Pergamon

Tetrahedron Letters 41 (2000) 2925–2928

TETRAHEDRON LETTERS

Studies on the nonmevalonate pathway: conversion of 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol to its 2-phospho derivative by 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase

Tomohisa Kuzuyama,[a,†] Motoki Takagi,[a,†] Kazuhide Kaneda,[a] Hiroyuki Watanabe,[a] Tohru Dairi [b] and Haruo Seto [a,*]

[a] *Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032, Japan*
[b] *Biotechnology Research Center, Toyama Prefectural University, Toyama 939-0398, Japan*

Received 18 January 2000; accepted 10 February 2000

Abstract

A nonmevalonate pathway intermediate, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol, is transformed to its 2-phospho-derivative in the presence of ATP by a novel *Escherichia coli* enzyme, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: isopentenyl diphosphate; 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase.

In many bacteria, green algae and chloroplasts of higher plants, isopentenyl diphosphate (IPP **1**), a building block of isoprenoids, is biosynthesized in the nonmevalonate pathway (Fig. 1).[1] The initial step of this pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) **2** by condensation of pyruvate and glyceraldehyde 3-phosphate catalyzed by DXP synthase.[1] The second step catalyzes the intramolecular rearrangement and reduction of **2** simultaneously to yield 2-*C*-methyl-D-erythritol 4-phosphate (MEP) **3**.[2,3]

Recently Zenk and co-workers purified an *E. coli* enzyme that catalyzed a cytidylyl transfer to **3** to synthesize 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME) **4**, and cloned the corresponding gene.[4] Although they insisted that this reaction was the third step in the nonmevalonate pathway, they could not exclude the possibility that **4** might be a shunt pathway product. We independently cloned the same gene that complemented the defect of a blocked mutant in synthesizing **1** from **3**, and unequivocally demonstrated that **4** synthesized from **3** by the gene product, MEP cytidylyltransferase, is a true intermediate in the nonmevalonate pathway.[5] The following reactions leading to **1** from **4**, however, remained unknown.

* Corresponding author. Tel: +81-3-5841-7839; fax: +81-3-5841-8485; e-mail: haseto@iam.u-tokyo.ac.jp (H. Seto)
† The first two authors contributed equally to this work.

0040-4039/00/$ - see front matter
PII: S0040-4039(00)00295-1

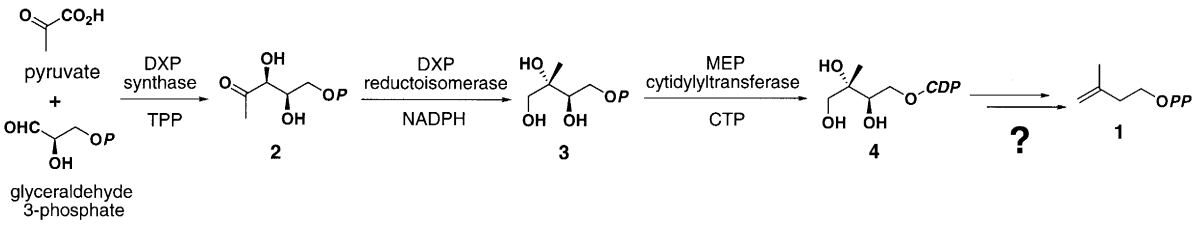

Fig. 1. The nonmevalonate pathway for IPP biosynthesis

In our previous paper[5] we reported the cloning of several genes that complemented the defects of blocked mutants in synthesizing **1** from **3**‡ and transformation of **4** to an unknown product by an enzyme encoded by one of these genes.[5] In this paper we describe the structure elucidation of this new compound.

Incubation of this gene product encoded by *ych*B (DDBJ/EMBL/GenBank accession number AB037116) (2 mg§) with **4** (1 mM) in the presence of 1 mM ATP at 37°C for 30 min in 100 ml of 5 mM Tris–HCl (pH 8.0) containing 1 mM $MgCl_2$ and 10 mM DL-dithiothreitol resulted in appearance of a new peak in HPLC with an Asahipak GS-320 HQ column (7.6×300 mm, Showa Denko, Tokyo, Japan).¶ The reaction product corresponding to this peak was purified with the assistance and guidance of this HPLC. Thus, the reaction mixture was diluted to 300 ml with H_2O, subjected to Dowex 1-X8 (Cl^- type, 2×6 cm) chromatography, washed with 1% NaCl aq. (100 ml), and then eluted with 2% NaCl aq. (100 ml). All fractions showing the peak with a retention time of 11.6 min were combined, concentrated and chromatographed on a Sephadex G-10 column (1.8×100 cm) and elution was carried out with water only. A fraction showing only the peak with the same retention time was freeze-dried to afford a pure reaction product (2.8 mg).

The structure of the purified product was deduced by spectroscopic methods. The molecular formula was determined to be $C_{14}H_{23}O_{17}N_3P_3Na_3$ by HR-FABMS [*m/z* 644.0057 $(M-Na)^-$, Δ+2.1 mmu]. The ^1H, ^{13}C, and ^{31}P NMR spectral features of the purified product showed close similarities to those of **4** (Table 1). The purified compound, however, showed an additional ^{31}P NMR signal at −2.4 ppm. These data suggested the reaction product to be a phosphorylated derivative of **4**. A noticeable difference of ^{13}C NMR spectra between **4** and the reaction product was found in the downfield shift of the C-2 signal; 74.8 ppm in **4** to 82.1 ppm in the reaction product. In addition, the methyl proton singlet in the reaction

‡ These mutants were derived from an *E. coli* transformant with the mevalonate kinase, phosphomevalonate kinase and pyrophosphomevalonate decarboxylase activities. Thus, this transformant can utilize the partial mevalonate pathway for IPP biosynthesis by supplementation of mevalonate. Therefore, these mutants, which can grow only in the presence of mevalonate, are concluded to be deficient in the nonmevalonate pathway (see Ref. 5).

§ Protein expression and purification of the CDP-ME kinase: The *E. coli* CDP-ME kinase gene, *ychB*, was cloned into the expression vector pQE30 (Qiagen) to give pQECMEK. *E. coli* M15 containing pREP4 [*neo*, *lacI*] (Qiagen) was used as a host for expression of the *ychB* gene. *E. coli* M15 (pREP4, pQECMEK) was cultured at 37°C in 100 ml of Luria–Bertani medium containing 25 μg/ml kanamycin and 200 μg/ml ampicillin for 5 hrs with 0.1 mM isopropyl-ß-D-thiogalactopyranoside upon reaching an optical density at 660 nm of 0.8. Cells were harvested by centrifugation and resuspended in 100 mM Tris-HCl (pH 8.0). After brief sonication, the lysate was centrifuged at 10,000×g for 20 min and the supernatant was collected. A 50% slurry of Ni-nitrilotriacetic acid (NTA) agarose resin (Qiagen) was added into the supernatant and stirred on ice for 60 min. The resin was washed with 50 mM imidazole in 100 mM Tris–HCl (pH 8.0) and then the protein which bound to the Ni–NTA agarose resin was eluted with 200 mM imidazole in 100 mM Tris–HCl (pH 8.0). The eluate was used as the purified CDP–ME kinase in the subsequent experiments. The purified enzyme afforded a homogeneous protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a subunit size of 32 kDa.

¶ Formation of the reaction product was monitored at 280 nm with an Asahipak GS-320 HQ column, eluted with 10 mM KH_2PO_4 (pH 2.5) at the flow rate of 1.0 ml/min at 30C. In this condition, **4** and the reaction product were eluted at 10.4 and 11.6 min, respectively.

product shifted downfield by 0.19 ppm. The UV spectral feature of the reaction product (λ_{max}=279 nm at pH 2.5) was identical to that of **4**. These spectral data unequivocally established the structure of the reaction product as 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME2P) **5**. Thus, **4** was proved to be converted into **5** in the presence of ATP by the enzyme (Fig. 2). This result clearly demonstrates that phosphorylation of the tertiary hydroxy group in the CDP-ME molecule follows the formation of **4** in the nonmevalonate pathway for IPP biosynthesis. We propose to designate this enzyme 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CDP-ME kinase), which is encoded by the *ychB* gene.

Table 1
^{1}H, ^{13}C and ^{31}P NMR spectra data of CDP-ME and the reaction product

	CDP-ME **4**	reaction product **5**
^{1}H-NMR	MEP moiety	MEP moiety
	H-1a 3.37 (d, 11.5)	H-1 3.58 (s, 2H)
	H-1b 3.49 (d, 11.5)	
	2-Me 1.03 (s, 3H)	2-Me 1.22 (s, 3H)
	H-3 3.73 (dd, 8.5, 2.5)	H-3 3.80 (m)
	H-4a 3.86 (m)	H-4a 4.06 (m)
	H-4b 4.11 (m)	H-4b 4.13 (m)
	CDP moiety	CDP moiety
	H-5 6.03 (d, 7.5)	H-5 6.00 (d, 7)
	H-6 7.88 (d, 7.5)	H-6 7.83 (d, 7)
	H-1' 5.89 (d, 4.3)	H-1' 5.85 (d, 4)
	H-2' 4.20 (dd, 5.0, 4.3)	H-2' 4.16 (dd, 5, 4)
	H-3' 4.25 (dd, 5.0, 5.0)	H-3' 4.21 (dd, 5, 5)
	H-4' 4.16 (m)	H-4' 4.13 (m)
	H-5'a 4.09 (m)	H-5'a 3.83 (m)
	H-5'b 4.16 (m)	H-5'b 4.13 (m)
^{13}C-NMR	MEP moiety	MEP moiety
	C-1 67.3	C-1 66.0 (d, 2)
	C-2 74.8	C-2 82.1 (d, 7)
	2-Me 19.1	2-Me 17.5
	C-3 74.3 (d, 6.7)	C-3 73.4 (dd, 7, 7)
	C-4 67.8 (d, 5.8)	C-4 65.0 (d, 6)
	CDP moiety	CDP moiety
	C-2 158.7	C-2 157.9
	C-4 167.1	C-4 166.4
	C-5 97.5	C-5 97.0
	C-6 142.4	C-6 141.9
	C-1' 90.1	C-1' 89.4
	C-2' 75.2	C-2' 74.6
	C-3' 70.2	C-3' 69.7
	C-4' 83.6 (d, 8.6)	C-4' 83.1 (d, 9)
	C-5' 65.5 (d, 5.8)	C-5' 67.4 (d, 6)
^{31}P-NMR	-11.2 (d, 20.2)	-11.2 (d, 19)
	-10.6 (d, 20.2)	-10.6 (d, 19)
		-2.4

All NMR data were collected with an A500 NMR spectrometer (JEOL) in D_2O. The ^{31}P chemical shifts are relative to phosphoric acid as external standard at 0 ppm.

Lange and Croteau reported recently that the partially purified *ychB* gene product catalyzed ATP-dependent phosphorylation of isopentenyl monophosphate (IP). The specific activity of the enzyme, however, was extremely low (178 pmol/s·g of protein).[7] In our study with the completely purified *ychB* gene product, no kinase activity was observed with IP as a substrate under the assay condition reported by them. On the other hand, the high kinase activity (6.7×10^{8} pmol/s·g of protein) of our enzyme was detected with **4** as a substrate.[6] Compound **4** is thus clearly shown to be a true substrate of the *ychB* gene product.

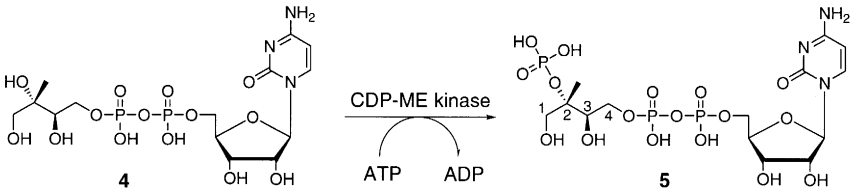

Fig. 2. CDP-ME kinase reaction

Acknowledgements

We thank Dr. K. Furihata and Dr. H. Kobayashi for collecting NMR and MS spectra, respectively. This work was supported in part by a grant, Research for the Future Program (RFTF) from the Japan Society for the Promotion of Science (JSPS), JSPS-RFTF96I00301 to H.S., by a Grant-in-Aid for Scientific Research (B) from The Ministry of Education, Science, Sports and Culture, Japan, 10460047 to H.S., by a Grant-in-Aid for Encouragement of Young Scientists from JSPS, 11760086 to T.K., and by a grant from Uehara Memorial Foundation to T.K.

References

1. Rohmer, M. Isoprenoids including carotenoids and steroids. In *Comprehensive Natural Products Chemistry*; Vol. 2, Barton, D. Nakanishi, K., Eds.; Elsevier: Amsterdam, 1999; pp. 45–67.
2. Kuzuyama, T.; Takahashi, S.; Watanabe, H.; Seto, H. *Tetrahedron Lett*. **1998**, *39*, 4509–4512.
3. Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9879–9884.
4. Rohdich, F.; Wungsintaweekul, J.; Fellermeier, M.; Sagner, S.; Herz, S.; Kis, K.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11 758–11 763.
5. Kuzuyama, T.; Takagi, M.; Kaneda, K.; Dairi, T.; Seto, H. *Tetrahedron Lett*. **2000**, *41*, 703–706.
6. Takagi, M.; Kuzuyama, T.; Kaneda, K.; Dairi, T.; Seto, H. In preparation.
7. Lange, B. M.; Croteau, R. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 13 714–13 719.