Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction studies of recombinant *Escherichia coli* 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthetase

Lauris E. Kemp, Charles S. Bond and William N. Hunter*

The Wellcome Trust Biocentre, University of Dundee, Dundee, DD1 5EH, Scotland

Correspondence e-mail: w.n.hunter@dundee.ac.uk

Received 11 April 2001
Accepted 19 June 2001

Diphosphocytidyl-methylerythritol (DPCME) synthetase is involved in the mevalonate-independent pathway of isoprenoid biosynthesis, where it catalyses the formation of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol from 2-*C*-methyl-D-erythritol 4-phosphate and CTP. The *Escherichia coli* enzyme has been cloned, expressed in high yield, purified and crystallized. Elongated tetragonal prismatic crystals grown by the hanging-drop vapour-diffusion method using polyethylene glycol (PEG) 4000 as the precipitant belong to space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = b = 73.60$, $c = 175.56$ Å. Diffraction data have been recorded to 2.4 Å resolution using synchrotron radiation.

1. Introduction

Isoprenoids are a diverse range of natural products including cholesterol, dolichols, triterpenes, ubiquinones and components of macromolecules such as the prenyl groups of prenylated proteins and isopentenylated tRNAs (Bochar *et al.*, 1999). Isoprenoids participate in varied and important biological functions such as electron transport or hormone-based signalling and also constitute components of membranes. The biosynthesis of this type of natural product depends on the condensation of a C5 precursor unit, isopentenyl diphosphate (IPP). In animals, fungi and certain bacteria IPP is synthesized through the mevalonate (MVA) pathway (Bach, 1995; Bloch, 1992; Qureshi & Porter, 1981). This begins with the conversion of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA, followed by reduction, phosphorylation and decarboxylation to generate IPP. IPP can be isomerized to dimethylallyl pyrophosphate; together, these compounds serve as the universal precursors of isoprenoids.

The MVA pathway was considered ubiquitous until the recent identification of an alternative isoprenoid biosynthetic route, the 1-deoxy-D-xylulose 5-phosphate (DOXP) or 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in plants, some bacteria and apicomplexans (Boucher & Doolittle, 2000; Eisenreich *et al.*, 1998; Lange *et al.*, 2000; Rohmer *et al.*, 1993). This mevalonate-independent pathway begins with the condensation of glyceraldehyde-3-phosphate and pyruvate to form DOXP, a reaction which is catalysed by the enzyme DOXP synthase (Lois *et al.*, 1998). The second step in the pathway consists of a reduction and isomerization catalysed by DOXP reductoisomerase (Takahashi *et al.*, 1998) to produce MEP.

The enzyme that follows the reductoisomerase in the pathway, DPCME synthetase, catalyses the addition of CTP to MEP to form 4-diphosphocytidyl-2-*C*-methylerythritol. The presence of this third activity was confirmed using radiolabelled assays both *in vitro* and *in vivo*, with the latter resulting in the radiolabelled 4-diphosphocytidyl-2-*C*-methylerythritol being incorporated into carotenoids in the chloroplasts of *Capsicum annum*, an observation consistent with the localization of the DOXP pathway to the plastid (Rohdich *et al.*, 1999). The first three enzymes of the pathway are well characterized and although a series of further reactions follow to eventually produce IPP, many details remain to be elucidated.

DOXP reductoisomerase has been shown to be inhibited by the antimicrobial compound fosmidomycin, which is efficacious in the treatment of rodent malaria (Jomaa *et al.*, 1999). Fosmidomycin has a short half-life in serum and is unsuitable as a human treatment; nevertheless, the study of Jomaa *et al.* (1999) has drawn attention to DOXP reductoisomerase and also to the DOXP pathway as a potential target for the development of more useful antimicrobial compounds (Ridley, 1999; Vial, 2000). Since the DOXP pathway is absent in humans and is found in many serious pathogens, the constituent enzymes offer the potential for therapeutic intervention. We have initiated a crystallographic study of DPCME synthetase to delineate structure–function relationships and to provide information in

© 2001 International Union of Crystallography
Printed in Denmark – all rights reserved

support of a structure-based inhibitor-design strategy to generate novel antimicrobial compounds.

2. Cloning, expression and purification of DPCME synthetas

The *E. coli ispd* gene that codes for DPCME synthetase was amplified from genomic DNA by a polymerase chain reaction (PCR) using the primers 5′-ACG-**CAT**-**ATG**-GCA-ACC-ACT-CAT-TTG-GAT-GTT-3′ and 5′-ACG-**CTC**-**GAG**-TTA-TGT-TAT-TCT-CCT-GAT-GGA-TGG-T-3′ (Sigma Genosys). The primers carried *Nde*I and *Xho*I restriction sites, respectively, which are in bold. The PCR product (726 bps) was gel purified (Qiagen) and cloned into the PCR4Blunt TOPO vector using the zero blunt TOPO PCR cloning system (Invitrogen). Positive clones were identified by restriction-enzyme digest and the resulting DNA fragment then ligated into a *Xho*I- and *Nde*I-digested pET15b expression vector (Novagen). The resulting pET15*b-ispd* construct was sequenced to confirm the integrity of the product.

E. coli strain BL21 (DE3) (Novagen) was heat-shock transformed with pET15b-*ispd* and selected on Luria–Bertani agar plates containing 0.1 mg ml^{-1} ampicillin. A single colony was cultured to an OD_{600} of approximately 1.00 in 1 l of LB Amp at 310 K, at which point 1 m*M* IPTG was added to induce protein expression and cell growth was allowed to continue overnight at room temperature.

The cells were harvested by centrifugation (2600*g*) at 277 K, resuspended in binding buffer (100 m*M* Tris–HCl pH 7.7, 100 m*M* NaCl) and, following addition of DNAseI and lysozyme, lysed using a French press. Insoluble debris was separated by centrifugation (35 000*g*) at 277 K for 25 min and the supernatant containing DPCME synthetase passed through a 0.2 µm syringe filter. The filtered supernatant was applied to a metal-chelating Hitrap column (Pharmacia) preloaded with nickel and equilibrated with binding buffer. The resin was washed with 25 m*M* bis-tris propane pH 7.7 and 10 m*M* imidazole to remove any non-specifically bound and histidine-rich proteins. DPCME synthetase was then recovered by an increasing imidazole gradient and eluted at a concentration of 120 m*M*. Fractions were analysed by SDS–PAGE and those containing DPCME synthetase were pooled and dialysed overnight against 100 m*M* Tris–HCl pH 7.7, 100 m*M* NaCl. The resulting protein concentration was measured, thrombin (Pharmacia) was added to a concentration of 6.7 units per milligram of synthetase and the mixture incubated overnight at room temperature. Following thrombin cleavage, the protein solution was filtered and applied to a Q-Sepharose anion-exchange column to remove the thrombin and any impurities which still remained. DPCME synthetase eluted from the Q-Sepharose column at 0.2 *M* NaCl. The resulting fractions were analysed by SDS–PAGE and those containing the synthetase were pooled and dialysed against 100 m*M* Tris–HCl pH 7.7, 100 m*M* NaCl and concentrated to approximately 9 mg ml^{-1}. The purity of the sample was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

3. Crystallization

Crystallization was achieved using the vapour-diffusion hanging-drop method. Initial trials were conducted at 293 and 277 K with Crystal Screens I and II (Hampton Research), with drops assembled using either 3 µl protein solution (9 mg ml^{-1}) and 1 µl reservoir or 2 µl protein solution and 2 µl reservoir. Needles which diffracted poorly on a rotating-anode X-ray source were obtained. These conditions were subsequently refined testing pH and the use of additives; this produced a marked improvement in crystal size and morphology. The final reservoir used contained 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 5.6 and 25% PEG 4000. The drops typically consisted of 3 µl protein solution, 0.4 µl 30%(*w*/*v*) 1,5-diaminopentane dihydrochloride and 0.6 µl reservoir. Well ordered blocks grew over a period of 4 d at 293 K (Fig. 1).

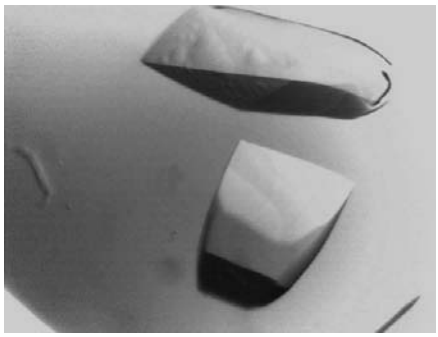

Figure 1
Typical crystals of DPCME synthetase, which attain dimensions of 0.1 × 0.1 × 0.5 mm.

4. X-ray analysis

A crystal was cryopreserved by soaking in substituted mother liquor (0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate, 25% PEG 4000 and 18% 2-methyl-2,4-pentanediol) for 10 s then maintained at 100 K in a stream of nitrogen gas. Data were collected to a resolution of 2.4 Å at beamline BM14 at the European Synchrotron Research Facility (ESRF), Grenoble, France. Diffraction data were processed using the *DENZO*/*SCALEPACK* programs (Otwinowski & Minor, 1997) and details are given in Table 1. The space group has been identified as $P4_12_12$ or entantiomer, with unit-cell parameters $a = b = 73.60$, $c = 175.56$ Å. DPCME synthetase functions as a homodimer with a subunit of 236 amino acids and a molecular mass of 25.7 kDa (Rohdich *et al.*, 1999). One molecule per asymmetric unit corresponds to a Matthews coefficient (Matthews, 1968) of 4.6 Å^3 Da^{-1} (solvent content 73%), whilst two molecules in the asymmetric unit corresponds to a coefficient of 2.23 Å^3 Da^{-1} (solvent content 46%). The lack of any identifiable peaks representing non-crystallographic symmetry in several self-rotation function calculations (*POLARRFN*; Collaborative Computational Project, Number 4, 1994) suggests the presence of only a single molecule per asymmetric unit. This is, however, not definitive and will be confirmed once the structure is solved. Since there are no homologous protein structures available from the Protein Data Bank, experimental phase information will now be sought to determine this structure.

Table 1
Data-collection and processing statistics.

Values in parentheses refer to the highest resolution shell, 2.46–2.40 Å.

X-ray source	ESRF-BM14
Detector	MAR CCD
Wavelength (Å)	0.97
Mosaicity (°)	0.46
No. of measured reflections	81222
No. of unique reflections	19619
Resolution range (Å)	30–2.4
Completeness (%)	99.5 (94.7)
R_{sym}	0.03 (0.27)
Average $I/\sigma(I)$	37 (3)

In summary, we have obtained crystals of *E. coli* DPCME synthetase, the third enzyme in the DOXP pathway of isoprenoid biosynthesis. The crystals are well ordered and diffract to 2.4 Å resolution using a synchrotron X-ray source and will facilitate a complete structural analysis to complement ongoing biochemical studies.

Funded by the Engineering and Physical Sciences Research Council (UK) and the Wellcome Trust. We thank the ESRF for

beamtime and Dr Gordon Leonard for support on BM14.

References

Bach, T. J. (1995). *Lipids*, **30**, 191–202.

Bloch, K. (1992). *Steroids*, **57**, 378–382.

Bochar, D. A., Friesen, J. A., Stauffacher, C. V. & Rodwell, V. W. (1999). *Comprehensive Natural Product Chemistry*, edited by D. Barton & K. Nakanishi, Vol. 2, pp. 15–44. Oxford: Pergamon Press.

Boucher, Y. & Doolittle, W. F. (2000). *Mol. Microbiol.* **37**, 703–716.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M. H. & Bacher, A. (1998). *Chem. Biol.* **5**, R221–R233.

Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Turbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H. K., Soldati, D. & Beck, E. (1999). *Science*, **285**, 1573–1575.

Lange, B. M., Rujan, T., Martin, W. & Croteau, R. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 13172–13177.

Lois, L. M., Campos, N., Putra, S. R., Danielson, K., Rohmer, M. & Boronat, A. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 2105–2110.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Qureshi, N. & Porter, J. W. (1981). *Biosynthesis of Isoprenoid Compounds*, edited by J. W. Porter & S. L. Spurgeon, Vol. 1, pp. 47–94. New York: John Wiley & Sons.

Ridley, R. G. (1999). *Science*, **285**, 1502–1503.

Rohdich, F., Wungsintaweekul, J., Fellermeier, M., Sagner, S., Herz, S., Kis, K., Eisenreich, W., Bacher, A. & Zenk, M. H. (1999). *Proc. Natl Acad. Sci. USA*, **96**, 11758–11763.

Rohmer, M., Knani, M., Simonin, P., Sutter, B. & Sahm, H. (1993). *Biochem. J.* **295**, 517–524.

Takahashi, S., Kuzuyama, T., Watanabe, H. & Seto, H. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 9879–84.

Vial, H. J. (2000). *Parasitol. Today,* **16**, 140–141.