Pergamon

Tetrahedron Letters 39 (1998) 4509–4512

TETRAHEDRON LETTERS

Direct Formation of 2-*C*-Methyl-D-Erythritol 4-Phosphate from 1-Deoxy-D-Xylulose 5-Phosphate by 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase, a New Enzyme in the Non-Mevalonate Pathway to Isopentenyl Diphosphate

Tomohisa Kuzuyama‡, Shunji Takahashi‡, Hiroyuki Watanabe and Haruo Seto*

Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

Received 18 March 1998; accepted 13 April 1998

Abstract.
1-Deoxy-D-xylulose 5-phosphate is biotransformed to 2-*C*-methyl-D-erythritol 4-phosphate in a single step in the presence of NADPH by a new recombinant enzyme named 1-deoxy-D-xylulose 5-phosphate reductoisomerase purified from *Escherichia coli*.

Keywords: Biosynthesis, Isopentenyl diphosphate, 2-*C*-methyl-D-erythritol 4-phosphate, 1-Deoxy-D-xylulose 5-phosphate reductoisomerase

Since the initial discovery of the mevalonate pathway in the 1950s, it was a widely accepted view that isopenteny diphosphate (IPP), the fundamental unit in terpenoid biosynthesis, was only formed in all living organisms by condensation of acetyl coenzyme A through the ubiquitous mevalonate pathway. However, it has been disclosed recently that some organisms including many bacteria, green algae and chroloplasts of higher plants can utilize an alternative mevalonate-independent pathway (non-mevalonate pathway)[1-4] for the formation of IPP. Several recent experimental findings lend additional support to the operation of this novel non-mevalonate pathway in terpenoid biosynthesis.[5-8]

According to Rohmer and co-workers, the initial step of this pathway is the formation of 1-deoxy-D-xylulose 4-phosphate (DXP) **1** by condensation of pyruvate and glyceraldehyde 3-phosphate. In the second step the intramolecular rearrangement of **1** to a hypothetical rearranged intermediate 2-*C*-methyl-D-erythrose 4-phosphate **2** is proposed to occur and is

FAX: +81-3-3816-0453; e-mail: c00402@simail.nc.jp

‡ The first two authors contributed equally to this work.

0040-4039/98/$19.00
PII: S0040-4039(98)00802-8

followed by an unspecified reduction process of this intermediate to afford 2-*C*-methyl-D-erythritol 4-phosphate **3**. Precise details about the reductive mechanism of formation of **3**, however, were not disclosed nor made available from these earlier studies.

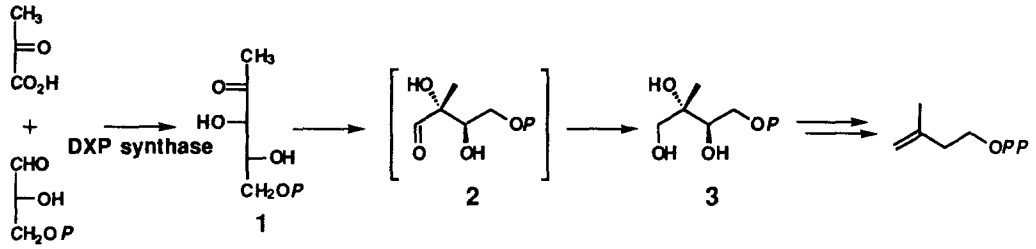

Figure 1. Hypothetical alternative non-mevalonate pathway for IPP biosynthesis

To further elucidate the details of this interesting mechanism of **3** formation, we initiated studies on the cloning of the gene responsible for synthesis of **3**. To this end, we employed the useful strategy of preparing and selecting mutants of *Esherichia coli* possessing a metabolic block(s) between **1** and **3**. Thus these mutants would require **3** for their growth and survival. Three mutants selected from *ca.* 20,000 colonies screened showed the expected properties; addition of the free alcohol of **3**[9], but not of 1-deoxyxylulose, to the minimal medium M9[10] facilitated the growth of these mutants.[11] With these mutants in hand, we cloned a gene which complemented IPP biosynthesis coding region of these blocked mutants.[11] To demonstrate that the cloned gene was actually involved in IPP biosynthesis, we constructed a plasmid for the overexpression of this gene product and succeeded in preparation of the recombinant enzyme in quantities sufficient for its characterization.[12] The purified recombinant enzyme afforded a homogeneous protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a subunit size of 42 kDa. The enzyme had a molecular mass of 165 kDa on native polyacrylamide gel electrophoresis. Thus the enzyme appears to form a homotetramer.

Incubation of this enzyme with enzymatically synthesized **1**[13] (0.15 mM) in the presence of 0.3 mM NADPH at 37 °C for 30 min in 200 ml of 100 mM Tris-HCl (pH 7.5) containing 1 mM $MgCl_2$ resulted in utilization of NADPH. Replacement of NADPH with NADH decreased the reaction rate to *ca.* 1/100 indicating that the enzyme utilizes only NADPH as a coenzyme for this reaction. The enzyme required Co^{2+}, Mg^{2+} or Mn^{2+} as well; no effect was observed with other divalent cations such as Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+}.

The reaction product was purified with the assistance and guidance of ^{31}P NMR spectroscopy. The reaction mixture was passed through an activated carbon column (2.2 X 10 cm) and after dilution to 1000 ml with H_2O, subjected to Dowex 1-X8 (Cl^- type, 3.5 X 20 cm) chromatography and eluted with 1 % NaCl (400 ml). All fractions showing a ^{31}P-signal was combined, concentrated and chromatographed on a Sephadex G-10 column (1.8 X 100 cm) and eluted with water only. Fractions showing a ^{31}P NMR signal were combined and freeze-dried to afford the pure reaction product.

The structure of the purified product was deduced by spectroscopic methods. The molecular formula was determined to be $C_5H_{12}O_7P$ by HR-FABMS [*m/z* 215.0276 $(M-H)^-$, Δ-4.5 mmu]. The ^1H and ^{13}C NMR spectral features of the compound[14] showed close similarities to those of a

chemically synthesized sample of 2-*C*-methylerythritol.[15] The isolated compound, however, showed one ^{31}P NMR signal at 4.1 ppm which was coupled to C-4 ($J_{C4\text{-}P}$=4.6 Hz) and C-3 ($J_{C3\text{-}P}$=6.5 Hz). Treatment of the reaction product with bacterial alkaline phosphatase (Takara) gave a free alcohol with ^{1}H and ^{13}C NMR spectral features in full accord with those of chemically synthesized 2-*C*-methylerythritol. The optical rotation[16] of the dephosphorylated product ($[\alpha]_D^{21}$ +6.0 (*c* 0.050, H_2O)) was identical with that of 2-*C*-methyl-D-erythritol ($[\alpha]_D^{25}$ +7.0 (*c* 0.13, H_2O)).[7] These spectral data unequivocally establish the structure of the reaction product produced by the recombinant enzyme as 2-*C*-methyl-D-erythritol 4-phosphate. Thus, the hypothetical rearranged intermediate **2** suggested by Rohmer was not yielded as the reaction product by the enzyme. This result clearly demonstrates that 2-*C*-methyl-D-erythritol 4-phosphate is synthesized in the presence of NADPH by rearrangement and reduction of **1** in a single step. We propose to designate this enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

Precedent for this biosynthetic rearrangement reaction is found in the biosynthesis of valine, isoleucine and leucine. The enzyme involved in this reaction, ketol acid reductoisomerase (EC1.1.1.86) catalyzes rearrangement of 2-acetolactate to yield 2,3-dihydroxyisovalerate with simultaneous reduction in the presence of NADPH (Figure 2).[17-20] It is to be noted that a hypothetical compound (Figure 2) with a keto function corresponding to **2** had been excluded as a possible reaction product intermediate because of its inhibitory action on this reaction.[18]

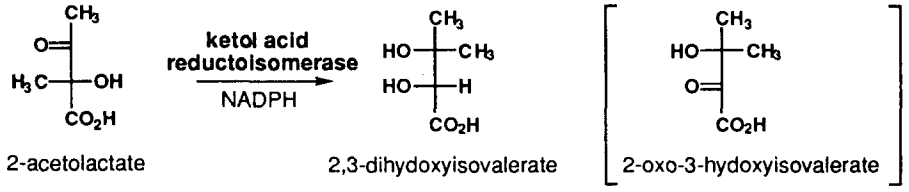

Figure 2. ketol acid reducoisomerase reaction

1 is a biosynthetic intermediate not only for IPP but also for thiamine and pyridoxol in *E. coli*.[21-23] Therefore, our result on the *in vitro* formation of **3** from **1** provides the first evidence that the reaction catalyzed by DXP reductoisomerase is the committed step in the IPP formation.

Acknowledgments. Mass spectra were collected by Dr. N. Morisaki of The Institute of Molecular and Cellular Biosciences, University of Tokyo. This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from The Ministry of Education, Science, Sports and Culture, Japan (09760114 to T. K.) and by Research for the Future Program from The Japan Society for the Promotion of Science (JSPS), JSPS-RFTF96100301 to H. S.

References and Notes

1. Flesch G, Rohmer M. Eur. J. Biochem. 1988;175:405-411.
2. Rohmer M, Knani M, Simonin P, Sutter B, Sahm H. Biochem. J. 1993;295:517-524.
3. Lichtenthaler HK, Schwender J, Disch A, Rohmer M. Biochem. J. 1996;316:73-80.
4. Seto H, Watanabe H, Furihata K. Tetrahedron Lett. 1996;37:7979-7982.
5. Rohmer M, Seeman M, Horbach S, Bringer-Meyer S, Sahm H. J. Am. Chem. Soc. 1996;118:2564-2566.

6. Duvold T, Bravo JM, Palc-Grosdemange C, Rohmer M. Tetrahedron Lett. 1997;38:4769-4772.
7. Duvold T, Cali P, Bravo JM, Rohmer M. Tetrahedron Lett. 1997;38:6181-6184.
8. Putra SR, Lois LM, Campos N, Boronat A, Rohmer M. Tetrahedron Lett. 1998;39:23-26.
9. 1-Deoxyxylulose[8] and 2-*C*-methylerythritol[7] were reported to be utilized by *E. coli* in place of **1** and **3**, respectively.
10. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A laboratory Manual (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed. 1989
11. Takahashi S, Kuzuyama T, Watanabe H, Seto H. to be published elsewhere.
12. Protein expression and purification of the recombinant DXP reductoisomerase. The coding region of the *E. coli* DXP reductoisomerase gene[11] was cloned into the expression vector pQE30 (Qiagen) to give pQEDXR. *E. coli* M15[24] containing pREP4 [*neo, lacI*] (Qiagen) was used as a host for expression of the DXP reductoisomerase gene. *E. coli* M15 (pREP4, pQEDXR) was cultured at 37 °C in 100 ml of Luria-Bertani medium[10] containing kanamycin and ampicillin for 5 hrs with 0.2 mM isopropylthiogalactoside 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 X g for 20 min and the supernatant was collected. A 50% slurry of Ni-NTA 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 binds to the Ni-NTA resin was eluted with 200 mM imidazole in 100 mM Tris-HCl (pH 8.0). The eluate was used as the purified DXP reductoisomerase in the subsequent experiments.
13. Enzymatic synthesis of 1-deoxy-D-xylulose 5-phosphate. The coding region of the *E. coli* DXP synthase gene[25] was cloned into the expression vector to give pQEDXS. *E. coli* M15 containing pREP4 was used as a host for expression of the DXP synthase gene. Protein expression and purification of the recombinant DXP synthase were done according to the identical procedures to those of DXP reductoisomerase. Production and purification of **1** were monitored at 195 nm by HPLC with a Senshu pak NH2-1251-N (4.6 X 250 mm) HPLC column (Senshu), eluted with 100 mM KH_2PO_4 (pH 3.5). The DXP synthase reaction was started by adding the enzyme solution at 37 °C for 12 hrs in 20 ml of 100 mM Tris-HCl (pH 7.5) containing 10 mM sodium pyruvate, 30 mM DL-glyceraldehyde 3-phosphate, 1.5 mM thiamine diphosphate, 10 mM $MgCl_2$ and 1 mM DL-dithiothreitol. The reaction mixture was diluted to 300 ml by adding H_2O and subjected to a column of activated carbon (2.2 X 8 cm). The passing fraction was applied to Dowex 1-X8 (Cl^- type, 3.5 X 25 cm) chromatography eluting with 1 % NaCl (500 ml). The eluate was concentrated and then chromatographed on Sephadex G-10 (1.8 X 100 cm) eluting with H_2O. The fractions containing **1** were freeze-dried to give a white powder of **1** as the disodium salt (*ca.* 50 mg). Then, the enzymatically synthesized **1** was analyzed by NMR spectroscopy. The chemical shifts and coupling constants of ^1H and ^{31}P NMR spectra (A-500 instrument, JEOL) of the enzymatic product were identical to those reported previously.[25]
14. ^1H NMR (D_2O, 500 MHz): δ4.03 (ddd, J = 11.5, 6.5, 2.5 Hz, 1H), 3.84 (ddd, J = 11.5, 8.0, 6.5 Hz, 1H), 3.78 (dd, J = 8.0, 2.5 Hz, 1H), 3.60 (d, J = 12.0 Hz, 1H), 3.50 (d, J = 12.0 Hz, 1H), 1.15 (s, 3H); ^{13}C NMR (D_2O, 125 MHz): δ75.1 (C-2), 74.8 (d, J = 6.5, C-3), 67.4 (C-1), 65.9 (d, J = 4.6, C-4), 19.4 (2-Me).
15. ^1H NMR (D_2O, 500 MHz): δ3.83 (dd, J = 11.5 and 2.5 Hz, 1H), 3.66 (dd, J = 8.5 and 2.5 Hz, 1H), 3.59 (dd, J = 11.5 and 8.5 Hz, 1H), 3.58 (d, J = 11.5 Hz, 1H), 3.47 (d, J = 11.5 Hz, 1H), 1.12 (s, 3H); ^{13}C NMR (D_2O, 125 MHz): δ76.1 (C-3), 75.2 (C-2), 67.4 (C-1), 63.1 (C-4), 19.5 (2-Me).
16. The optical rotation of the reaction product (2-*C*-methyl-D-erythritol 4-phosphate): $[\alpha]_D^{21}$ +6.4 (*c* 0.10, H_2O).
17. Umbarger HE, Brown B, Erying EJ. J. Biol. Chem. 1960;235:1425-1432.
18. Satyanarayana T, Radhakrishnan AN. Biochim. Biophys. Acta. 1965;110:380-388.
19. Kiritani K, Narise S. J. Biol. Chem. 1966;241:2047-2051.
20. Arfin SM, Umbarger HE. J. Biol. Chem. 1969;244:1118-1127.
21. Julliard JH, Douce R. Proc. Natl. Acad. Sci. 1991;88:2042-2045.
22. Himmeldirk K, Kennedy IA, Hill RE, Sayer BG, Spencer ID. Chem. Commun. 1996:1187-1188.
23. Hill RE, Sayer BG, Spencer ID. J. Am. Chem. Soc. 1989;111:1916-1917.
24. Zamenhof PJ, Villarejo M. J. Bacteriol. 1972;110:171-178.
25. Sprenger GA, Schorken U, Wiegert T, Grolle S, Graaf AA, Taylar SV, Begley TP, Bringer-Meyer S, Sahm H. Proc. Natl. Acad. Sci. 1997;94:12857-12862.