (nmol/h/mg)
1L-Ino-1P	76.2 ± 1.2
1D-Ino-1P	< 0.2

The assay conditions are described in Materials and Methods. Source of enzyme was *E. coli*-pET21a-BCG-PIPS membrane fraction. 1L-Ino-1P: 1L-myo-inositol 1-phosphate, 1D-Ino-1P: 1D-myo-inositol 1-phosphate

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ホスファチジルイノシトールリン酸合成酵素の基質1L-イノシトール1リン酸の調製法

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要 旨：結核菌を含めた病原性抗酸菌に特異的な活性作用機序を持った新薬の開発が求められている。我々は2010年に膜リン脂質のホスファチジルイノシトールの生合成経路がヒトと抗酸菌とで異なる事を発見した。相違点の鍵となる酵素がホスファチジルイノシトールリン酸(PIP)合成酵素である。この酵素は放線菌門の一部の細菌のみに存在し、ヒトやヒトの常在細菌には存在しない。この酵素活性を阻害する化合物を見つけることができれば病原性抗酸菌に特異的な新薬の開発に繋がる。PIP合成酵素活性測定には、基質としてアイソトープでラベルされた1L-イノシトール1リン酸(1L-Ino-1P)が必要である。この基質は市販されていないので、これまでは[¹⁴C]グルコース6リン酸([¹⁴C]Glc-6P)からメタン生成古細菌の粗酵素を1L-Ino-1P合成酵素として使って調製していた。この粗酵素の活性は低く、粗酵素中の不純物の影響で生成物の1L-Ino-1Pの定量が不正確だった。本研究では、超好熱性古細菌 *Aeropyrum pernix* の1L-Ino-1P合成酵素組換え体溶液を酵素として用いて、1L-Ino-1P調製の最適条件を検討し、1L-Ino-1Pの簡易定量法が正確な濃度を示している事をガスクロマトグラフィーで確認した。また、1D-イノシトール1リン酸(1D-Ino-1P)はPIP合成酵素の基質にならないことを確認した。

キーワード：イノシトール, リン脂質, ホスファチジルイノシトールリン酸.

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