Enzymes of the Alternative Terpenoid Pathway

in *Escherichia coli*

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ABBREVIATIONS

APS	ammonium persulfate
ATP	adenosine 5´-triphosphate
BSA	bovine serum albumin
CDP	cytidine 5´-diphosphate
CMP	cytidine 5´-monophosphate
CoA	coenzyme A
cpm	counts per minute
CTP	cytidine 5´-triphosphate
Da	dalton
DEAE	diethylaminoethyl
DMAPP	dimethylallyl diphosphate
dpm	disintegrations per minute
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fig.	figure
FPLC	fast protein liquid chromatography
GSH	glutathione (reduced form)
GTP	guanosine 5´-triphosphate
h	hour
HPLC	high-pressure liquid chromatography
IMAC	immobilized metal affinity chromatography
IMP	isopentenyl monophosphate
IPP	isopentenyl diphosphate
IPTG	isopropyl-β-D-thiogalactoside
ITP	inosine 5´-triphosphate
IspD (YgbP)	4-diphosphocytidyl-2C-methyl-D-erythritol synthase
IspE (YchB)	4-diphosphocytidyl-2C-methyl-D-erythritol kinase
IspF (YgbB)	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
min	minute

MWCO	molecular weight cut off
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
NMR	nuclear magnetic resonance
PC	paper chromatography
PMSF	phenylmethanesulfonyl fluoride
R_f	retention factor
RP	reverse phase
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBAS	tetrabutylammonium sulfate
TEMED	N,N,N´,N´-tetramethylethylenediamine
TLC	thin layer chromatography
Tris	tris-(hydroxymethyl)aminomethane
UTP	uridine 5´-triphosphate
UV	ultraviolet

1. INTRODUCTION : Isoprenoid Biosynthesis

Isoprenoids are a large family of natural products with important representatives in all taxonomic groups. More than 30,000 structurally different terpenoids have been described (Connolly and Hill 1992). Terpenoids have a wide variety of biological functions and many have potential medicinal applications. All isoprenoids share a common feature: they are derived biosynthetically from 5-carbon compound, namely, isopentenyl diphosphate and dimethylallyl diphosphate. The biosynthesis of isopentenyl diphosphate was investigated by many research groups. They demonstrated that at least two different pathways for the biosynthesis of isopentenyl diphosphate exist, namely, the mevalonate pathway and the alternative deoxyxylulose phosphate pathway.

1.1 THE MEVALONATE PATHWAY

The formation of isopentenyl diphosphate via the mevalonate pathway has been documented exclusively. The pioneering work of Bloch, Lynen, Cornforth, and many others allowed an understanding of how living cells synthesize their isoprenoids from acetate (for review see Spurgeon and Porter, 1981). This pathway starts from acetyl-CoA as precursor. Sequentially, two molecules of acetyl-CoA (**1**, Fig. 1.1) are condensed to acetoacetyl-CoA (**2**) by thiolase. The latter compound then is condensed with another molecule of acetyl-CoA to HMG-CoA (**3**) by HMG-CoA synthase. In a subsequent step, HMG-CoA is reduced to mevalonic acid (**4**) in the presence of NADPH by HMG-CoA reductase. This enzyme attracted and great interest since it catalyzes the rate-limiting step in mevalonate pathway thus became a key target for interruption of cholesterol biosynthesis. The series of HMG-CoA reductase inhibitors such as mevastatin, lovastatin (mevinolin), simvastatin and pravastatin are highly effective hypocholesterolemic agents and are therefore of a great value in treating patients with high risk of coronary disease.
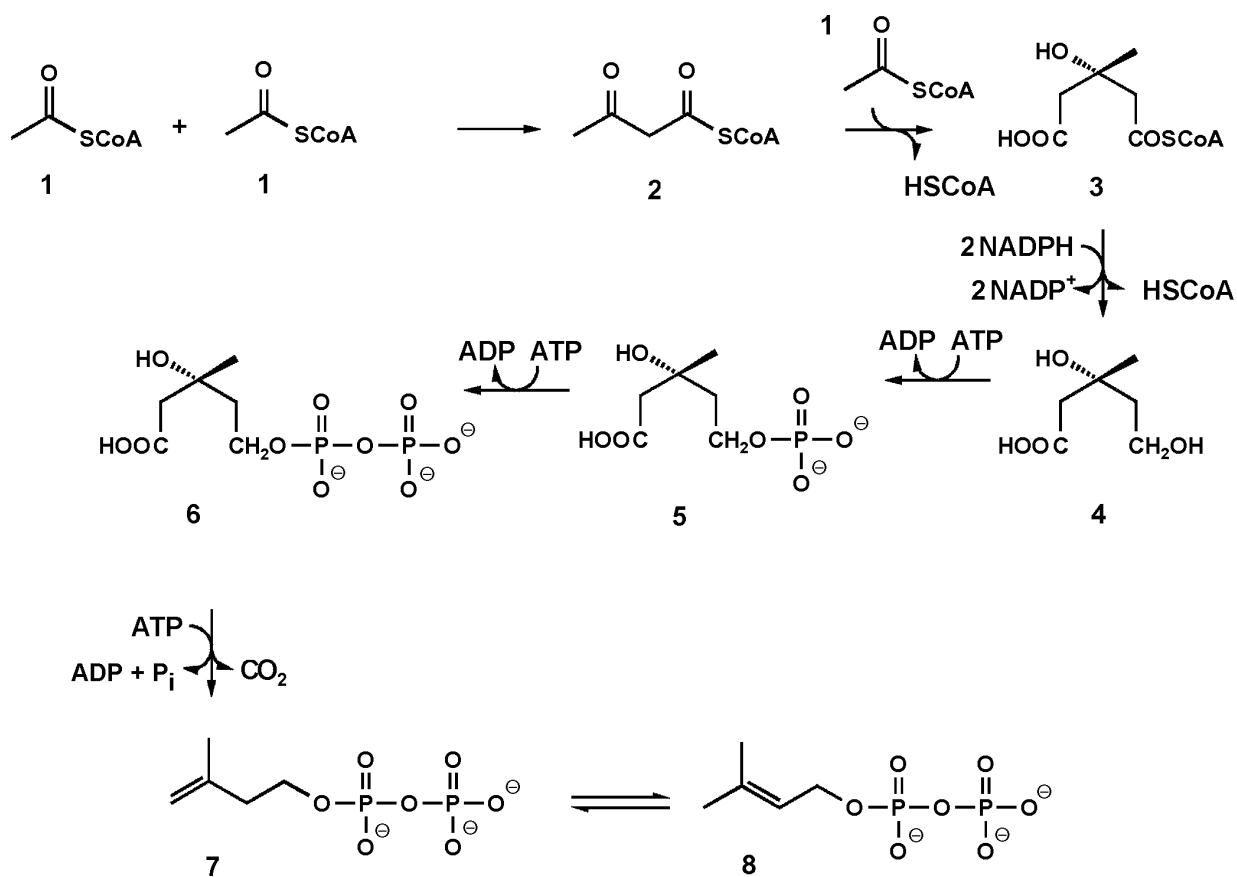
Figure 1.1   The mevalonate pathway

The six-carbon compound mevalonic acid (**4**) is phosphorylated twice to form mevalonic 5-diphosphate (**6**) via the monophosphate (**5**). Mevalonic 5-diphosphate is then decarboxylated and dehydrated to form isopentenyl diphosphate (**7**). These reaction steps are catalyzed by mevalonate kinase, mevalonate 5-phosphate kinase and mevalonate 5-diphosphate decarboxylase. Isopentenyl diphosphate is isomerized to dimethylallyl diphosphate (**8**) by the catalytic action of isopentenyl diphosphate isomerase. All of these enzymes are localized in the cytosolic compartment, with the exception of HMG-CoA reductase, which is located in the endoplasmic reticulum (in liver) and in mitochondria (in yeast) (Qureshi and Porter 1981, Spurgeon and Porter 1981, Bloch 1992).

Evidence for the existence of an alternative isoprenoid biosynthetic pathway emerged from independent incorporation studies in the research groups of Rohmer and Arigoni who found that the isotopic labeling patterns observed in their studies could not be explained in terms of the mevalonate pathway (for reviews see Eisenreich et al. 1998, Lichtenthaler 1999, Rohmer 1999).

Different ^{13}C-labelled glucose samples were fed to *Escherichia coli*, *Zymomonas mobilis*, *Methylobacterium fujisawaense*, *Alicyclobacillus acidoterrestris* and *Ginkgo biloba* (Broers 1994, Schwarz 1994, Rohmer et al. 1993, 1996). Terpenoids were isolated, and the labeling patterns were elucidated by NMR experiments. The data independently showed that these terpenoids were not formed via the mevalonate route. This discovery was the starting point for re-investigating plant and microbial isoprenoid biosynthesis. Various labeled precursors such as acetate and glucose were studied in *Scenedesmus obliquus*, *Lemna gibba*, *Hordeum vulgare*, *Daucus carota* and cell cultures of *Taxus chinensis* and *Mentha x piperita* (Schwender et al. 1996, Lichtenthaler et al. 1997, Eisenreich et al. 1996, 1997). These results show that green algae and plants possess two biosynthetic routes. Notably, cytoplasmic sterols were formed via the mevalonate pathway, whereas isoprenoids formed in chloroplast were synthesized via an alternative pathway.

1.2 THE DEOXYXYLULOSE PHOSPHATE PATHWAY
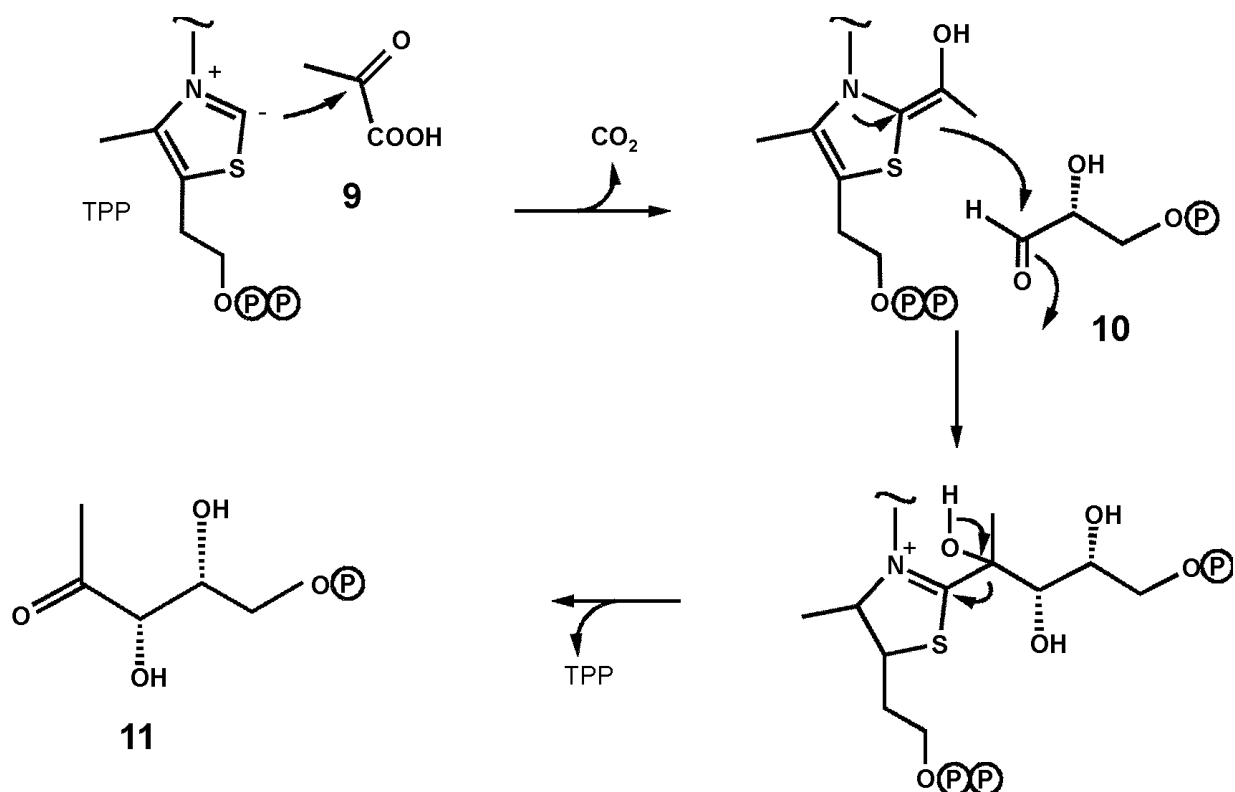
Figure 1.2   Formation of 1-deoxyxylulose 5-phosphate

Arigoni's group suggested a head-to-head condensation of glyceraldehyde 3-phosphate (**10**, Fig. 1.2) and „activated acetaldehyde" generated from pyruvate (**9**) by a thiamin-diphosphate-dependent decarboxylation, yielding 1-deoxy-D-xylulose or its 5-phosphate as the first intermediate (**11**) (Broers 1994, Schwarz 1994). Evidence for this hypothesis was the incorporation of 1-deoxy-D-xylulose into the isoprenoid side chain of ubiquinone in *E. coli*, into ginkgolides of *Ginkgo biloba* and into ferruginol of *Salvia miltiorrhiza* (Broers 1994, Schwarz 1994). Subsequent studies in different research groups confirmed the incorporation of isotope-labeled 1-deoxy-D-xylulose into terpenoids in green cell culture of *Catharanthus roseus*, in the plants *Populus nigra*, *Chelidonium majus*, *Salix viminalis*, *Lemna gibba*, *Liriodendron tulipifera*, and the algae *Cyanidium caldarium*, *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* (Arigoni et al. 1997, Schwender et al. 1997, Putra et al. 1998, Sagner et al. 1998). Notably, 1-deoxy-D-xylulose had already been shown to be a precursor for the biosynthesis of thiamin and pyridoxal (Himmeldirk et al. 1996).

An intramolecular rearrangement reaction was expected to occur with 1-deoxy-D-xylulose 5-phosphate (Arigoni et al. 1997, Duvold et al. 1997, Sagner et al. 1998). The rearrangement step via breaking the C-3–C-4 bond of the 1-deoxy-D-xylulose skeleton (**12**, Fig. 1.3) and closure of a new bond between C-2 and C-4 result in the formation of 2*C*-methyl-D-erythrose 4-phosphate (**13**). The latter intermediate is then reduced yielding the branched chain polyol 2*C*-methyl-D-erythritol 4-phosphate (**14**) (Kuzuyama et al.1998b). From a structural point of view, further transformation of 2*C*-methyl-D-erythritol 4-phosphate into isopentenyl diphosphate must involve two reductions, two dehydrations and at least one phosphorylation step.
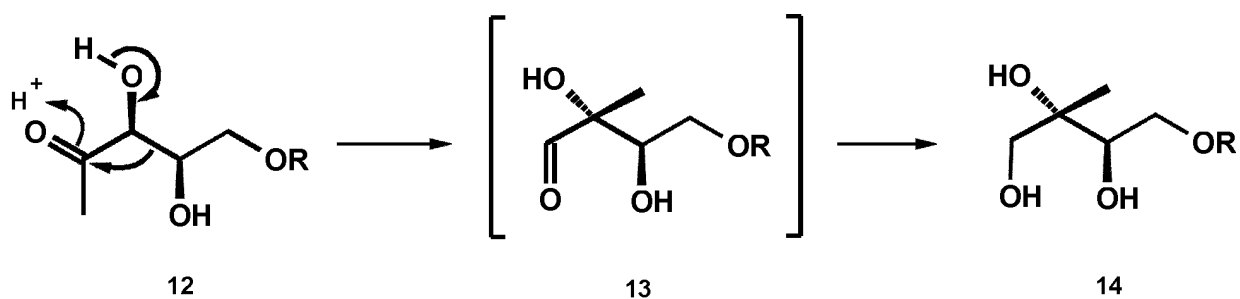
Figure 1.3   The proposed mechanism of 1-deoxy-D-xylulose skeletal rearrangement

1.2.1 1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE (DXS)

The enzyme that catalyzes the formation of 1-deoxy-D-xylulose 5-phosphate (**17**, Fig 1.4) from pyruvate (**15**) and glyceraldehyde 3-phosphate (**16**) was first identified in *E. coli* (Sprenger et al. 1997). The cognate *dxs* gene from *E. coli* was cloned and the corresponding gene product, 1-deoxy-D-xylulose 5-phosphate synthase was hyperexpressed, purified and characterized in some detail (Sprenger et al. 1997, Lois et al. 1998). Later, 1-deoxy-D-xylulose 5-phosphate synthases were also cloned from *Capsicum annuum* (Bouvier et al. 1998), *Mentha x piperita* (Lange et al. 1998), *Synechococcus leopoliensis* (Miller et al. 1999), *Bacillus subtilis*, *Synechocystis sp*. (Harker and Bramley 1999), *Streptomyces sp*. (Kuzuyama et al. 2000b) and *Pseudomonas aeruginosa* (Altincicek et al. 2000).

The enzyme requires thiamin diphosphate (TPP) as cofactor. 1-Deoxy-D-xylulose 5-phosphate synthase from *E. coli* is a homodimer consisting of 2 subunits of 65 kDa. Interestingly, the N-terminal amino acid sequences of 1-deoxy-D-xylulose 5-phosphate from *M. piperita*, and *Arabidopsis thaliana* carry plastid targeting sequences. Translocation into chloroplasts was shown for 1-deoxy-D-xylulose 5-phosphate synthases of *A. thaliana* (Estevez et al. 2000) and *Lycopersicon esculentum* (Lois et al. 2000). 1-Deoxy-D-xylulose 5-phosphate synthase from *E. coli* and *P. aeruginosa* were inhibited by fluoropyruvate with an IC_{50} of 80 µM and 400 µM, respectively (Altincicek et al. 2000). Fluoropyruvate is supposed to bind covalently to the active site of 1-deoxy-D- xylulose 5-phosphate synthase as already demonstrated for the pyruvate dehydrogenase component (E1) in the pyruvate dehydrogenase complex (Altincicek et al. 2000, Flournoy and Frey 1989).

1.2.2 1-DEOXY-D-XYLULOSE 5-PHOSPHATE REDUCTOISOMERASE (DXR)

Studies with *E. coli* mutants which were blocked in the biosynthesis of 2*C*-methyl-D-erythritol 4-phosphate revealed 1-deoxy-D-xylulose 5-phosphate reductoisomerase specified by *yaeM* gene (designated now as *dxr*) (Takahashi et al. 1998, Kuzuyama et al. 1998b). The recombinant Dxr gene product of *E. coli* was shown to catalyze the intramolecular rearrangement and reduction of 1-deoxy-D-xylulose 5-phosphate (**17**) yielding 2*C*-methyl-D-erythritol 4-phosphate (**19**). Based on its catalytic activity, this enzyme was named 1-deoxy-D-

xylulose 5-phosphate reductoisomerase. It requires divalent cations, preferably Mn^{2+} or Mg^{2+},

and   accepts
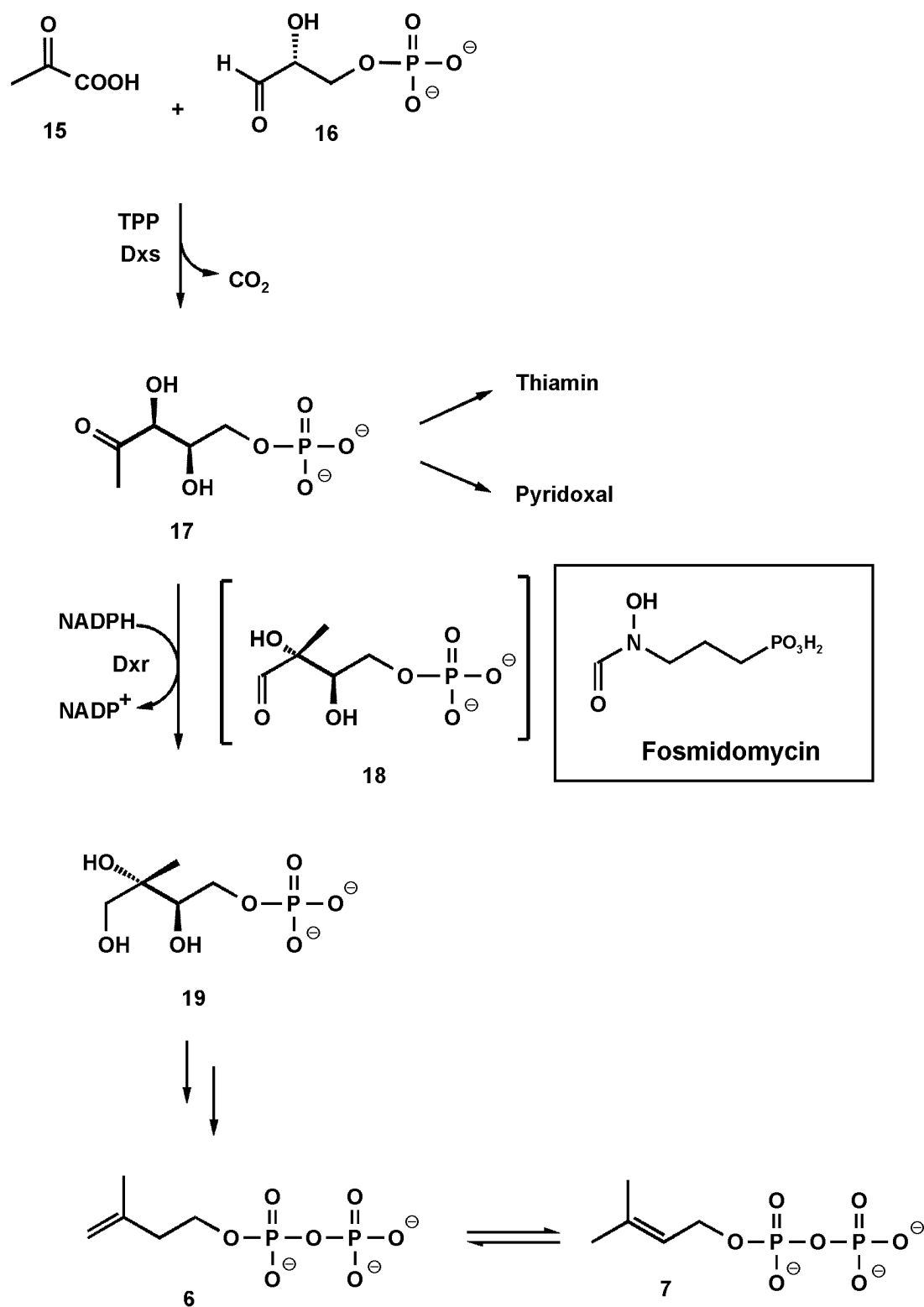
Figure 1.4   The deoxyxylulose phosphate pathway : the early steps of isopentenyl diphosphate biosynthesis

1. INTRODUCTION

only NADPH as electron donor. Only the phosphorylated 1-deoxy-D-xylulose is utilized as substrate. Recently, 1-deoxy-D-xylulose 5-phosphate reductoisomerases were also cloned from *Mentha x piperita* (Lange and Croteau 1999a), *Arabidopsis thaliana* (Schwender et al. 1999), *Synechocystis sp.* (Proteau et al. 1999), *Synechococcus leopoliensis* (Miller et al. 2000), *Plasmodium falciparum* (Jomaa et al. 1999), *Zymomonas mobilis* (Charon et al. 1999, Grolle et al. 2000) and *Pseudomonas aeruginosa* (Altincicek et al. 2000). Deduced amino acid sequences of plant 1-deoxy-D-xylulose 5-phosphate reductoisomerases carry plastid leader sequences which is in line with the localization on the deoxyxylulose phosphate pathway in the plastids.

Fosmidomycin was reported as a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase. It is a structural analogue of 2*C*-methyl-D-erythrose 4-phosphate (**4**). It inhibited the *E. coli* enzyme with a K_I value of 38 nM and it was shown to be a mixed-type (competitive and noncompetitive) inhibitor (Kuzuyama et al. 1998a). In a mouse malaria model, fosmidomycin administered orally or intraperitoneally after infection prevented parasitemia (Jomaa et al. 1999). Therefore, fosmidomycin is not only a potential antibiotic and herbicide but is also a candidate for treatment of human malaria (for review see Lichtenthaler 1999, 2000).

This study investigated novel enzymes which are involved in „the alternative terpenoid pathway“. Recombinant enzymes from *Escherichia coli* were purified to homogeneity by techniques of column chromatography. These enzymes were characterized in some detail and were also used for the large scale-up synthesis of new intermediates. The information about enzyme properties obtained by this study will be useful for protein structural determination and will also benefit the development of new enzyme inhibitors for the purpose of medicinal applications and plant biotechnology.

2   MATERIALS AND METHODS

2.1   INSTRUMENTS

FPLC instrumentation (Amersham Pharmacia Biotech, Freiburg, Germany)

      Columns for FPLC System

      Conductivity Monitor

      FRAC-100 Fraction Collector

      LCC-500 PLUS Controller

      MV-7 Motor Valve

      P-500 Pump

      REC100 Recorder

      Superloop 10 ml

      UV-1 Monitor

HPLC instrumentation

Knauer HPLC system (Zehlendorf, Berlin, Germany) with ChromGate^{TM} software

      Diode array photometer (J&M TIDAS, Aalen, Germany)

      Interface Box WellChrom

      K-1001 WellChrom HPLC Pump

      K-2600 WellChrom Spectrophotometer

      Radiomonitor β-RAM Flow-Through System Model 2 (Biostep GmbH, Jahnsdorf, Germany) with solid cell (CaF or YtSi)

      Refractometer Optical unit (Optilab Multiref 902B)

Radioactive instrumentation

      Automatic TLC-Linear Analyzer Tracemaster 20 (Berthold, Wildbad, Germany)

      Liquid Scintillation Counter (Beckman LS 7800, Fullerton, USA)

      PhosphorImager : Strom860 (Molecular Dynamics, Sunnyvale, CA, USA) with Kodak Identifying Screen (Molecular Dynamics, Sunnyvale, CA, USA)

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Additional equipment

Centrifuge : A14 LabTop Microcentrifuge (Jouan, Unterhaching, Germany), Servall Superspeed RC-2B and RC5B Plus with rotors GS3, GSA and SS34 (Dupont Instruments, Bad Homburg, Germany), GS-15R Refrigerated centrifuge with rotor S4180 and F2402 (Beckman , Fullerton, USA)

Electrophoresis : SE 250 Mighty Small II (Hoefer Scientific, San Francisco, USA)

Power supply : PHERO-stab. 200 (Reiskirchen, Germany), Biocapt camera with software LTF Labortechnik (Wasserburg, Germany)

Heatblock : Techne DRI.BLOCK DB-2A (Wertheim, Germany)

Incubator: Thermostat 340 (Eppendorf, Hamburg, Germany)

Lyophylizer : CHRIST ALPHA 1-4 (Osterode am Harz, Germany)

NMR : AVANCE DRX500 Spectrometer (Bruker, Karlstruhe, Germany)

pH Meter : E512 Metrohm AG CH-9100 (Herisau, Switzerland)

Protein concentrator : Amicon (Witten, Germany), PALL Filtron (Dreieich, Germany)

Shaker : Lab Shaker and Certomat MO (B.Braun, Melsungen, Germany)

Spectrophotometer : Ultrospec 2000 (Amersham Pharmacia Biotech, Freiburg, Germany) equipped with control temperature holder

Ultrasonicator : Sonifier B-12 (Branson SONIC Power Company, USA)

2.2   MATERIALS

2.2.1   Chemicals

All chemical substances were purchased from Merck (Darmstadt), Sigma (Deisenhofen), Serva (Heidelberg), Aldrich (Steinheim), Boehringer Mannheim (Mannheim), Gibco-BRL (Eggenstein), Biomol (Hamburg), and Fluka (Neu-Ulm) as shown in Table 1 (Appendix).

2.2.2   Substrates and cofactors

Substrates for enzyme activity tests were synthesized and kindly provided from the laboratory of Prof. Dr. M.H. Zenk, Biozentrum-Pharmazie, Universität Halle, Halle/Saale, Germany and the laboratory of Prof. Dr. A. Bacher, Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Garching, Germany as listed in Table 2 (Appendix).

2.2.3   Enzymes

Commercial available enzymes were purchased from Sigma (Deisenhofen, Germany), Boehringer-Mannheim (Mannheim, Germany). Enzymes used in this study were also purified from recombinant strains (Table 2.2). All enzymes are listed in Table 3 (Appendix).

2.2.4   Chromatographic materials

FPLC	Sepharose Q FF, Phenyl Sepharose 6FF, Source 15 Q, Superdex 200 HR 26/60, Superdex 75 HR 26/60, Resource PHE, Mono Q HR5/5, Red Sepharose CL-6B, Chelating Sepharose FF (Amersham Pharmacia Biotech, Freiburg, Germany.)
	Cibacron Blue 3GA type 3000, Reactive Yellow 86, Reactive Green 19 (Sigma, Deisenhofen, Germany)
	Macro-Prep Hydroxyapatite, type I, 40 µm (Biorad, Munich, Germany)
HPLC	Rezex RPM Monosaccharide (Phenomenex, Hösbach, Germany)
	Nucleosil 10 SB, Nucleosil 5 SB (Macherey Nagel, Düren, Germany)
	Multospher 120 RP18-5 (CS: Chromatography Service, Langerwehe, Germany)
TLC	POLYGRAM® CEL 300 PEI, POLYGRAM® Sil N-HR/UV$_{254}$ (Macherey Nagel, Düren, Germany)
	Silica gel 60 F$_{254}$ (Merck, Darmstadt, Germany)
PC	Whatman No.3 (Whatman, Springfield Mill, England)

2.2.5   Buffers and solutions

Protein determination

Bradford reagent	0.1 g of Serva Blue G was dissolved in 10 ml of ethanol and 25 ml of H_3PO_4, stirred thoroughly for 10 min. The mixture was adjusted to a volume of 250 ml with distilled water, filtered and stored in a dark colored bottle at room temperature.
BSA solution	10 mg of Bovine Serum Albumin (BSA) was dissolved in distilled water. The solution was adjusted to a volume of 100 ml with distilled water, using as protein standard solution.

2. MATERIALS AND METHODS

Protein purification

Buffer A	50 mM Tris hydrochloride, pH 7.4
	5 mM $MgCl_2$
	1 mM DTE
	0.02 % NaN_3
Buffer B	100 mM Tris hydrochloride, pH 8.0
	1 mM DTE
	0.02 % NaN_3
Buffer C	50 mM Tris hydrochloride, pH 7.4
	1 mM $MnCl_2$
	1 mM DTE
	0.02 % NaN_3
Buffer D	100 mM Tris hydrochloride, pH 8.0
	0.5 M NaCl
Buffer E	100 mM Tris hydrochloride, pH 8.0
	5 mM DTE
	0.02% NaN_3
Buffer F	50 mM Potassium Phosphate, pH 7.0
	0.5 M NaCl
Buffer G	20 mM Potassium phosphate buffer, pH 7.0
	1 mM DTE
	0.02 % NaN_3

Radioactivity determination

Fluid scintillation reagent	5.5 g Permablend® (Hewlett Packard)
	180 ml 2-Phenyl ethylamine
	250 ml Distilled methanol
	570 ml Toluene

SDS-Polyacrylamide gel electrophoresis

Acrylamide Solution 40%	38.9 g Acrylamide
	1.2 g Bis-acrylamide

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	The volume was adjusted with distilled water to 100 ml
Coomassie blue staining	2.5 g Coomassie-Blue R-250
	454 ml Methanol
	92 ml Acetic acid
Destaining solution	250 ml Methanol
	100 ml Acetic acid
	650 ml H_2O
Stacking gel buffer	0.25 M Tris hydrochloride, pH 6.8
	0.2 % SDS
SDS-PAGE running buffer	192 mM Glycine
	25 mM Tris hydrochloride, pH 8.3
	0.1 % SDS
SDS-PAGE sample buffer	60 mM Tris hydrochloride, pH 6.8
	5% SDS
	3% 2-Mercaptoethanol
	30% Glycerin
	0.02% Bromphenol Blue
	10% Saccharose
Separating gel buffer	1.5 M Tris hydrochloride, pH 8.8
	0.4 % SDS

Thin layer chromatography

Anisaldehyde/H_2SO_4	0.5 ml Anisaldehyde
	10 ml Acetic Acid
	5 ml Sulfuric Acid (conc.)
	85 ml Methanol

2.2.6   Culture Media

The components of media were dissolved in deionized water. The medium was sterilized by autoclaving (121 ^{o}C, 1.3 bar, 25 min). The medium was cooled to a temperature about 50 ^{o}C. Supplements were added as defined.

2.2.6.1   Medium for *Escherichia coli* DH5**a**

The medium for growing of *Escherichia coli* DH5α contained per l : 7.0 g Na_2HPO_4 x 2 H_2O, 4.5 g of anhydrous KH_2PO_4, 1.0 g of $MgSO_4$ x 7 H_2O, 1.0 g of NH_4Cl, 2.0 g of casein hydrolysate, 30 mg of $CaCl_2$, 10 ml of glycerol 99.5 % and 1.0 mg of thiamin hydrochloride.

2.2.6.2   Complete medium

LB-medium (Luria-Bertani) contained per l : 10 g of casein hydrolysate, 5 g of yeast extract and 5 g of NaCl.

LB amp contained per l : 180 mg ampicillin trihydrate, dissolved in 1 ml of sterilized water and added to 1 l of sterilized LB-medium.

LB amp/kan contained per l : 180 mg ampicillin trihydrate and 50 mg kanamycin, dissolved in 1 ml of sterilized water and added to 1 l of sterilized LB-medium.

2.3   *Escherichia coli* Strains

2.3.1   Wild type strains

Table 2.1   *E. coli* strains used in this study.

Strain	Genotype	Reference
DH5α	*F', endA1, hadR17($R_K^- m_K^+$), supE44, thi-1, recA1, gyrA(NaI$^+$)relA1**D**(lacZYA-argF)U169,deoR(**f**80dlac**D**(lacz)M15)*	DSM[a]
XL1-Blue	*RecA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac*, [F´, *proAB, lacI^{q}Z**D**M15, Tn10 (tet^r)*]	Stratagene, La Jolla, USA
M15[pREP4]	*Lac, ara, gal, mtl, recA$^+$, uvr$^+$,* [pREP4, *lacI, kan^r*]	Zamenhof, P.J. & Villarejo, M. (1972)

[a]Deutsche Stammsammlung von Mikroorganismen, Braun-schweig

2.3.2 Recombinant strains

Table 2.2   Recombinant strains from *E. coli* used in this study

Strain	Enzyme name	Source
XL1-pNCO-xylB	D-Xylulokinase	Dr. S. Herz, TU, München
XL1-pNCO-ychB	4-Diphosphocytidyl-2*C*-methyl-D-erythritol kinase	Dr. F. Rohdich, TU, München
XL1-pNCO-ygbP	4-Diphosphocytidyl-2*C*-methyl-D-erythritol synthase	Dr. F. Rohdich, TU, München
XL1-pQE-dxr	1-Deoxy-D-xylulose 5-phosphate reductoisomerase	Dr. F. Rohdich, TU, München
XL1-pQE-ygbB	2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	Dr. S. Herz, TU, München

2.4   METHODS

2.4.1   Culture conditions

2.4.1.1   Wild type *E. coli* cells

The suspension culture of *E. coli* DH5α was prepared by inoculation of *E. coli* cells from agar plate into medium (2.2.6.1). The suspension culture was stirred at 37 $^{\circ}$C (200 rpm) overnight. The overnight culture was subcultured to flasks (5 x 0.8 l) in the same medium. The cells were grown at 37 $^{\circ}$C (200 rpm) for 5 hr. The grown medium was transferred to the fermenter in 20 l of medium. *E. coli* strain DH5α was grown with stirring (100 rpm) and aeration (4 m^3 min^{-1}) in 200 l of medium. Cells were harvested by centrifugation, washed in saline (0.9% NaCl) and stored at –20 $^{\circ}$C. The yield of cells was 3 g wet weight per liter.

2.4.1.2   Recombinant *E. coli* strains

The recombinant *E. coli* strains were stored at –70 $^{\circ}$C in LB-medium containing 30 % glycerol. Overnight cultures were prepared in LB-amp/LB-amp/kan as appropriate. The *E. coli* cells were grown overnight (37 $^{\circ}$C, 200 rpm). The overnight culture was transferred to the same

medium at a ratio 1:50. The cells were incubated at 37 $^{\circ}$C by shaking (200 rpm). Cell growth was monitored by measuring the optical density at 600 nm (OD$_{600}$).

2.4.2   Proteinchemical methods

2.4.2.1   Expression of protein from recombinant *E. coli* strain

The genes of interest were cloned and overexpressed in the high copy vectors pNCO113 or pQE30 (Qiagen) under the control of a T5 promoter and a *lac* operator in *E. coli* host strains. The T5 promoter is a strong promoter which is recognized by the RNA polymerase of *E. coli*. Expression of recombinant proteins encoded by these vectors is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and inactivates it. Once the *lac* repressor is inactivated, the host cell's RNA polymerase can transcribe the sequences downstream from the promoter. The *E. coli* host cells strain M15[pREP4] contain multiple copies of the plasmid pREP4 which confers kanamycin resistance and carries the *lacI* gene (encoding the lac repressor). The *E. coli* host strain K12 XL1-Blue contain a mutated *lacI* gene (*lacI^q*) (encoding the lac repressor).

For overexpression, the recombinant *E. coli* cells were grown as described under 2.4.1.2. At an OD$_{600}$ of 0.6-0.7, IPTG was added to a final concentration of 2 mM. The cells were further grown with shaking at 37 $^{\circ}$C, 5 h, 200 rpm. The cells were harvested by centrifugation (rotor GS3, 5000 rpm, 4 $^{\circ}$C, 20 min), washed with extraction buffer and stored at –20 $^{\circ}$C.

2.4.2.2   Cell extraction

2.4.2.2.1   Extraction of *E. coli* DH5**a** cells

E. coli DH5α cells (400 g) were suspended in 1.5 l of buffer A. Lysozyme (1 mg per g *E. coli* cells) and DNaseI (0.1 mg per g of *E. coli* cells) were added. The mixture was stirred and incubated at 37 $^{\circ}$C for 30 min. Ultrasonication was performed (70 % duty cycle, output 4, 10 x 1 min, 1 min pause) during cooling on ice. Cell debris were centrifuged (rotor GSA, 13,000 rpm, 4 $^{\circ}$C, 1 h). The supernatant was used as cell extract.

2.4.2.2.2   Extraction of recombinant *E. coli* cells

The recombinant *E. coli* cells were suspended in 1:5 volumes of extraction buffer. The *E. coli* cells carrying a pNCO113 vector were suspended in buffer B, whereas the *E. coli* cells carrying a pQE30 vector were suspended in buffer D or F containing 20 mM of imidazole. The suspension was cooled in an ice bath for 15 min. Ultrasonication was then performed (70 % duty cycle, output 4, 10 x 15 s, 30 s pause) during cooling on ice. Cell debris were centrifuged (rotor SS34, 15,000 rpm, 4 $^{\circ}$C, 40 min) and the supernatant was used as cell extract.

2.4.2.3   Protein purification

Proteins used in this study were purified by column chromatography. The proteins were loaded on column as clear solution. The buffer contained sodium azide (0.02 %) as bacterial growth inhibitor, dithioerythritol or dithiothreitol (1-5 mM) as antioxidant and/or sodium chloride (100 mM). All buffers were filtered and degassed before use. The flow rate was 1-5 ml min^{-1}. The effluent was monitored photometrically at 280 nm. The fraction size was 5-10 ml. All procedure of purification were carried at 4 $^{\circ}$C.

2.4.2.3.1   Anion exchange chromatography

Ion exchange chromatography separates molecules based on differences in their surface charges. The protein can bind on anion exchanger due to its negative charge if the pH of buffer is higher than its isoelectric point (pI). Anion exchange chromatography used in this study include Sepharose Q FF, Source 15 Q and Mono Q (Amersham Pharmacia Biotech, Freiburg, Germany). They are strong anion exchangers with quaternary amines as functional groups. Proteins can be eluted from the column by increasing the salt concentration in the buffer.

General procedure

The column was first equilibrated with 5-10 column volumes of buffer A, B or C. Then the protein was loaded on top of the column. The column was washed with 5-10 column volumes of starting buffer. The protein was then eluted from the column with a linear gradient of 5 column volumes of 0-1 M NaCl (Sepharose Q FF) ,16 column volumes of 0-0.5 M NaCl or KCl (Source 15Q), or 60 column volumes of 0-0.5 M NaCl or KCl (Mono Q) in buffer A, B or C respectively. The flow rate was at 2-5 ml min^{-1}.

2.4.2.3.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography provides a separation based on hydrophobicity on the surface of protein. In general, protein is loaded onto the matrix in high-salt buffer, and eluted by decreasing the salt concentration, either by gradient or in a stepwise fashion. Phenyl Sepharose 6FF and Resource PHE (Amersham Pharmacia Biotech, Freiburg, Germany) were used in this study.

General procedure

The column was equilibrated with 5 volumes of 0.5 M or 1 M ammonium sulfate in buffer A or B as starting buffer at the flow rate of 1 ml min^{-1} (Resource PHE) or 3 ml min^{-1} (Phenyl Sepharose 6FF), respectively. Saturated ammonium sulfate solution (4 M) was added to the protein solution to a final concentration of 0.5-1 M, respectively. Then protein was loaded on top of the column which was washed with 5 column volumes of starting buffer and was eluted with a linear gradient of 10 column volumes (Phenyl Sepharose 6FF) or 30 column volumes (Resource PHE) of 0.5 M or 1 M to 0 M ammonium sulfate in buffer A or B, respectively. The strongly bound protein was eluted isocratically with 5 column volumes of glycerol (10 %) in buffer.

2.4.2.3.3 Size-exclusion chromatography

Size-exclusion (gel filtration) chromatography is a technique for separating proteins on the basis of molecular size. Size-exclusion is often incorporated as a final purification step to remove aggregated proteins and acts as a buffer exchange mechanism into the final solution. The elution buffer contains 0.1 M NaCl (in excess of ionic strength) to minimize the protein-matrix interactions. Superdex 200 HR 26/60 and Superdex 75 HR 26/60 (Amersham Pharmacia Biotech, Freiburg, Germany) are gel filtration matrices which the proteins separate in the molecular weight range 10-600 kDa and 3-70 kDa, respectively.

General procedure

The column was equilibrated with buffer B containing 100 mM NaCl as starting buffer at the flow rate of 3 ml min^{-1}. The protein sample was concentrated by ultrafiltration (Amicon, MWCO 10 kDa) and loaded on top of the column (size 2.6 x 60 cm) in maximum volumes of 3-4 ml. The elution was performed with the same buffer.

2.4.2.3.4 Immobilized metal affinity chelating chromatography (IMAC)

IMAC is also referred to as metal chelating chromatography. It involves the utilizing of strong interaction between certain amino acid residues in proteins, primarily histidines, and metal ions that are immobilized on an absorbent matrix. A tag of 5-6 histidines can be added at the end of a protein by genetic engineering to facilitate the purification. Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech, Freiburg, Germany) used in this study has a iminodiacetate as a chelating group.

General procedure

To immobilize the metal ion on Chelating Sepharose Fast Flow, a solution of 50 mM $NiSO_4$ was passed through the column, washed with distilled water to get rid of an excess $NiSO_4$. The column was equilibrated with 10 column volumes of 20 mM imidazole in buffer D or F at the flow rate of 3 ml min^{-1}. The protein in the starting buffer was loaded on the column and the column was washed with 5 column volumes of starting buffer. The protein was eluted with a linear gradient of 10 column volumes of 20-500 mM imidazole in buffer D or F. The imidazole in the protein solution was removed by dialysis against buffer B in order to protect the protein from fragmentation.

2.4.2.3.5 Hydroxyapatite chromatography

Hydroxyapatite is a crystalline form of calcium phosphate with the molecular formula $Ca_{10}(PO_4)(OH)_2$. The mechanism of binding appeared to be that positively charged proteins interact with the general negative charge on the column generated by the immobilized phosphate ions. The protein can then be eluted by increasing the phosphate concentration in the buffer solution. Macro-Prep Ceramic Hydroxyapatite (type I, size 40 µm, BioRad, Munich, Germany) was used in this study.

General procedure

The column was equilibrated with 5 column volumes of 20 mM potassium phosphate pH 7.0 as starting buffer at the flow rate of 3 ml min^{-1}. The protein was loaded on top of the column which was washed with 5 column volumes of starting buffer. The protein was eluted with a linear gradient of 20-1000 mM of potassium phosphate pH 7.0.

2.4.2.3.6 Dye-ligand affinity chromatography

Dye-ligand chromatography is based on the ability of reactive dyes to bind proteins in a selective and reversible manner. The dye is immobilized on the support matrix, such as Sepharose or agarose. Elution of the bound protein is generally carried out by increasing ionic strength or by using a competing ligand. Cibacron Blue 3GA agarose type 3000-CL, Reactive Yellow 86, Reactive Green 19 (Sigma, Deisenhofen, Germany) and Red Sepharose CL-6B (Amersham Pharmacia Biotech, Freiburg, Germany) were used in this study.

General procedure

The column was equilibrated with 5 column volumes of buffer A or B as starting buffer at the flow rate of 2-3 ml min^{-1}. The protein was loaded on top of the column which was then washed with 5 column volumes of the same buffer. The protein was eluted with a linear gradient of 10 column volumes of 0-1 M NaCl in starting buffer.

2.4.2.4 Protein determination

The amount of protein was determined by the dye-binding method (Bradford assay) modified by Read and Northcote (1981).

950 µl of Bradford reagent was added to a 50 µl protein sample or standard protein solution (0.01- 0.1 mg ml^{-1}) in a 1 ml cuvette. As a reference, 50 µl of buffer without protein was mixed with 950 µl of Bradford reagent. The absorbance was measured at 578 nm. The amount of protein was estimated from a standard curve using bovine serum albumin as standard protein.

2.4.2.5 Polyacrylamide gel electrophoresis

The determination of the purity of protein fractions was measured using discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the system of Laemmli (1970). SDS is an anionic detergent which solubilizes and denatures proteins, SDS confers net negative charges to proteins. The proteins have then a mobility which is inversely proportional to their size or molecular mass.

The gel was prepared using the SDS-PAGE discontinuous buffer system with vertical slab gels (Table 2.3). The components of the separating gel solution were mixed together and then loaded into the slab deposited between two glass plates on the gel caster. The top of the gel was overlayed with isopropanol. The polymerization of acrylamide was completed after 1 h. After removing of the isopropanol, the stacking gel solution was prepared and loaded on top of the separating gel (avoided air bubbles) in the presence of 10 wells comb. The stacking gel completed its polymerization after 20 min. The slab gel was placed on the SE 250 Mighty Small II electrophoresis system (Hoefer Scientific, San Francisco, USA) which was connected to a cooling system. Proteins sample were mixed with SDS-sample buffer at a ratio of 1:1. The mixtures were heated at 95 ^{o}C for 5 min. Then the protein samples were loaded into the wells. Standard proteins were used as weight markers. The electrophoresis system was powered from a power supply with 20 mA per gel. After running for 2 h, the gel was removed carefully and stained in staining solution for 30 min. The gel was then destained with destaining solution for 1 h.

Table 2.3   SDS-PAGE recipe (using the SDS-PAGE discontinuous buffer system)

Stock solution	Stacking gel	15 % Acrylamide in separating gel
Acrylamide (40 % T, 3 % C)	0.5 ml	3.75 ml
Stacking gel buffer	2.5 ml	-
Separating gel buffer	-	2.5 ml
Water	2.0 ml	3.75 ml
10% w/v APS	50 µl	75 µl
TEMED	5 µl	5 µl

2.4.2.6   Enzyme assays

2.4.2.6.1   Determination of polyol kinase activity

The polyol kinase activity was measured by a photometric assay that measured the rate of formation of ADP from ATP (Simpson, 1966). The assay reaction was coupled with pyruvate kinase and lactate dehydrogenase. Assay mixtures, total volume 1 ml, contained 50 mM Tris hydrochloride, pH 8.0, 50 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM phosphoenolpyruvate, 2 mM ATP, 0.12 mM NADH, 2 mM polyol substrate, 2.5 U of pyruvate kinase (EC 2.7.1.40)

and 2.5 U of rabbit muscle lactate dehydrogenase (EC 1.1.1.27). The mixtures were preincubated at 37 ^{o}C for 5 min. The reaction was started by adding the protein and the absorbance at 340 nm was measured with an Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech, Freiburg, Germany). The extinction coefficient at 340 nm (ε) of NADH is 6.2 x 10^3 M^{-1} cm^{-1}. The ratio of NADH oxidation was corrected by subtraction of the ratio of control without substrate.

One unit of polyol kinase activity was defined as the amount of enzyme required to phosphorylate 1 µmol of polyol substrate per minute.

2.4.2.6.2 Determination of 1-deoxy-D-xylulose kinase activity

A. Radiometric assay

Radiometric assay of 1-deoxy-D-xylulose kinase activity was found conveniently to follow an enzyme activity during purification of wild type *E. coli* protein. The assay mixtures contained 50 mM Tris hydrochloride pH 7.4, 40 mM MgCl$_2$, 40 mM ATP, 20 mM glutathione, 20 mM sodium fluoride, 3.5 µM [1,2-^{14}C]1-deoxy-D-xylulose (24,000 dpm, specific activity 62.5 µCi µmol^{-1}) and protein in a total volume of 50 µl. The mixtures were incubated at 37 ^{o}C for 1 h. Aliquots (10 µl) were applied to a piece of anion thin layer plates, POLYGRAM$^®$ CEL 300 PEI (1 x 1 cm, Macherey Nagel, Düren, Germany). The plates were dried at 65 ^{o}C, washed twice with distilled water to get rid of the unphosphorylated substrate whereas the phosphorylated product, [1,2-^{14}C]1-deoxy-D-xylulose 5-phosphate was bound on the thin layer plates. The plates were then transferred to scintillation vials, 15 ml of fluid scintillation reagent was added and radioactivity was counted with a liquid scintillation counter (Beckman LS 7800, Fullerton, USA). Control assays were performed without addition of protein.

B. Photometric assay

1-Deoxy-D-xylulose kinase activity was measured by a photometric assay coupled with 1-deoxy-D-xylulose 5-phosphate reductoisomerase. Assay mixtures (1 ml) contained 100 mM Tris hydrochloride, pH 8.0, 4 mM MgCl$_2$, 1 mM MnCl$_2$, 5 mM ATP, 0.5 mM NADPH, 1 mM DTT, 5 mM 1-deoxy-D-xylulose, 5 µg of 1-deoxy-D-xylulose 5-phosphate reductoisomerase,

and protein. The reaction was initiated by adding protein to the complete assay mixture. The oxidation of NADPH was monitored with a Ultrospec 2000 spectrophotometer equipped with cell holders for adjusting temperature at 37 ^{o}C. The extinction coefficient at 340 nm (ε) of NADPH is 6.2 x 10^3 M^{-1} cm^{-1}.

One unit of 1-deoxy-D-xylulose kinase activity was defined as the amount of enzyme that caused the oxidation of 1 µmol of NADPH per min.

2.4.2.6.3 Determination of 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity

A. Radiometric assay

The radiometric assay was used for screening the enzyme activity. Assay mixtures contained 50 mM Tris hydrochloride, pH 8.4, 1 mM MnCl$_2$, 5 mM NADPH, 40 mM sodium fluoride, 2 µM [1,2-^{14}C]1-deoxy-D-xylulose 5-phosphate (13,000 dpm, specific activity 62.5 µCi µmol^{-1}) and protein in total volume of 50 µl. The assay were incubated at 37 ^{o}C for 30 min. The assays were subsequently heated at 95 ^{o}C for 1 min to stop the reaction. A solution containing 50 mM MgCl$_2$, 50 mM ZnCl$_2$ and 2 units of alkaline phosphatase (EC 3.1.3.1) was added and the assays were incubated at 37 ^{o}C for 30 min. Aliquots (30 µl) were spotted on Silica gel 60 F$_{254}$ plates and developed in a system of SS1 (Table 2.4). The radiochromatogram was developed and evaluated as described in 2.4.4.

B. Photometric assay

1-Deoxy-D-xylulose 5-phosphate reductoisomerase activity was determined photometrically according to a modified method of Takahashi (1998). The assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 0.2 mM NADPH, 0.2 mM 1-deoxy-D-xylulose 5-phosphate and protein in a total volume of 1 ml. The reaction was initiated by adding protein to the complete assay mixture. The oxidation of NADPH was monitored in a Ultrospec 2000 spectrophotometer equipped with cell holders adjusting temperature at 37 ^{o}C. The extinction coefficient at 340 nm (ε) of NADPH is 6.2 x 10^3 M^{-1}cm^{-1}.

One unit of 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity was defined as the amount of enzyme that caused the oxidation of 1 µmol of NADPH per min.

2.4.2.6.4 Determination of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase activity

A. Radiometric assay

Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 20 mM sodium fluoride, 10 mM MgCl$_2$, 100 µM CTP, 11.4 µM [2-^{14}C]2*C*-methyl-D-erythritol 4-phosphate (17.5 mCi mmol^{-1}) and 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase. The assay mixtures were incubated at 37 °C for 20 min. The reactions were terminated by the addition of methanol (20 µl). The mixtures were centrifuged. Aliquots were spotted on Sil N-HR thin layer plates which were developed with the mixture SS2 (Table 2.4). Radioactivity was monitored with a PhosphorImager as described in 2.4.4.

B. Photometric assay

4-Diphosphocytidyl-2*C*-methyl-D-erythritol synthase activity was determined by a spectrophotometric assay, in which the inorganic pyrophosphate formed from 2*C*-methyl-D-erythritol 4-phosphate and CTP is used in a cascade of downstream reactions leading to the reduction of NADP$^+$ measured at 340 nm. The reaction contained 50 mM Tris hydrochloride, pH 8.0, 5 mM MgCl$_2$, 1 mM DTT, 200 µM 2*C*-methyl-D-erythritol 4-phosphate, 200 µM CTP, 1 µM glucose-1,6-bisphosphate, 500 µM UDP-glucose, 174 µM NADP$^+$, 0.125 units of UDP-glucose pyrophosphorylase, 0.16 units of phosphoglucomutase, 1 unit of glucose 6-phosphate dehydrogenase, and 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase in a total volume of 1 ml.

One unit of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase activity was defined as the amount of enzyme catalyzing the conversion of 1 µmol of 2*C*-methyl-D-erythritol 4-phosphate per min under standard assay conditions at 37 °C.

C. HPLC Assay

Assay mixtures for direct detection of the enzyme product via HPLC contained 100 mM Tris hydrochloride, pH 8.0, 5 mM MgCl$_2$, 1 mM CTP, 1 mM 2*C*-methyl-D-erythritol 4-phosphate,

1 mM DTT, 0.1 unit of inorganic pyrophosphatase (EC 3.6.1.1), and protein in a total volume of 100 µl. The mixtures were incubated at 37 $^{\circ}$C for 20 min, heated to 80 $^{\circ}$C for 5 min, and centrifuged. Aliquots (10 µl) of the supernatant were applied to a Nucleosil RP 18 column (4.6 x 250 mm, Macherey-Nagel, Düren, Germany) that was developed as described in Isocratic No.1 (2.4.3.3.3). The effluent was monitored photometrically at 270 nm.

One unit of 4-diphosphocytidyl-2C-methyl-D-erythritol synthase activity was defined as the amount of enzyme which produced 1 µmol of 4-diphosphocytidyl-2C-methyl-D-erythritol per min.

2.4.2.6.5 Determination of 4-diphosphocytidyl-2C-methyl-D-erythritol kinase activity

A. Radiometric assay

Assay mixtures containing 100 mM Tris hydrochloride, pH 8.0, 5 mM MgCl$_2$, 100 µM ATP, 5 mM DTT, 11.4 µM [2-^{14}C]4-diphosphocytidyl-2C-methyl-D-erythritol (specific activity 17.5 mCi mmol^{-1}), and protein in a total volume of 50 µl were incubated at 37 $^{\circ}$C for 30 min. Aliquots (40 µl) were spotted on POLYGRAM$^{®}$ Sil N-HR thin layer plates (Macherey-Nagel, Düren, Germany), which were developed with a system of SS2 (Table 2.4). Radioactivity was monitored with a PhosphorImager (Storm 860, Molecular Dynamics, Sunnyvale, CA, USA) as described in 2.4.4.

B. HPLC assay

Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 5 mM MgCl$_2$, 5 mM DTT, 2 mM ATP, 2 mM 4-diphosphocytidyl-2C-methyl-D-erythritol and protein in a total volume of 100 µl. The mixtures were incubated at 37 $^{\circ}$C for 15 min. The reaction was terminated by heating at 80 $^{\circ}$C for 5 min. The samples were centrifuged and the supernatant was analyzed by reverse phase HPLC using a column of Multospher 120 RP 18-AQ-5 (4.6 x 250 mm, CS-Chromatographic Service GmbH). The column was developed as Gradient No. 1 (2.4.3.3.3). The effluent was monitored photometrically (270 nm).

One unit of 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase activity was defined as the amount of protein which catalyzed the formation of 1 µmol of 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate per min.

2.4.2.6.6 Determination of isopentenyl monophosphate kinase activity

Assay mixtures contained 100 mM Tris hydrochloride buffer, pH 8.0, 5 mM DTT, 5 mM ATP, 1 mM [4-^{14}C]isopentenyl monophosphate (0.5 µCi µmol^{-1}), and 100 µg protein in a volume of 100 µl. The mixtures were incubated at 37 °C for 60 min. The reaction was terminated by heating at 80 °C for 5 min, and the mixtures were centrifuged. The supernatants were analyzed by reversed phase HPLC using a column of Multospher 120 RP 18-AQ-5 (4.6 x 250 mm) that had been equilibrated and run with Gradient No. 2 (2.4.3.3.3). The effluent was monitored by a continuous-flow radiodetector (Beta-RAM, Biostep GmbH, Jahnsdorf, Germany).

One unit of isopentenyl monophosphate kinase activity was defined as amount of protein which catalyzed the formation of 1 µmol of isopentenyl monophosphate per min.

2.4.2.6.7 Determination of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase activity

A. Radiometric assay

The assay mixtures containing 100 mM Tris hydrochloride, pH 8.0, 5 mM MnCl$_2$, 5 mM DTT, 11.4 µM [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate (specific activity 17.5 mCi mmol^{-1}) (or 11.4 µM [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol) and protein in a total volume of 50 µl were incubated at 37 ^{o}C for 10 min. Aliquots (40 µl) were applied to thin layer plates (POLYGRAM$^®$ Sil NH-R, Macherey-Nagel, Düren, Germany) that were developed in system of SS2 (Table 2.4). Radioactivity was monitored by a PhosphorImager (Storm 860, Molecular Dynamics, Sunnyvale, CA, USA) as described in 2.4.4.

B. HPLC assay

Assay mixtures contained 50 mM potassium phosphate, pH 7.0, 2 mM DTT, 2 mM MgCl$_2$, 2 mM   4-diphosphocytidyl-2*C*-methyl-D-erythritol   (or   4-diphosphocytidyl-2*C*-methyl-D-

erythritol 2-phosphate) and protein in a total volume of 100 µl. The mixtures were incubated at 37 $^{\circ}$C for 20 min. The reactions were terminated by adding EDTA in a final concentration of 4 mM. The samples were centrifuged and the supernatant was analyzed by HPLC using a column of Multospher 120 RP 18-AQ-5 (4.6 x 250 mm, CS-Chromatographic Service GmbH) that had been equilibrated and run with Gradient No. 3 (2.4.3.3.3). The effluent was monitored photometrically (270 nm).

One unit of 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase activity was defined as the amount of protein which produced 1 µmol of CMP per min.

2.4.3   Chromatographic methods

2.4.3.1   Thin layer chromatography

The qualitative analysis of enzyme reactions was performed by thin layer chromatography. Sugar derivatives were separated on thin layer plates (Silica gel F_{254}, 20 x 20 cm ;Merck, Darmstadt, Germany) using solvent system SS1 (Stahl and Kaltenbach 1962, Table 2.4). Sugar phosphates were separated on Silica gel N-HR/UV_{254} (POLYGRAM$^{\circledR}$ Sil N-HR, UV_{254}, Macherey-Nagel, Düren, Germany) using the solvent system SS2 (Table 2.4).

Table 2.4   Solvent systems used in this study

Solvent system	Ratio	Stationary phase
SS1 Ethyl acetate:Isopropanol:Water	6:2:1	Silica gel 60
SS2 N-Propanol:Ethyl acetate:Water	6:1:3	Silica gel N-HR
SS3 $tert$-Butanol:Formic acid:Water	50:13:20	Whatman Paper No.3

2.4.3.2   Paper chromatography

Whatman Chromatography Paper No. 3 was used as the stationary phase which was washed with distilled water before use. The enzymatic reaction mixture was subjected on the paper (size 400 x 450 mm), dried and run in the solvent system SS3 (Table 2.4) overnight. The product band was detected with a TLC-Radioscanner (Automatic TLC-Linear Analyzer Tracemaster 20, Berthold, Wildbad, Germany). The band of product was excised and eluted with 50 ml of distilled water. The eluate was lyophilized.

2.4.3.3 HPLC

2.4.3.3.1 Semipreparative HPLC for sugar compounds

Column:	Rezex RPM Monosaccharide, 300 x 7.8 mm, particle size 10 µm (Phenomenex)
Precolumn:	Rezex RPM Monosaccharide, 50 x 7.8 mm, particle size 10 µm (Phenomenex)
Mobile phase:	Distilled Water
Temperature:	65 ^{o}C
Flow rate:	0.4 ml min^{-1}
Detection	Refractometer, Optical unit (Optilab Multiref 902B)
	LB 503 HPLC Radiactivity Monitor (Berthold, Wildbad, Germany)
Injection volume	50-200 µl

2.4.3.3.2 Semipreparative HPLC for sugar phosphate compounds

Column:	Nucleosil 10 SB, 250 x 4.6 mm, particle size 10 µM (Macherey-Nagel, Düren, Germany)	
Mobile phase	Eluent A:	100 mM Ammonium formate in 40 % methanol (vol/vol)
	Eluent B:	1 M Ammonium formate
Elution No.1	Isocratic elution with eluent A	
Elution No.2	Gradient elution	

Time (min)	A [%]	B [%]
0	100	0
15	100	0
30	50	50
35	0	100
40	100	0
50	100	0

Flow rate	1 ml min^{-1}
Detection:	LB 503 HPLC Radiactivity Monitor (Berthold, Wildbad, Germany)
	Diode array photometer (J&M TIDAS, Aalen, Germany)
	K-2600 WellChrom Spectrophotometer
Injection volume	20-100 µl

2.4.3.3.3 Analytical HPLC for quantitative determination

Quantitative determination of enzyme activity was performed using a ion-pair reversed phase HPLC method (McCaskill and Croteau 1993). A stock solution (200 mM) of the ion-pair reagent tetra-*N*-butylammonium hydrogen sulfate (TBAS) was prepared. The pH was adjusted to 6.0 with solid Na_2HPO_4.

Column:		Multospher 120 RP 18-5 AQ, 250 x 4.6 mm, particle size 5 µm (CS: Chromatographic Service GmbH, Langerwehe, Germany)
		Nucleosil RP-18, 250 x 4.6 mm, particle size 5 µm (Macherey-Nagel, Düren, Germany)
Mobile phase:	Eluent A	10 mM TBAS in distilled H_2O
	Eluent B	10 mM TBAS in 70 % (v/v) Methanol
Isocratic No.1	Isocratic elution with 4 mM TBAS in 20 % (v/v) Methanol	

Gradient No.1

Time (min)	A [%]	B [%]
0	100	0
20	100	0
80	40	60
81	100	0
100	100	0

Gradient No.2

Time (min)	A [%]	B [%]
0	100	0
30	0	100
35	100	0
40	100	0

Gradient No.3

Time (min)	A [%]	B [%]
0	100	0
20	40	60
25	0	100
26	100	0
30	100	0

Flow rate: Isocratic No.1   2 ml min^{-1}

                 Gradient No.1   0.75 ml min^{-1}

                 Gradient No.2   1 ml min^{-1}

                 Gradient No.3   1 ml min^{-1}

Detection: Radiomonitor β-RAM Flow-Through System Model 2 (Biostep GmbH, Jahnsdorf, Germany) with solid cell (CaF or YtSi)

                 K-2600 WellChrom Spectrophotometer

Injection volume: 10-20 µl

2.4.4   Determination of radioactivity

Radioactivity of solutions containing of ^{14}C or ^{3}H labeled compounds were measured by adding of 15 ml of fluid scintillation reagent and by counting with a Liquid Scintillation Counter (Beckman LS 7800, Fullerton, USA).

$$\text{Radioactivity } (\mu Ci) \quad = \quad \frac{\text{Disintegration per minute (dpm)}}{2.2 \times 10^{6}}$$

For qualitative and quantitative analysis, the radio-chromatogram on thin layer chromatography was analyzed by PhosphorImager (Storm 860, Molecular Dynamics, Sunnyvale, CA, USA). The thin layer plates were exposed on a Kodak Identifying Screen for 10-15 h. The radiochromatogram was evaluated by the ImageQuant™ 1.0 software. In the case of paper chromatograms, the radiochromatogram was developed with a TLC-Scanner (Automatic TLC Linear Analyzer Tracemaster 20, Berthold).

3 RESULTS

3.1 1-DEOXY-D-XYLULOSE KINASE

1-Deoxy-D-xylulose was shown to be formed from pyruvate and glyceraldehyde *in vitro* by pyruvate dehydrogenase of *Escherichia coli* and *Bacillus subtilis* (Yokota and Sasajima 1986). The compound is diverted to many terpenoids via the deoxyxylulose phosphate pathway as shown by many research groups (see introduction). From this evidence, it was proposed (Eisenreich et al. 1998) that 1-deoxy-D-xylulose can enter into the deoxyxylulose phosphate pathway via phosphorylation catalyzed by an unspecific carbohydrate kinase (Fig. 3.1.1). In this study, this hypothesis was confirmed by incubation experiments using radiolabeled 1-deoxy-D-xylulose and cell extracts from wild type *E. coli* cells in the presence of ATP and Mg^{2+} ions as described under methods (2.4.2.6.2 A). The reaction mixtures were separated by paper chromatography (see method 2.4.3.2) and the radiochromatograms were analyzed with a TLC-radioscanner (see method 2.4.4). The *Rf* values of 1-deoxy-D-xylulose and the reaction product were 0.71 and 0.35, respectively indicating that the reaction product had a higher polarity than 1-deoxy-D-xylulose. Therefore, it was assumed to be the phosphorylated product of 1-deoxy-D-xylulose (Fig. 3.1.1). The enzyme catalyzing this reaction was named 1-deoxy-D-xylulose kinase.
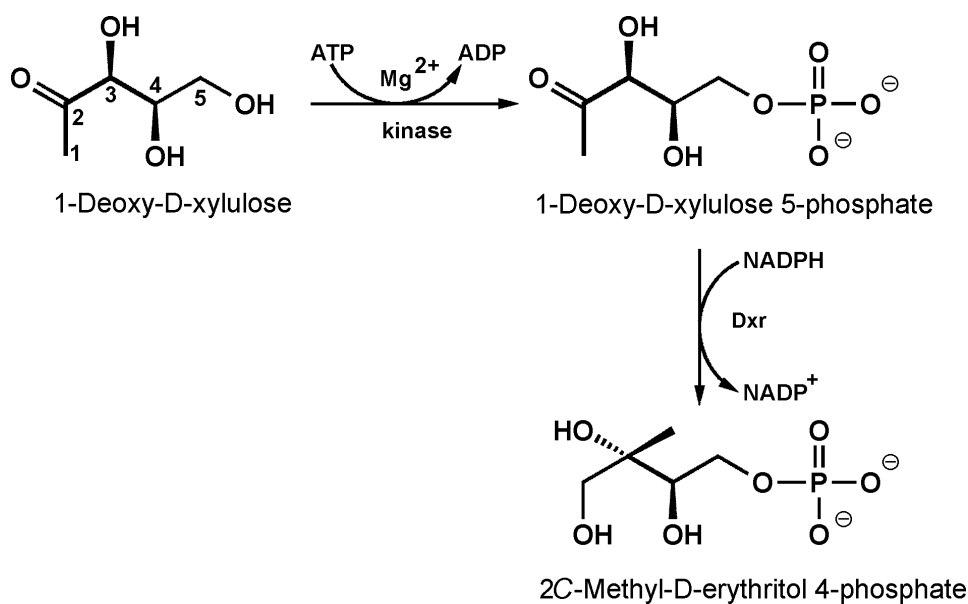
Figure 3.1.1 Proposed phosphorylation of 1-deoxy-D-xylulose

3. RESULTS

3.1.1 Purification of 1-deoxy-D-xylulose kinase from wild type *E. coli* DH5**a**

A radiochemical assay was used for monitoring 1-deoxy-D-xylulose kinase activity from wild type *E. coli* (2.4.2.6.2 A). Based on the different polarity of 1-deoxy-D-xylulose and the phosphorylated reaction product, a detection method for this reaction was developed. It was proposed that the putative reaction product 1-deoxy-D-xylulose 5-phosphate and the substrate 1-deoxy-D-xylulose could be separated by anion exchangers. Therefore, different anion exchanger matrices such as DEAE paper, CEL 300 PEI (Macherey Nagel), DOWEX 1x4 (formate form, SERVA) were tested. The CEL 300 PEI was shown to be an appropriate matrix to trap the reaction product. Aliquots (10 µl) of the reaction mixtures (2.4.2.6.2 A) were subjected on pieces of CEL 300 PEI layers (size 1x1 cm) and fixed by heating at 65 ^{o}C. The reaction product bound on the CEL 300 PEI, whereas the substrate [1,2-^{14}C]1-deoxy-D-xylulose, could be washed out with distilled water and the radioactivity was counted by scintillation counting. The enzyme activity was determined as relative activity per min using a control reaction without protein.

$$\text{Relative enzyme activity} = \frac{\text{Counts of reaction (dpm)} - \text{Counts of control (dpm)}}{\text{Time of incubation (min)}}$$

The assay conditions were optimized according to the rate of product formation. Assay mixtures contained ATP as phosphate group donor, Mg^{2+} as cofactor, glutathione as antioxidant and sodium fluoride as phosphatase inhibitor (see 2.4.2.6.2 A). Highest rates were found at temperatures between 35 ^{o}C and 40 ^{o}C, and at pH values between 7.0 and 7.5.

The purification of 1-deoxy-D-xylulose kinase by column chromatography is summarized in Table 3.1.1.

400 g of *E. coli* DH5α cells were suspended in 1.5 l of buffer A (2.2.5). Cell extract was prepared as described under 2.4.2.2.1. The total protein of the cell extract was 21.8 g.

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The cell extract (21.8 g protein) was loaded on top of a Sepharose Q FF column (4.6 x 24 cm, Amersham Pharmacia Biotech) which had been equilibrated with buffer A. The flow rate was 5 ml min^{-1}. The column was washed with 300 ml of buffer A and developed by a linear gradient of 0-1 M NaCl in buffer A (total volume 600 ml). Active fractions were eluted at 0.3-0.5 M NaCl and combined. A chromatogram of this step is shown in Fig. 3.1.2 A (Table 3.1.1).

Saturated ammonium sulfate solution was added to the protein solution to a final concentration of 0.2 M. The solution (8085 mg protein) was applied to a column of Phenyl Sepharose 6 FF (high substance, 4.6 x 12 cm, Amersham Pharmacia Biotech) which had been equilibrated with 0.2 M ammonium sulfate in buffer A. The flow rate was 5 ml min^{-1}. The column was developed by a linear gradient of 0.2-0 M ammonium sulfate in buffer A (total volume, 300 ml). Protein was eluted with 10% glycerol in buffer A as shown in Fig. 3.1.2 B. Fractions containing activity were combined and dialyzed against buffer A. The protein amount was 871 mg corresponding to a yield of 28%. The purification factor was 7-fold (Table 3.1.1).

The dialyzed protein fraction (871 mg) was loaded on top of a Cibacron Blue 3GA agarose 3000-CL column (2.6 x 10 cm, Sigma) which was developed with buffer A at a flow rate of 3 ml min^{-1}. The enzyme was in the flow through fraction and was purified 20-fold with 27% yield (Fig. 3.1.2 C, Table 3.1.1).

The protein fraction (324 mg) was then applied to a Source 15Q column (1.5 x 10 cm, Amersham Pharmacia Biotech) which had been equilibrated with buffer A at a flow rate of 3 ml min^{-1}. The column was developed by a linear gradient of 0-0.5 M NaCl in buffer A (total volume 180 ml). The enzyme was eluted at 20-100 mM NaCl as shown in Fig 3.1.2 D. This step gave 50-fold purification with 18% yield (Table 3.1.1). Fractions containing the enzyme activity were combined and concentrated to ca. 3 ml by ultrafiltration (Amicon UF-10, MWCO 10 kDa).
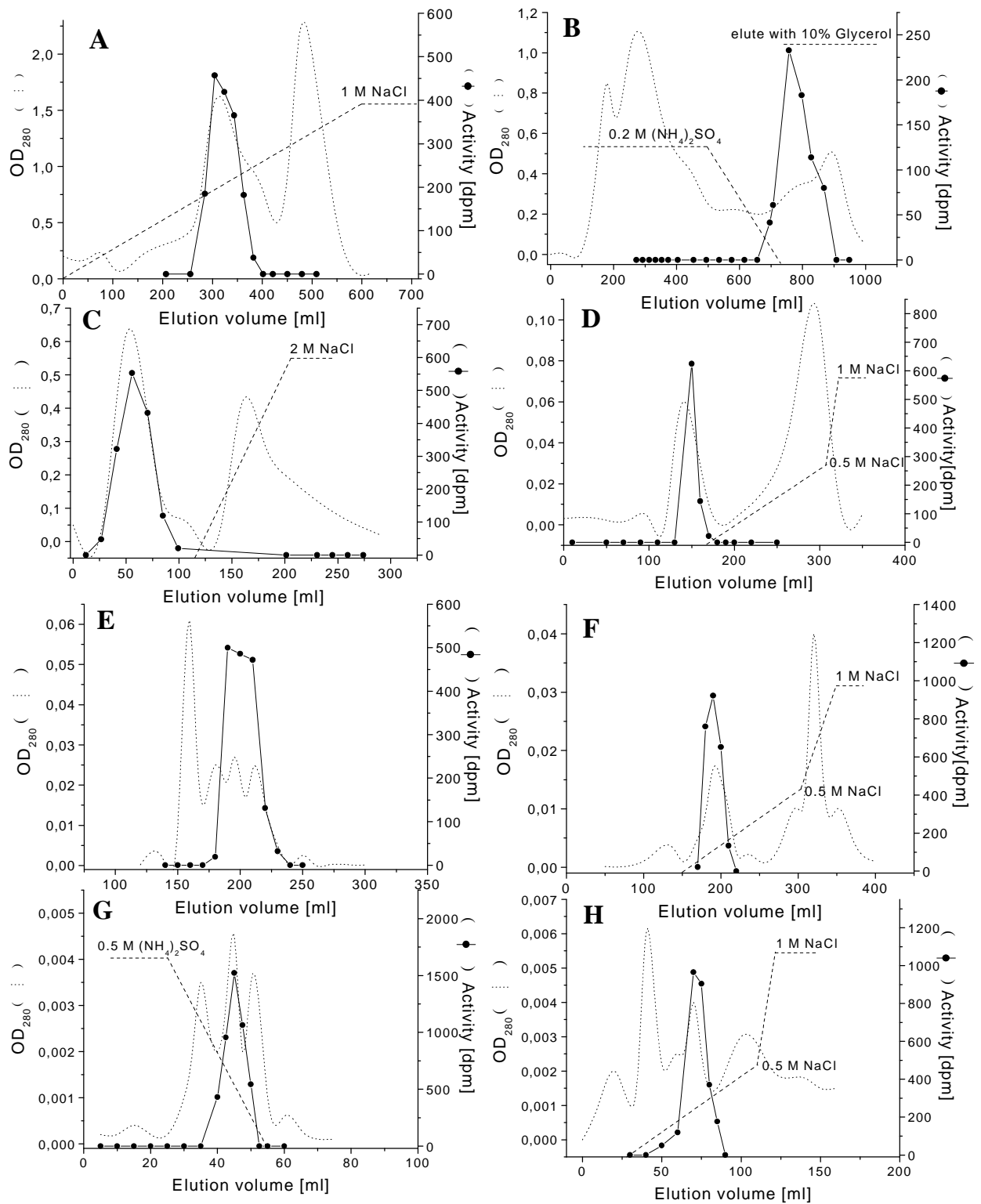
Figure 3.1.2 Chromatographic steps for the purification of 1-deoxy-D-xylulose kinase from *E. coli* DH5α; A : Sepharose Q FF; B: Phenyl Sepharose 6FF; C: Cibacron Blue 3GA; D: 1st Source 15Q; E: Superdex 200 HR; F: 2nd Source 15Q; G: Resource PHE; H: Mono Q HR

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The concentrated protein solution (76 mg) was applied to a Superdex 200 HR column (2.6 x 60 cm, Amersham Pharmacia Biotech) which was developed with 50 mM NaCl in buffer A at a flow rate of 3 ml min^{-1}. The enzyme was eluted at 185 ml. The chromatogram of this step is shown in Fig 3.1.2 E. This step gave 120-fold purification with 11% of protein recovery (Table 3.1.1). The active fractions were combined and dialyzed against buffer A.

The dialyzed protein fraction (19 mg) was then applied to a Source 15 Q column (1.5 x 10 cm, Amersham Pharmacia Biotech) which was developed by a linear gradient of 0-0.5 M NaCl in buffer A (total volume, 180 ml). The flow rate was 3 ml min^{-1}. The enzyme was eluted at 75-120 mM NaCl as shown in Fig. 3.1.2 F. The enzyme was purified 400-fold with 10% recovery (Table 3.1.1).

The active fractions were combined and saturated ammonium sulfate solution was added to a final concentration of 0.5 M. The protein solution (5.7 mg) was applied to a Resource PHE column (column volume 1 ml, Amersham Pharmacia Biotech) which had been equilibrated with 0.5 M ammonium sulfate in buffer A at a flow rate of 1 ml min^{-1}. The column was developed by a linear gradient of 0.5-0 M ammonium sulfate in buffer A (total volume, 30 ml). The enzyme was eluted at 0.2-0.1 M ammonium sulfate. The chromatogram is shown in Fig. 3.1.2 G. The recovery of protein was 1%. This step gave a 2500 fold purification (Table 3.1.1).

The active fractions were combined and dialyzed against buffer A. The protein solution (90 µg) was loaded on top of a Mono Q HR column (0.5 x 5 cm, Amersham Pharmacia Biotech) which had been equilibrated with buffer A at a flow rate of 2 ml min^{-1}. The column was developed by a linear gradient of 0-0.5 M NaCl in 60 ml of buffer A. The enzyme was eluted at 140-160 mM NaCl as shown in Fig 3.1.2 H. The active fractions were combined. This final step gave a 3400-fold enrichment with 0.5% protein recovery (30 µg)(Table 3.1.1). The purification of 1-deoxy-D-xylulose kinase is also demonstrated by SDS-PAGE as shown in Fig 3.1.3. The protein migrated as single band with a molecular mass of approximately 52 kDa.

Table 3.1.1   Purification of 1-deoxy-D-xylulose kinase from wild type *E. coli* DH5α

Procedure	Relative activity (dpm min^{-1})	Relative specific activity (dpm min^{-1}mg^{-1})	Yield %	Purification factor
Cell extract	218000	10	100	1
Sepharose Q FF	80800	10	37	1
Phenyl Sepharose 6FF	61000	70	28	7
Cibacron Blue 3GA	58300	180	27	20
1st Source 15Q	39500	500	18	50
Superdex 200 HR	23400	1200	11	120
2nd Source15Q	22600	4000	10	400
Resource PHE	2200	24900	1	2500
Mono Q HR	1000	33600	0.5	3400
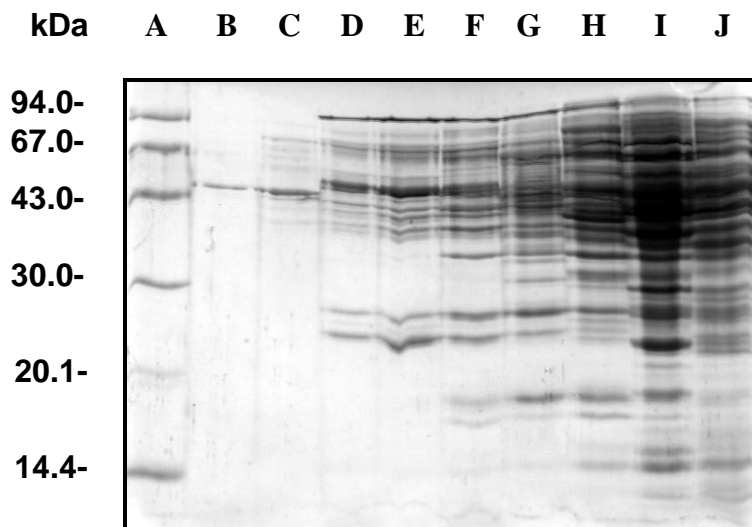
Figure 3.1.3   SDS-PAGE : purification of 1-deoxy-D-xylulose kinase from *E. coli* DH5α; A: molecular mass markers; B: after Mono Q HR5/5; C: after Resource PHE; D: after 2nd Source 15Q; E: after Superdex 200 HR 26/60; F: after 1st Source 15Q; G: after Cibacron Blue 3GA 3000 CL; H: after Phenyl Sepharose 6 FF; I: after Sepharose Q FF; J: cell free extract of *E. coli* DH5α.

3.1.2 Amino acid sequence analysis

The N-terminal amino acid sequence of 1-deoxy-D-xylulose kinase was determined by Edman degradation (Yarwood, 1989) at the Lehrstuhl für Lebensmittelchemie, TU München. The N-terminal sequence was as follows:

MYIGIDLGTSGVKVILLNEQG-

A BLASTP search in the *E. coli* genome showed that the N-terminal sequence of the purified enzyme was identical with the N-terminus of *E. coli* D-xylulokinase (EC 2.7.1.17) specified by the *xylB* gene. Moreover, the molecular mass of the purified enzyme, estimated by SDS-PAGE to about 52 kDa, corresponded very well to the calculated mass (52.6 kDa) of D-xylulokinase of *E. coli*. These data indicated that both enzymes are identical. For confirming these results, the *xylB* gene of *E. coli* was cloned, the cognate enzyme was expressed, purified, and studied enzymatically.

3.1.3 Overexpression of the *xylB* gene of *E. coli*

The *xylB* gene was cloned into the expression plasmid pNCO113 under the control of a T5 promoter and *lac* operator (Wungsintaweekul et al. 2001). The procedure of *xylB* gene expression was described under 2.4.2.1. Cells of the recombinant *E. coli* strain XL1-pNCO-xylB were prepared as described under 2.4.1.2. Cell extracts of this strain contained a 52 kDa peptide accounting for 20% of total soluble protein as shown by SDS-PAGE (Fig. 3.1.5).

3.1.4 Purification of recombinant D-xylulokinase of *E. coli*

For the determination of enzyme activity, a method originally described by Simpson was used and optimized (Simpson 1966). In this assay, the rate of ADP formation is determined by its rephosphorylation using pyruvate kinase and phosphoenolpyruvate yielding ATP and pyruvate (see 2.4.2.6.1). This reaction is coupled with the reduction of pyruvate by lactate dehydrogenase
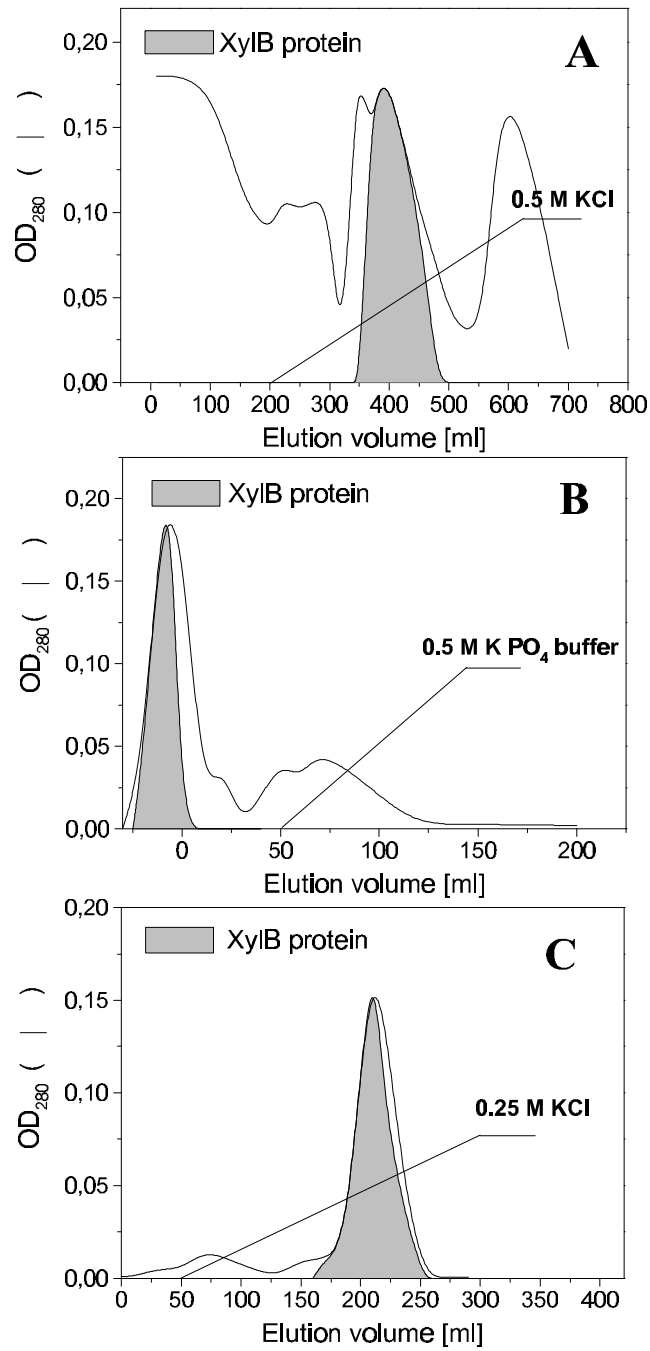
Figure 3.1.4 Chromatographic steps for the purification of recombinant D-xylulokinase of *E. coli* ; A: Sepharose Q FF; B: Hydroxyapatite (Macro Prep); C: Source 15 Q

and the oxidation of NADH to NAD^+. The oxidation of NADH is determined photometrically at 340 nm. This method is rapid and easily performed. It was used for the measurement of recombinant polyol kinase activities and determination of kinetic properties. Controls without substrate were performed to avoid the interference of NADH oxidases present in protein preparations (2.4.2.6.1).

1-Deoxy-D-xylulose kinase activity could also be measured by coupling with 1-deoxy-D-xylulose 5-phosphate reductoisomerase (see method 2.4.2.6.2 B and results 3.2). The oxidation of NADPH is proportional to the formation of 1-deoxy-D-xylulose 5-phosphate and was determined photometrically at 340 nm.

Cells of the recombinant *E. coli* strain XL1-pNCO-xylB (7.8 g) were suspended in 40 ml of buffer B (2.2.5). The purification of D-xylulokinase is summarized in Table 3.1.2. Cell extract was prepared as described under 2.4.2.2.2. It had a specific 1-deoxy-D-xylulose kinase activity of 0.6 µmol min^{-1} mg^{-1}. The cell extract (240 mg) was loaded on top of a Sepharose Q FF column (2.8 x 15 cm, Amersham Pharmacia Biotech) which had been equilibrated with buffer B at a flow rate of 5 ml min^{-1}. The column was washed with 100 ml of buffer B and was developed by a linear gradient of 0-1 M KCl in 800 ml of buffer B. The enzyme was eluted at 140-220 mM KCl as shown in Fig. 3.1.4 A. Fractions were combined according to SDS-PAGE. The purification factor was 2-fold with a protein recovery of 52% (Table 3.1.2).

The protein (70 mg) was concentrated to a volume of 7 ml by ultrafiltration (PALL Macrosep 30 kDa). The concentrated fraction was diluted to 40 ml with buffer B. The solution was then loaded on top of a hydroxyapatite column (Macro-Prep, 40 µM, type I, 2.8 x 5 cm, BioRad) which had been equilibrated with buffer B at a flow rate of 4 ml min^{-1}. The enzyme was in the flow through fractions, whereas contaminants were bound on the column and eluted with 0-0.5 M potassium phosphate pH 7.0 (total volume 200 ml). The chromatogram is shown in Fig. 3.1.4 B. The specific activity of enzyme was 1.4 µmol $min^{-1}mg^{-1}$ and the yield was 51% (55 mg) (Table 3.1.2).

The protein (55 mg) was then loaded on top of a Source 15Q column (1.5 x 10 cm. Amersham Pharmacia Biotech) which was developed by a linear gradient of 0-0.5 M KCl in 600 ml of buffer B at a flow rate of 4 ml min^{-1}. The chromatogram is shown in Fig. 3.1.4 C. Fractions were combined and concentrated by ultrafiltration (PALL Macrosep, MWCO 30 kDa). The

protein was purified 3-fold with 51% protein recovery (Table 3.1.2). The protein migrated as a single band on SDS-PAGE (Fig. 3.1.5). The specific activity of the purified enzyme was determined to 1.6 and 1.5 µmol min^{-1}mg^{-1} by the first (2.4.2.6.1) and second (2.4.2.6.2 B) coupled enzyme assay, respectively.

Table 3.1.2   Purification of recombinant D-xylulokinase from *E. coli*

Procedure	Total activity (µmol min^{-1})	Specific activity (µmol min^{-1}mg^{-1})	Yield %	Purification factor
Cell extract	161	0.6	100	1
Sepharose Q FF	84	1.2	52	2
Hydroxyapatite	82	1.4	51	2
Source 15Q	83	1.6	51	3
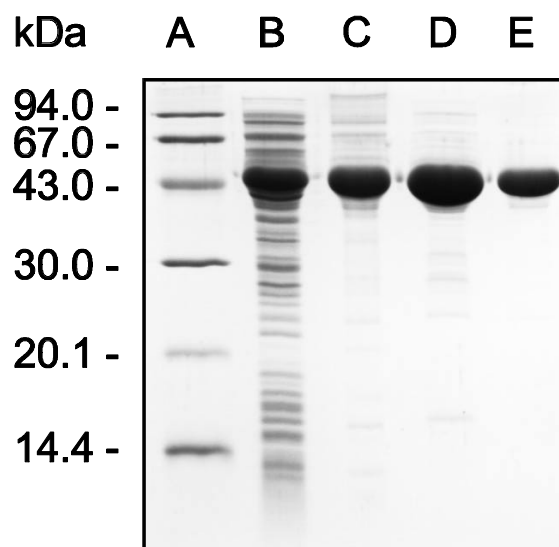
Figure 3.1.5   SDS-PAGE : purification of recombinant D-xylulokinase of *E. coli* ; A: molecular mass markers; B: cell free extract; C: after Sepharose Q FF; D: after Hydroxyapatite (Macro Prep); E: after Source 15Q.

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3.1.5   Characterization of the enzyme product

1-Deoxy-D-xylulose was treated with partially purified enzyme. Reaction mixtures contained 50 mM Tris hydrochloride, pH 7.4, 40 mM $MgCl_2$, 40 mM ATP, 20 mM glutathione, 20 mM NaF, 10 mM 1-deoxy-D-xylulose, 0.2 µCi [1,2-^{14}C]1-deoxy-D-xylulose (62.5 µCi µmol^{-1}) and 22.5 mg of partially purified 1-deoxy-D-xylulose kinase in a total volume of 7.5 ml. The reaction mixtures were incubated at 37 ^{o}C for 14 h. The reaction was monitored and analyzed as described under 2.4.4. The product with an *Rf* value of 0.35 was purified by paper chromatography as described under 2.4.3.2. The product band was excised, eluted with distilled water and lyophilized. The structure was determined in NMR experiments by comparing with authentic 1-deoxy-D-xylulose 5-phosphate. The ^{31}P-NMR spectrum showed a signal at 4.2 ppm indicating to a monophosphate of 1-deoxy-D-xylulose 5-phosphate.

In a further NMR experiments, [3,4,5-^{13}C$_3$]1-deoxy-D-xylulose was used as substrate with recombinant D-xylulokinase. [3,4,5-^{13}C$_3$]1-deoxy-D-xylulose (3 mg) was incubated with 70 µg of purified D-xylulokinase in the presence of 10 mM ATP, 10 mM $MgCl_2$ and 1 mM glutathione at 37 ^{o}C for 1 h (in a total volume of 1 ml). The reaction product was analyzed directly in the reaction mixture by NMR spectroscopy. ^{13}C^{31}P coupling of the reaction product confirmed that the phosphate moiety is located at the position 5-hydroxy group of 1-deoxy-D-xylulose (Table 3.1.3). These data clearly showed that D-xylulokinase phosphorylates 1-deoxy-D-xylulose at C-5 hydroxy group yielding 1-deoxy-D-xylulose 5-phosphate (Fig. 3.1.1).

Table 3.1.3   ^{13}C NMR data of 1-deoxy-D-xylulose and 1-D-xylulose 5-phosphate

	[3,4,5-[13]C$_3$]1-Deoxy-D-xylulose		[3,4,5-[13]C$_3$]1-Deoxy-D-xylulose 5-phosphate		
Position	Chemical shifts (ppm)	Coupling constants (Hz) J_{CC}	Chemical shifts (ppm)	Coupling constants (Hz) J_{CC}	J_{CP}
3	76.8 (d)	39.6	76.7 (d)	39.6	
4	71.1 (dd)	39.6, 42.5	70.4 (ddd)	39.8, 43.6	7.2
5	61.9 (d)	42.2	63.8 (dd)	43.6	4.7

3.1.6   Characterization of D-xylulokinase from *E. coli*

3.1.6.1   Substrate specificity

The phosphorylation activity of recombinant D-xylulokinase was investigated with the photometric assay system using different polyol compounds as substrates (2.4.2.6.1). Highest phosphorylation rates were determined with D-xylulose as substrate (51 µmol min^{-1}mg^{-1}) (Table 3.1.4). The phosphorylation rates of other polyols are significantly lower. Only 1-deoxy-D-xylulose could serve as substrate at significant rates (10% as compared to D-xylulose). D-Ribulose and D-threitol could serve as substrates at low rates and the phosphorylation rates of L-erythrulose and D-erythrose are even lower. Three-carbon or branched sugars could not serve as substrates (Table 3.1.4).

Table 3.1.4   Substrate specificity of D-xylulokinase of *E. coli*

Substrate	Relative activity (%)
D-Xylulose	100
1-Deoxy-D-xylulose	10
D-Ribulose	4.0
D-Threitol	2.4
L-Erythrulose	0.6
D-Erythrose	0.4
D-Xylose	0
(D,L)-Glyceraldehyde	0
D-Ribose	0
Dihydroxyacetone	0
L-Arabinose	0
2*C*-Methyl-D-erythritol	0

3.1.6.2   Nucleoside triphosphate dependency

Recombinant D-xylulokinase requires Mg^{2+} and ATP for the phosphorylation of 1-deoxy-D-xylulose (2.4.2.6.1). Highest rates were determined with ATP as phosphorylation donor group, but several other nucleoside triphosphate could substitute for ATP at reduced catalytic rates as shown in Table 3.1.5.

Table 3.1.5   Nucleoside triphosphate dependency of D-xylulokinase using 1-deoxy-D-xylulose as substrate

Nucleoside triphosphate	Relative activity (%)
ATP	100
GTP	38
ITP	26
CTP	18
UTP	8

3.1.6.3   Kinetic parameters of D-xylulokinase

The kinetic parameters of D-xylulokinase were determined with the photometric assay under steady state conditions (2.4.2.6.1). The plot 1-deoxy-D-xylulose concentrations [S] versus initial velocities (V) obeys the Michaelis-Menten equation. The reciprocal plot exhibited K_M and V_{max} values of 1.4 mM and 32 µmol min^{-1}mg^{-1}, respectively (Fig. 3.1.6).
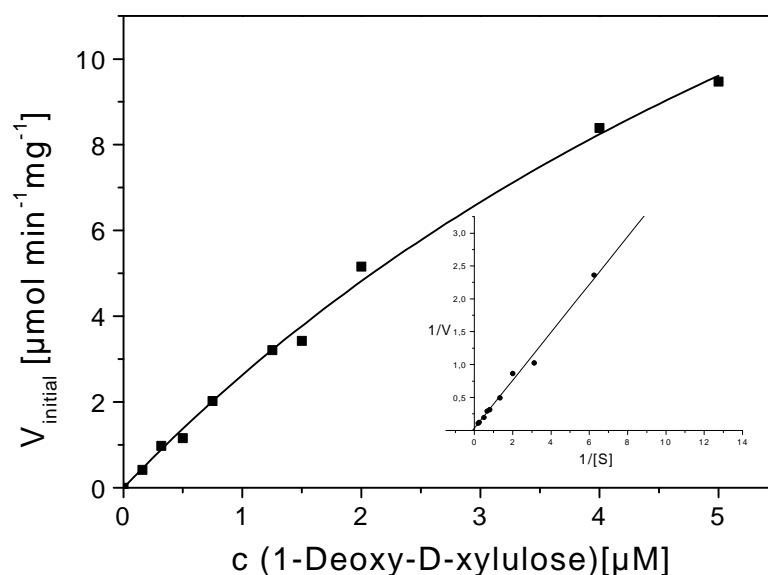
Figure 3.1.6   Michaelis-Menten plot of D-xylulokinase (inset: Lineweaver-Burk plot)

3.2   1-DEOXY-D-XYLULOSE 5-PHOSPHATE REDUCTOISOMERASE

An intramolecular rearrangement reaction was expected to occur with 1-deoxy-D-xylulose 5-phosphate yielding 2*C*-methyl-D-erythritol 4-phosphate (see introduction). In order to search for enzymes involved in the deoxyxylulose phosphate pathway downstream from 1-deoxy-D-xylulose 5-phosphate, radiolabeled 1-deoxy-D-xylulose 5-phosphate was incubated with wild type *E. coli* cell extracts as described under methods 2.4.2.6.3 A. Preliminary data showed that a new intermediate was formed in reaction mixtures containing NADPH and Mg^{2+}. The results agreed with the expected formation of 2*C*-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate (Arigoni et al. 1997, Sagner et al. 1998, Duvold et al. 1997) which was expected to depend on the presence of reduction equivalents such as NADPH or NADH. Thus, a putative enzyme catalyzing this reaction was purified from cell extracts of wild type *E. coli*. The amino acid sequence of the protein was analyzed and the gene specifying this protein was cloned. The recombinant protein was characterized in some detail.

3.2.1   Optimization of assay conditions for 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity determination

In order to purify 1-deoxy-D-xylulose 5-phosphate reductoisomerase from wild type *E. coli*, a radiochemical assay for the determination of enzyme activity was developed using [1,2-^{14}C]1-deoxy-D-xylulose 5-phosphate as substrate (2.4.2.6.3 A). This assay was optimized during protein purification and some properties of 1-deoxy-D-xylulose 5-phosphate reductoisomerase were determined using the partially purified protein. Reaction mixtures were prepared and incubated as described under 2.4.2.6.3 A. The mixture was separated by thin layer chromatography (2.4.3.1) and the radiochromatograms were analyzed (2.4.4). The *Rf* values of the dephosphorylated substrate 1-deoxy-D-xylulose and the dephosphorylated putative reaction product 2*C*-methyl-D-erythritol were 0.56 and 0.44, respectively. The enzyme activity was determined as pmol of [1,2-^{14}C]2*C*-methyl-D-erythritol, formed.

3.2.1.1 Stability of 1-deoxy-D-xylulose 5-phosphate reductoisomerase

The stability of the enzyme was tested at temperatures of 20, 4 and –20 $^{\circ}$C in 50 mM Tris hydrochloride, pH 7.4, containing 5 mM $MgCl_2$, 1 mM DTE and 0.02% NaN_3 (Fig 3.2.1). The enzyme activity was reduced after 4 days at 20 $^{\circ}$C and after 2 weeks at 4 $^{\circ}$C. At –20 $^{\circ}$C, the enzyme activity was reduced moderately after 7 weeks. The enzyme retained activity during storing at –70 $^{\circ}$C for at least 6 months. Thawing and freezing caused inactivation.
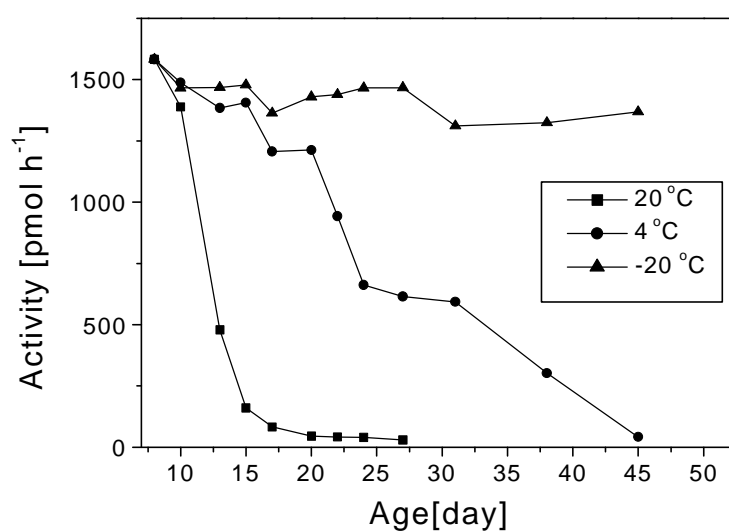
Figure 3.2.1 Stability curve of 1-deoxy-D-xylulose 5-phosphate reductoisomerase

3.2.1.2 pH-Dependency

The enzyme activity was determined in different buffers (see below). The enzyme was active in a wide pH range with an optimum between pH 7.5 and 8.5 as shown in Fig. 3.2.2.

Buffers used :   50 mM Sodium potassium phosphate, pH 6.5

200 mM Tris hydrochloride, pH 7.3

200 mM Tris hydrochloride, pH 7.5

200 mM Tris hydrochloride, pH 8.3

200 mM Tris hydrochloride, pH 8.6

200 mM Tris hydrochloride, pH 9.2

200 mM Tris hydrochloride, pH 10.2
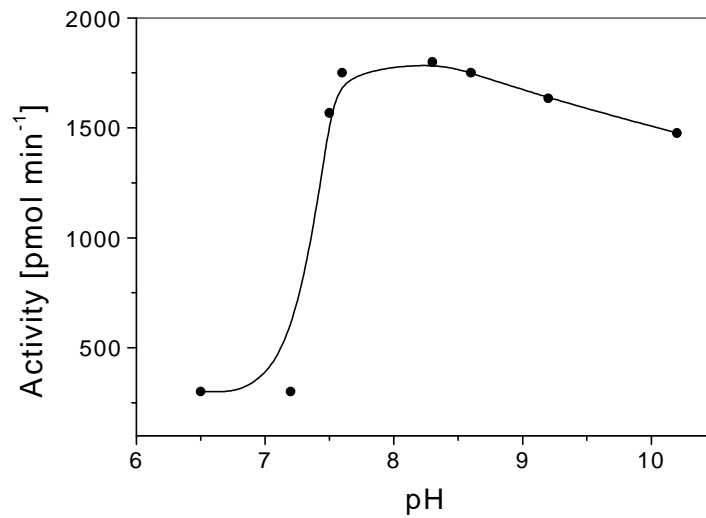
Figure 3.2.2   Effect of different pH values on 1-deoxy-D-xylulose 5-phosphate
reductoisomerase activity

3.2.1.3   Temperature dependency

The enzyme activity was determined at different temperatures. The optimum temperature was
between 30-40 $^{\circ}$C as shown in Fig 3.2.3.
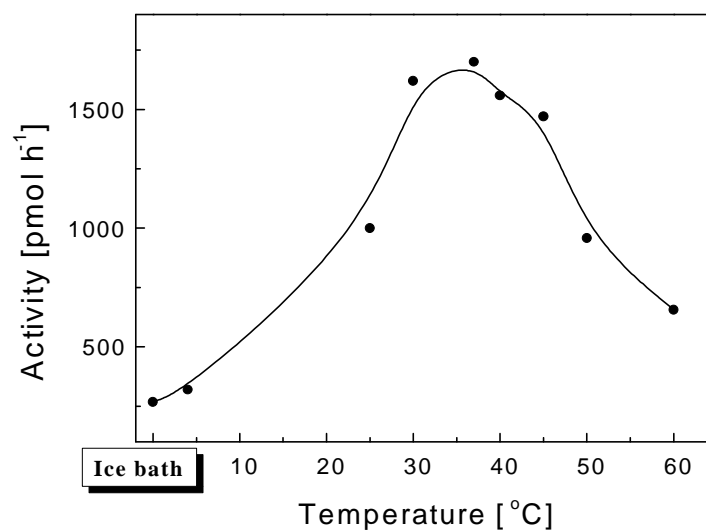
Figure 3.2.3   Effect of temperature on 1-deoxy-D-xylulose 5-phosphate reductoisomerase
activity

3.2.1.4   Metal dependency

The enzyme activity was determined using various divalent cations at concentrations of 5 mM (Table 3.2.1). Highest rates were measured with Mn^{2+} and Mg^{2+} ions. Other divalent cations could also serve as cofactors albeit at lower rates. The enzyme is active without addition of any metal ion indicating that the protein still carried metal ions. The enzyme could be inactivated by the addition of EDTA. However, the addition of metal ions recovered the enzyme activity.

Table 3.2.1   Effect of divalent cations on 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity

Divalent cation (5 mM)	Relative activity (%)
plus 1 mM EDTA	2
Mg^{2+}	97
Ca^{2+}	22
Mn^{2+}	100
Ni^{2+}	10
Fe^{2+}	49
Cu^{2+}	9

Additionally, the concentration dependency of 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity for Mn^{2+} and Mg^{2+} ions was determined (Fig. 3.2.4). For Mn^{2+} ions, highest rates were measured at about concentrations of 1 mM, whereas Mg^{2+} gave the highest rates at concentrations of about 7 mM.
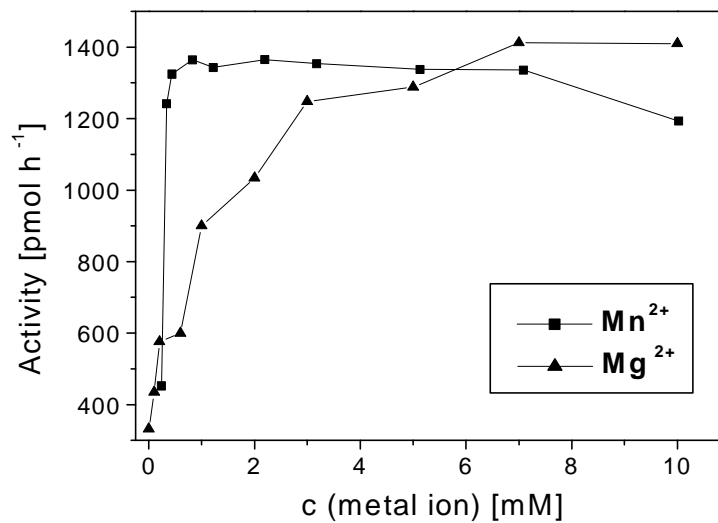
Figure 3.2.4   Concentration dependency of 1-deoxy-D-xylulose 5-phosphate
reductoisomerase activity for Mn^{2+} and Mg^{2+} ions

3.2.1.5   Pyridine nucleotide dependency

The pyridine nucleotides NADH and NADPH were tested in various concentrations for proofing their capabilities as electron donors (Fig. 3.2.5). Higher rates were observed in the assay mixtures containing NADPH at 50 µM. The enzyme showed only 5% activity when NADPH was replaced with NADH (100 µM). These data clearly showed that only NADPH efficiently serves as cofactor for 1-deoxy-D-xylulose 5-phosphate reductoisomerase.
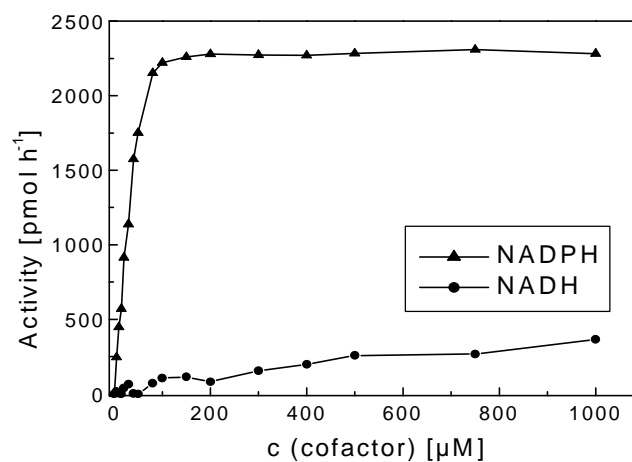
Figure 3.2.5 Pyridine nucleotide dependency

3.2.2 Purification of 1-deoxy-D-xylulose 5-phosphate reductoisomerase from wild type

E. coli **DH5a**

1-Deoxy-D-xylulose 5-phosphate reductoisomerase was purified from wild type *E. coli* DH5α and the enzyme activity was measured with a radiochemical method. The assay conditions of a radiochemical method were optimized as described above (3.2.1). Assay mixtures were performed at 37 ^{o}C in Tris hydrochloride, pH 8.0, containing NADPH and Mn^{2+} as cofactors and sodium fluoride as phosphatase inhibitor (see methods 2.4.2.6.3 A). These conditions were used as standard conditions for monitoring the 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity during protein purification from wild type *E. coli*.

400 g *E. coli* cells were suspended in 1.2 l of buffer C (2.2.5). Cell extract was prepared as described under 2.4.2.2.1. The total protein of the cell extract was 21.8 g. The purification of 1-deoxy-D-xylulose 5-phosphate reductoisomerase is summarized in Table 3.2.2.

The protein solution was loaded on top of Sepharose Q FF column (4.6 x 24 cm, Amersham Pharmacia Biotech) which had been equilibrated with buffer C. The flow rate was 5 ml min^{-1}. The column was developed by a linear gradient of 0-1 M NaCl in buffer C (total volume 600 ml). The chromatogram of this step is shown in Fig. 3.2.6 A. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase was eluted at 0.5-0.7 M NaCl. Fractions were combined. The enzyme was purified 1-fold with 53% enzyme recovery (Table 3.2.2).

Saturated ammonium sulfate was added to the protein solution to a final concentration of 0.5 M. The protein solution (11 g) was applied on top of a Phenyl Sepharose 6FF column (high substance, 4.6 x 10 cm, Amersham Pharmacia Biotech) which had been equilibrated with 0.5 M ammonium sulfate in buffer C. The column was developed at a flow rate of 5 ml min^{-1}. Proteins were eluted by a linear gradient of 0.5-0 M ammonium sulfate in buffer C (total volume 300 ml). 1-Deoxy-D-xylulose 5-phosphate reductoisomerase was eluted isocratically with a 10% glycerol solution in buffer C as shown in Fig.3.2.6 B. Active fractions were combined and dialyzed against buffer C. This step gave 3-fold purification and 25% yield (Table 3.2.2).
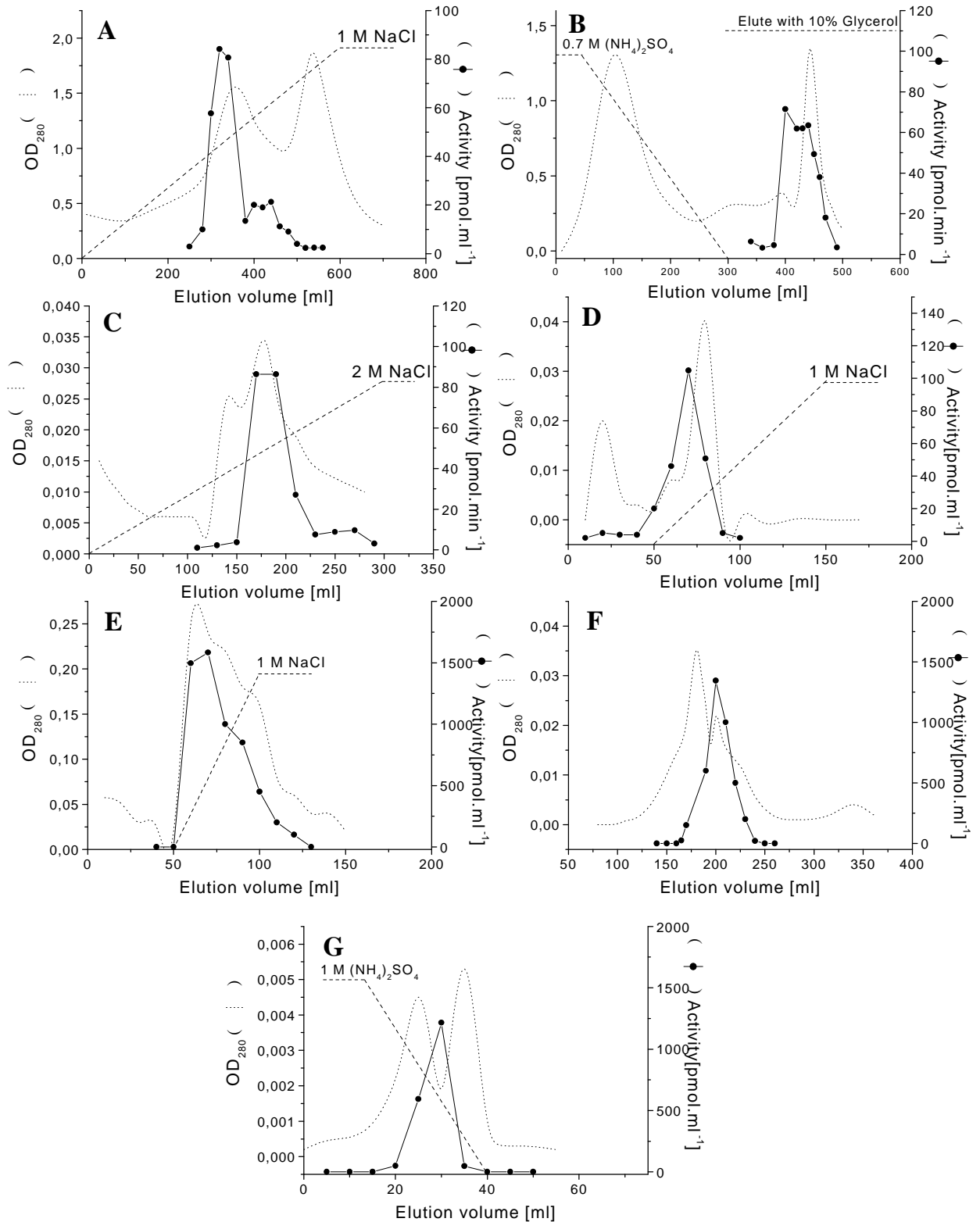
Figure 3.2.6 Chromatographic steps for the purification of 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *E. coli* DH5α; A: Sepharose Q FF; B: Phenyl Sepharose 6FF; C: Cibacron Blue 3GA; D: Reactive Yellow 86; E: Reactive Green 19; F: Superdex 200 HR; G: Resource PHE

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The protein (1.9 g) was then loaded on top of a Cibacron Blue 3GA 3000CL column (2.6 x 10 cm, Sigma) which had been equilibrated with buffer C at a flow rate of 3 ml min^{-1}. The column was developed by a linear gradient of 0-2 M NaCl in buffer C (total volume 300 ml). The enzyme was eluted at 0.7-1.5 M NaCl as shown in Fig. 3.2.6 C. Active fractions were combined, concentrated by ultrafiltration (Amicon UF 30, MWCO 30 kDa) and dialyzed against buffer C. The yield was 3% in this step (Table 3.2.2).

Protein (280 mg) was applied on top of a Reactive Yellow 86 column (2.5 x 10 cm, Sigma) which had been equilibrated with buffer C at a flow rate of 3 ml min^{-1}. The column was developed by a linear gradient of 0-1 M NaCl in 100 ml of buffer C. The enzyme was eluted at 0-0.3 M NaCl as shown in Fig. 3.2.6 D. Active fractions were combined and dialyzed against buffer C. The enzyme was purified 5-fold with 1.5% yield (Table 3.2.2).

Protein (60 mg) was then loaded on top of a Reactive Green 19 column (1.2 x 10 cm, Sigma) which had been equilibrated with buffer C. The flow rate was 3 ml min^{-1}. The column was developed by a linear gradient of 0-1 M NaCl in a total volume of 100 ml. The enzyme was eluted at 0-0.3 M NaCl as shown in Fig. 3.2.6 E. Active fractions were combined and concentrated by ultrafiltration (Amicon UF-30). This step gave a 29-fold purification with a 1.6% yield (Table 3.2.2).

Protein (12 mg) was loaded on top of a Superdex 200 HR column (2.6 x 60 cm, Amersham Pharmacia Biotech) which had been equilibrated with buffer C containing 50 mM NaCl at a flow rate of 3 ml min^{-1}. The enzyme was eluted at a volume of 197 ml, corresponding to a molecular mass of 83 kDa. The chromatogram of this step is shown in Fig. 3.2.6 F. The enzyme was purified 34-fold with 0.8% enzyme recovery (Table 3.2.2). The fractions were combined and saturated ammonium sulfate was added to a final concentration of 1 M.

The protein solution (5 mg) was applied to a column of Resource PHE (column volume, 1 ml) which had been equilibrated with buffer C containing 1 M ammonium sulfate at a flow rate of 1 ml min^{-1}. Proteins were eluted by a linear gradient of 1-0 M ammonium sulfate in buffer C (total volume 30 ml). The enzyme was eluted at 0.3-0.1 M ammonium sulfate as shown in Fig 3.2.6 G. Active fractions were combined and dialyzed against buffer C. The enzyme was purified to 61-fold with 0.2% yield (Table 3.2.2).

The enzyme (0.5 mg) was further purified on a column of Mono Q HR (0.5 x 5 cm) which had been equilibrated with buffer C at a flow rate of 2 ml min^{-1}. The column was developed by a linear gradient of 0-0.5 M NaCl in buffer C (total volume 60 ml). The enzyme was eluted at 150-200 mM NaCl. The active fraction contained 36 µg protein.

Table 3.2.2   Purification of 1-deoxy-D-xylulose 5-phosphate reductoisomerase from wild type *E. coli* DH5α

Procedure	Total activity (pmol h^{-1})	Specific activity (pmol h^{-1}mg^{-1})	Yield %	Purification factor
Cell extract	785000	36.0	100	1
Sepharose Q FF	417000	38.0	53	1
Phenyl Sepharose 6 FF	196600	104	25	3
Cibacron Blue 3GA	22900	81.8	3	2
Reactive Yellow 86	11800	197	1.5	5
Reactive Green 19	12500	1042	1.6	29
Superdex 200 HR	6110	1222	0.8	34
Resource PHE	1100	2200	0.2	61

3.2.3   Amino acid sequence analysis

Sequence analysis of the purified enzyme was performed by TOBLAB (Gesellschaft für angewandte Biotechnologie mbH). After tryptic cleavage (Bieman, 1989), the resulting peptide fragments were analyzed by mass spectroscopy. The (M+H)-peaks of the fragment ions were used as queries for searching matches in the *E. coli* database. 11 of 51 Fragment ions of the peptide sequence matched the gene product of the *yaeM* gene, rather recently renamed as *dxr* gene specifying 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Kuzuyama et al. 1998b, Takahashi et al. 1998) (Table 3.2.3). In order to confirm these findings, a recombinant *E. coli* *dxr* clone was constructed, and the recombinant enzyme was purified and studied enzymatically (3.2.4-3.2.7).

Table 3.2.3   Matched 11 from 51 peptide sequences of 1-deoxy-D-xylulose 5-phosphate reductoisomerase

(M+H) data submitted	(M+H) matched	Peptide sequences	Modification
805.4168	805.4242	(K)EVMRLAS(-)	
823.4232	823.4388	(R)LFMDAVK(Q)	
936.4640	936.4440	(R)HNPEHFR(V)	
950.4920	950.4947	(K)GLEYIEAR(W)	
984.4698	984.4474	(R)HPNWSMGR(K)	
1000.4330	1000.4423	(R)HPNWSMGR(K)	1 Met-ox
1048.5280	1048.5210	(K)TMLQQQGSR(T)	
1410.7370	1410.6952	(R)TPIAHTMAWPNR(V)	1-Met-ox
1533.8710	1533.8528	(R)FTDIAALNLSVLEK(M)	
1778.8580	1778.8383	(R)YQDGSVLAQLGEPDNR(T)	
1794.8270	1794.8332	(R)YQDGSVLAQLGEPDMR(T)	1-Met-ox

3.2.4   Overexpression of the *yaeM* gene of *E. coli*

The *dxr* gene was cloned into the expression plasmid pQE30 under the control of a T5 promoter and *lac* operator. The procedure of *dxr* gene expression is described under 2.4.2.1. Cells of the recombinant *E. coli* strain M15-pQE-dxr were prepared as described under 2.4.1.2. Cell extracts of the recombinant *E. coli* strain M15-pQE-dxr contained a 43 kDa peptide accounting for 50% of the total soluble protein as shown by SDS-PAGE (Fig 3.2.7).

3.2.5   Purification of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli*

A photometric assay was used to determine the activity of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase as described under 2.4.2.6.3 B. The purification of the recombinant 1-Deoxy-D-xylulose 5-phosphate reductoisomerase is summarized in Table 3.2.3.

Cells of the recombinant *E. coli* strain M15-pQE-dxr (2 g) were suspended in 15 ml of buffer D (2.2.5) containing 20 mM imidazole. Cell extract was prepared as described under 2.4.2.2.2. The specific activity of the cell extract was 3.1 μmol min^{-1}mg^{-1}. The cell extract (192 mg) was loaded on top of a Ni^{2+}-Chelating Sepharose (2 x 8.5 cm, Amersham Pharmacia Biotech) which had been equilibrated with 20 mM imidazole in buffer D at a flow rate of 3 ml min^{-1}. The column was washed with 60 ml of 20 mM imidazole in buffer D and protein was eluted by a linear gradient of 20-500 mM imidazole in buffer D (total volume 250 ml). The enzyme was eluted at 150-200 mM imidazole. Fractions containing 1-deoxy-D-xylulose 5-phosphate reductoisomerase were combined according to SDS-PAGE and dialyzed against buffer B (2.2.5). The specific enzyme activity was 6.8 μmol min^{-1}mg^{-1}. The protein migrated as a single band on SDS-PAGE (Fig. 3.2.7).

Table 3.2.4   Purification of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *E. coli*

Procedure	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1}mg^{-1})	Yield %	Purification factor
Cell extract	279	3.1	100	1
Ni^{2+}-Sepharose FF	374	6.8	134	2
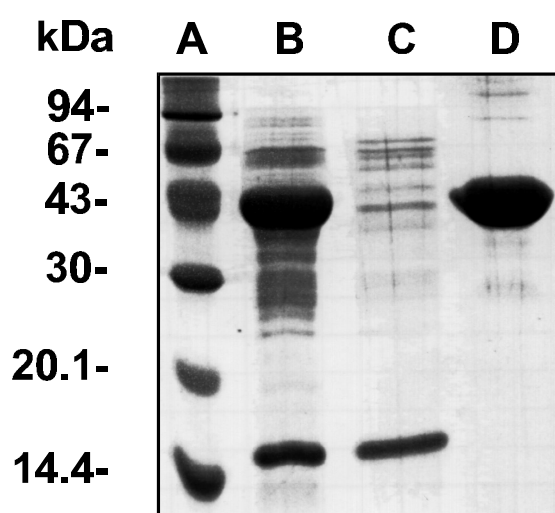
Figure 3.2.7   SDS-PAGE : purification of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli* ; A: molecular mass markers; B: cell extract of *E. coli* strain M15-pQE-dxr ; C: flow through after Ni^{2+}-Sepharose column ; D: after Ni^{2+}-Sepharose column

3.2.6   Characterization of the enzyme product

The enzyme catalyzed reaction was confirmed by enzymatic studies using partially purified 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *E. coli* and [2-^{13}C]1-deoxy-D-xylulose 5-phosphate as substrate (Hecht et al. 2001).

The reaction mixture contained 50 mM Tris hydrochloride, pH 8.3, 5 mM MgCl$_2$, 100 mM NADPH, 40 mM NaF, [2-^{13}C]1-deoxy-D-xylulose 5-phosphate and partially purified 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli* in a total volume of 3 ml. The reaction mixture was incubated at 37 ^{o}C and the reaction was monitored via detection of the ^{13}C signal of C-2 of 2*C*-methyl-D-erythritol 4-phosphate at 72 ppm (Kuzuyama et al. 1998b). After 18 h, the reaction was about 90% completed.

The enzyme product was purified by HPLC on a column of Nucleosil 10SB (size 4.6 x 250 mm) using 50 mM ammonium formate in 0.5 M formic acid as eluent at a flow rate of 1 ml min^{-1}. The effluent was monitored with a refractometer (2.4.3.3.2). The elution volume of 2*C*-methyl-D-erythritol 4-phosphate was 15 ml. Fractions were combined and lyophilized. The structure of the product was confirmed by NMR spectroscopy by comparing with an authentic sample of 2*C*-methyl-D-erythritol 4-phosphate (Kis et al. 2000). A sample of 2*C*-methyl-D-erythritol 4-phosphate was dephosphorylated with alkaline phosphatase and the dephosphorylated product was purified on a column of Rezex RPM Monosaccharide (Phenomenex) as described under 2.4.3.3.1. The retention volume of dephosphorylated reaction product and 2*C*-methyl-D-erythritol were identical (15 ml). Fractions were combined and lyophilized. Data from NMR spectroscopy and HPLC analysis confirmed that the reaction product was 2*C*-methyl-D-erythritol 4-phosphate. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate into 2*C*-methyl-D-erythritol 4-phosphate in a NADPH dependent reaction (Fig 3.2.8, Kuzuyama et al. 1998b).

1-Deoxy-D-xylulose 5-phosphate

2C-Methyl-D-erythrose 4-phosphate

2C-Methyl-D-erythritol 4-phosphate
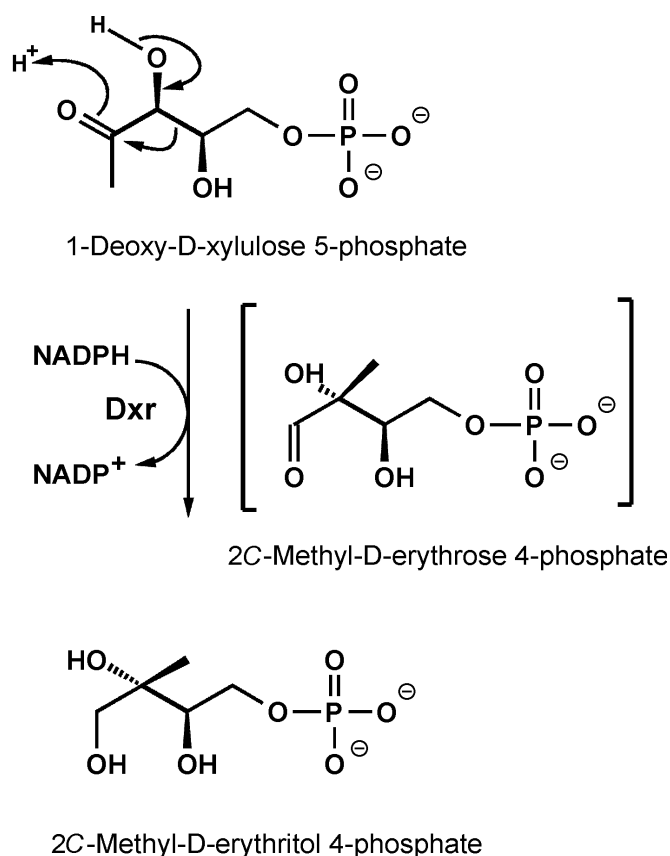
Figure 3.2.8 The skeletal rearrangement and reduction of 1-deoxy-D-xylulose 5-phosphate by 1-deoxy-D-xylulose 5-phosphate reductoisomerase

3.2.7 Characterization of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli*

3.2.7.1 Kinetic parameters of 1-deoxy-D-xylulose 5-phosphate reductoisomerase

The kinetic parameters of 1-deoxy-D-xylulose 5-phosphate reductoisomerase were determined with a photometric assay (2.4.2.6.3 B) under steady state conditions. The Michaelis-Menten plots of the initial velocities versus substrate concentrations (0-300 µM) are shown in Fig 3.2.9. The maximum velocity (V_{max}) of the reaction was 18 µmol $min^{-1}mg^{-1}$. The K_M values of 1-deoxy-D-xylulose 5-phosphate and NADPH were 171 µM and 25 µM, respectively, indicating a higher affinity of the enzyme to NADPH than to 1-deoxy-D-xylulose 5-phosphate.
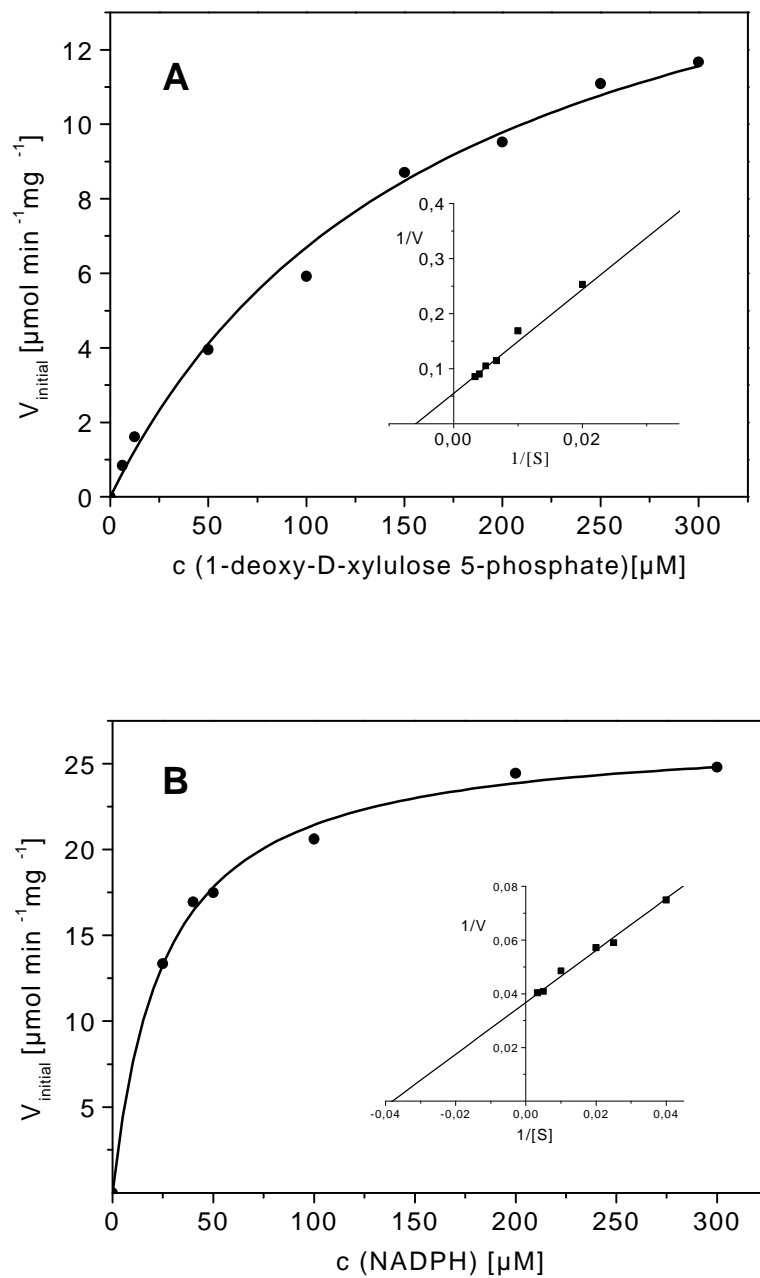
Figure 3.2.9 Michaelis-Menten plots of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (inset : Lineweaver-Burk plot); A: versus 1-deoxy-D-xylulose 5-phosphate concentrations; B: versus NADPH concentrations

3.2.7.2   Inhibition effect of fosmidomycin and fosfomycin on 1-deoxy-D-xylulose 5-phosphate reductoisomerase

Fosmidomycin and fosfomycin had been reported as antibiotics of *E. coli* (Iguchi et al. 1980). Fosfomycin acting as phosphoenolpyruvate analogue, irreversibly inhibits the enolpyruvyl transferase of peptidoglycan biosynthesis of *E. coli* (Kahan et al. 1974) and fosmidomycin was assumed to inhibit prenyltransferases in the biosynthesis of β-carotene in plants (Shigi 1989). Therefore, it had been proposed that the lethal effect of this antibiotics is related to the biosynthetic pathway of terpenoids. Moreover, the structure of fosmidomycin is similar to 2*C*-methyl-D-erythrose 4-phosphate, a putative intermediate of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase reaction (Fig. 3.2.8 and Fig 3.2.10). From these evidences, fosmidomycin and fosfomycin were assumed as putative inhibitors of the deoxyxylulose phosphate pathway, especially 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

2*C*-Methyl-D-erythrose 4-phosphate   Fosmidomycin   Fosfomycin
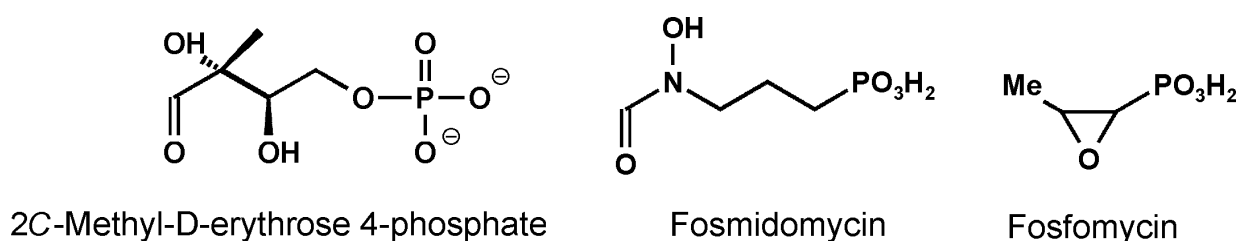
Figure 3.2.10   Structures of 2*C*-methyl-D-erythrose 4-phosphate, fosmidomycin and fosfomycin

In order to evaluate the inhibition effect of fosmidomycin and fosfomycin, 1-deoxy-D-xylulose 5-phosphate reductoisomerase activities were determined in the presence of fosmidomycin (0.01-4 µM) or fosfomycin (0.1-40 µM) as described under 2.4.2.6.3 A. As shown in Fig. 3.2.11 A, fosfomycin does not inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity. In contrast, fosmidomycin inhibits the enzyme with an IC_{50} of 56 nM (Fig 3.2.11 B).
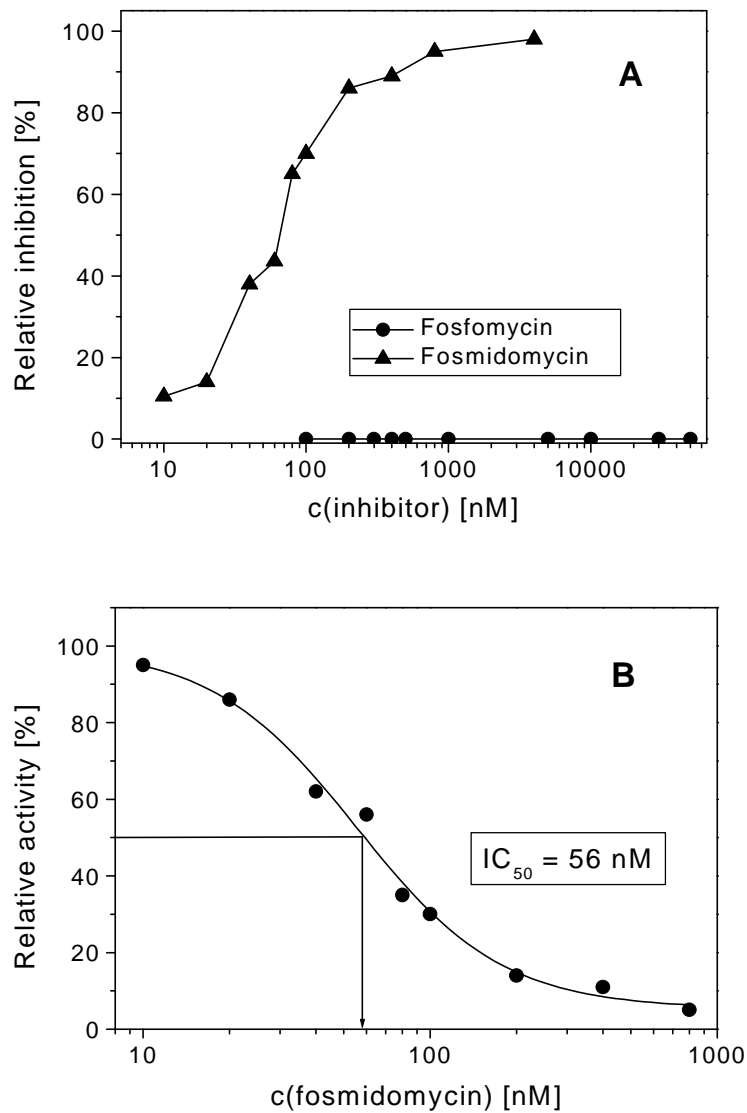
Figure 3.2.11   Inhibition of 1-deoxy-D-xylulose 5-phosphate reductoisomerase by fosmidomycin and fosfomycin; A: compared between fosfomycin and fosmidomycin; B: IC_{50} plot of fosmidomycin

3.3   4-DIPHOSPHOCYTIDYL-2*C*-METHYL-D-ERYTHRITOL SYNTHASE

In search for downstream intermediates and enzymes of the deoxyxylulose phosphate pathway, radiolabeled 2*C*-methyl-D-erythritol 4-phosphate was incubated with cell extracts from wild type *E. coli*. A radioactive product was observed when the reaction mixture contained ATP. It was assumed as a putative intermediate downstream from 2*C*-methyl-D-erythritol 4-phosphate in this pathway. From this evidence, the putative enzyme catalyzing this reaction was purified from wild type *E. coli* by column chromatography as described below.

3.3.1   Partially purification of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase from wild type *E. coli* DH5**a**

At the beginning of the purification procedure, the enzyme activity was determined using assay mixtures containing ATP (2.4.2.6.4 A), but the purification was accompanied by a severe reduction of activity. It was suggested that an essential compound required for the reaction was lost during the purification procedure. For improving the assay system, different nucleoside triphosphate were tested using partially purified enzyme of wild type *E. coli* (see method 2.4.2.6.4 A). Rates of product formation in the presence of ATP, UTP, GTP and ITP appeared only at a level of about 20% when compared to the rate of product formation in the presence of CTP. The data indicated that CTP could serve more efficiently than ATP as cosubstrate. Therefore, CTP was used as cosubstrate instead of ATP in the assay system.

Enzyme activity tests in crude enzyme fractions were performed using [2-^{14}C]2*C*-methyl-D-erythritol 4-phosphate as substrate (2.4.2.6.4 A). Aliquots of the reaction mixtures were separated by thin layer chromatography (2.4.3.1). The radiochromatograms were developed and analyzed as described under 2.4.4. The *Rf* values of [2-^{14}C]2*C*-methyl-D-erythritol 4-phosphate and the reaction product were 0.50 and 0.36, respectively. The enzyme activity was determined as pmol of product formation per time unit. The partially purification of this enzyme is summarized in Table 3.3.1.

330 g of *E. coli* DH5α cells were suspended in 1.5 l of buffer A (2.2.5). Cell extract was prepared as described under 2.4.2.2.1. The total protein amount of the cell extract was 6.7 g with a specific activity of 37 pmol min^{-1} mg^{-1}.

The cell extract was loaded on top of a Sepharose Q FF column (4.6 x 24 cm, Amersham Pharmacia Biotech) at a flow rate of 5 ml min^{-1}. The column was washed with 300 ml of buffer A and was subsequently developed by a linear gradient of 0-1.0 M NaCl in buffer A (total volume, 600 ml). Active fractions were combined. This step gave 24% yield (Table 3.3.1).

Saturated ammonium sulfate solution was added to a final concentration of 1 M. The solution (2.1 g) was applied to a column of Phenyl Sepharose 6FF (high substance, 5.6 x 10 cm, Amersham Pharmacia Biotech) which had been equilibrated with 1 M ammonium sulfate in buffer A. The column was developed by a linear gradient of 1.0-0 M ammonium sulfate in buffer A (total volume, 200 ml). The flow rate was 5 ml min^{-1}. Active fractions were combined and concentrated to 30 ml by ultrafiltration (Amicon UF-10). The enzyme was purified 9-fold with 30% protein recovery (Table 3.3.1).

The protein solution (210 mg) was dialyzed against buffer A and applied to a column of Cibacron Blue 3GA (type 3000CL, 2.6 x 10 cm, Sigma) at a flow rate of 3 ml min^{-1}. The flow through fractions were combined and concentrated by ultrafiltration (Amicon UF-10, MWCO 10 kDa membrane). The enzyme was purified 23-fold and gave 25% yield (Table 3.3.1).

Table 3.3.1 Partially purification of putative 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase from wild type *E. coli* DH5α

Procedure	Total activity (pmol min^{-1})	Specific activity (pmol min^{-1} mg^{-1})	Yield %	Purification factor
Cell extract	246000	37	100	1
Sepharose Q FF	59220	28	24	1
Phenyl Sepharose 6FF	73590	350	30	9
Cibacron Blue 3GA	61350	840	25	23

In further studies, it was shown that radioactivity from $[\alpha\text{-}^{32}P]CTP$ but not $[\gamma\text{-}^{32}P]CTP$ was incorporated into the enzyme product, indicating that the enzyme catalyzes a transfer of a cytidyl group on 2C-methyl-D-erythritol 4-phosphate (Rohdich et al. 1999). A database search retrieved the unannotated *ygbP* gene family with significant similarity to other nucleotidylyl transferases. *YgbP* orthologs were found many eubacteria as well as in *Arabidopsis thaliana*.

It was assumed that the YgbP gene product was identical with the enzyme purified from wild type *E. coli* as described above. Indeed, this hypothesis was confirmed in enzymatic studies with the recombinant *E. coli* YgbP gene product, which was shown to catalyze the formation of 4-diphosphocytidyl-2C-methyl-D-erythritol from 2C-methyl-D-erythritol 4-phosphate (see below).

3.3.2 Overexpression of the *ygbP* gene of *E. coli*

The *ygbP* gene specifying 4-diphosphocytidyl-2C-methyl-D-erythritol synthase from *E. coli* was cloned into the plasmid pNCO113 under the control of a T5 promoter and a *lac* operator. The procedure for *ygbP* gene expression is described under 2.4.2.1. Cells of the recombinant *E. coli* strain XL1-pNCO-ygbP were prepared as described under 2.4.1.2. The recombinant strain produced large amounts of a polypeptide with an apparent molecular mass of 26 kDa as judged by SDS-PAGE (Fig. 3.3.2)

3.3.3 Purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase from *E. coli*

A photometric assay for the determination of enzyme activity was developed according to a method as described by Follens et al. 1999. In this assay, the inorganic pyrophosphate formed in the reaction is consumed in a cascade of reactions conducive to the reduction of $NADP^+$ (Follens et al. 1999). This reaction is consecutively coupled with UDP-glucose pyrophosphorylase, phosphoglucomutase and glucose 6-phosphate dehydrogenase. Assays were performed as described under 2.4.2.6.4 A. Controls without substrate were performed to avoid the interference from oxidases present in protein preparations.

3. RESULTS

The purification procedure of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase is summarized in Table 3.3.2.

Cells of the recombinant *E. coli* strain XL1-pNCO-ygbP (5 g) were suspended in 50 ml of buffer B (2.2.5). Cell extract was prepared as described under 2.4.2.2.2. The cell extract (360 mg) was loaded on top of a Sepharose Q FF column (2.6 x 10 cm, Amersham Pharmacia Biotech). The column was washed with 300 ml of buffer B. Proteins were eluted by a linear gradient of 0-0.5 M NaCl in 300 ml of buffer B at a flow rate of 4 ml min^{-1} (Fig 3.3.1 A). Fractions were combined according to SDS-PAGE and dialyzed against buffer B. The enzyme was purified 8-fold in this step.

The protein solution (63 mg) was loaded on top of a Red Sepharose column (1.6 x 8 cm, Amersham Pharmacia Biotech), which was developed with buffer B at a flow rate of 2 ml min^{-1}. The enzyme was in the flow through fractions (Fig. 3.3.1 B) and was purified 5-fold with 74% yield. The protein solution (50 mg) was then loaded on top of a Source 15Q column (1.5 x 10 cm, Amersham Pharmacia Biotech), which was developed by a linear gradient of 0-0.5 M NaCl in 250 ml of buffer B at a flow rate of 3 ml min^{-1} (Fig 3.3.1 C). Fractions containing 4-diphosphocytidyl-2*C*methyl-D-erythritol synthase were combined. The enzyme was purified 11-fold with 44% protein recovery (Table 3.3.2). The protein migrated as single band at about 26 kDa indicating high homogeneity (Fig. 3.3.2).

Table 3.3.2 Purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase from *E. coli*

Procedure	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1}mg^{-1})	Yield %	Purification factor
Cell extract	770	2.1	100	1
Sepharose Q FF	1015	16.2	132	8
Red Sepharose	570	11.3	74	5
Source 15 Q	340	22.7	44	11
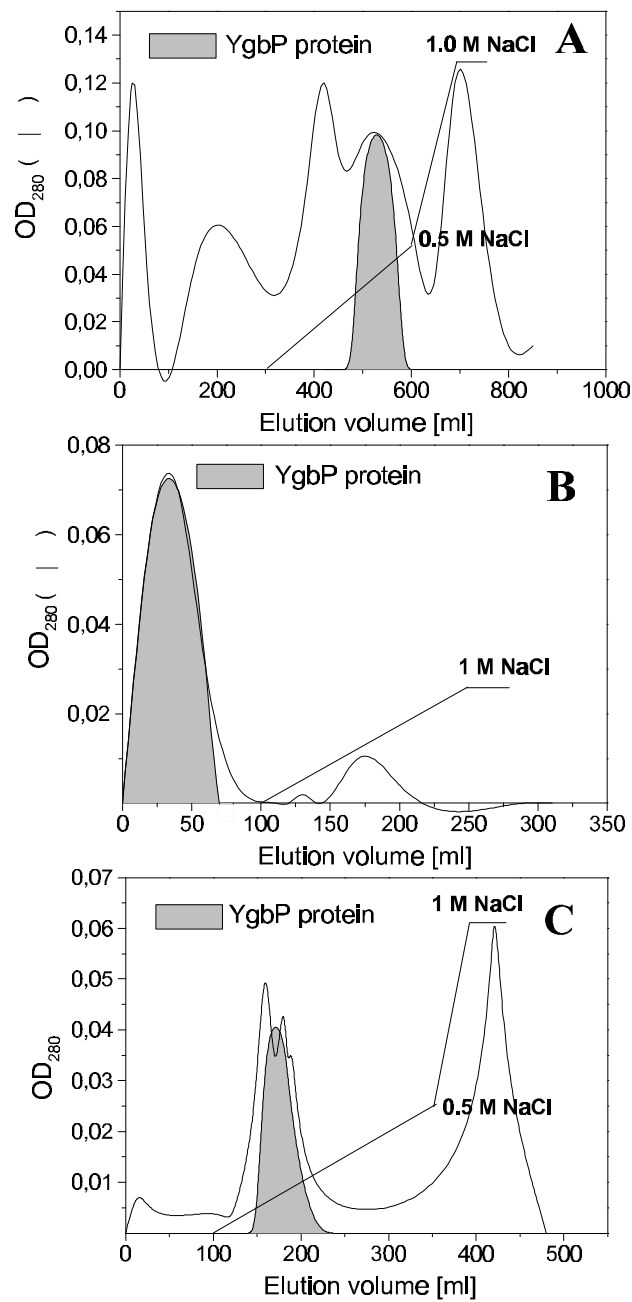
Figure 3.3.1 Chromatographic steps for the purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase of *E. coli*; A: Sepharose Q FF; B: Red Sepharose; C: Source 15Q
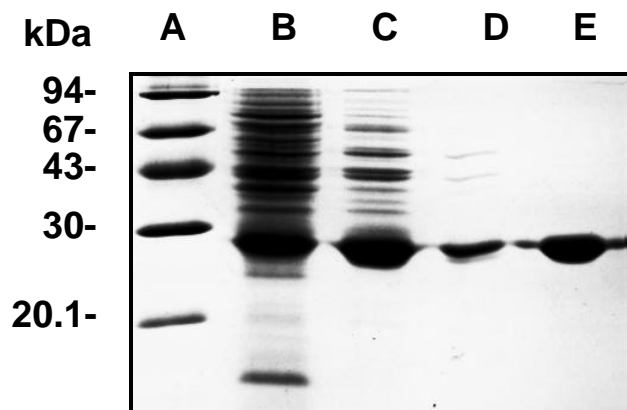
Figure 3.3.2 SDS-PAGE: purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase from *E. coli* ; A: molecular mass markers; B: cell free extract of the recombinant *E. coli* strain; C: after Sepharose Q FF; D: after Red Sepharose; E: after Source 15Q

3.3.4 Reaction product of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase

In order to determine the structure of the YgbP reaction product, it was synthesized enzymatically, isolated chromatographically and analyzed by NMR spectroscopy. The synthesis reaction contained 100 mM Tris hydrochloride pH 8.0, 10 mM $MgCl_2$, 46 mM CTP, 46 mM [2-^{14}C]2*C*-methyl-D-erythritol 4-phosphate (3.7 µCi mmol^{-1}) and 225 µg of purified recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol from *E. coli* in a total volume of 0.7 ml. The mixture was incubated at 37 ^{o}C for 1 h and the reaction was monitored by ^{31}P NMR. The product was purified by HPLC using a column of Nucleosil 10SB with the Elution No.1 (2.4.3.3.2). The effluent was monitored using a diode array photometer and a radiomonitor (2.4.3.3.2). The reaction product eluted at 30 ml. Fractions were collected and lyophilized (yield, 7 µmol).

The ^{31}P NMR spectrum of the reaction product was characterized by two signals at -7.2 ppm and -7.8 ppm (doublets with ^{31}P^{31}P coupling constants of 20 Hz) (Rohdich et al. 1999). This spectroscopic signature was attributed to a pyrophosphate motif that was also reflected in the ^{13}C NMR spectrum where 4 out of 14 signals show ^{31}P ^{13}C coupling with coupling constants in the range of 5 to 9 Hz. Two-dimensional COSY and HMQC experiments identified the spin networks of cytidine and 2*C*-methyl-D-erythritol motifs. On the basis of these data, the product structure was assigned as 4-diphosphocytidyl-2*C*-methyl-D-erythritol (Rohdich et al. 1999).

It was concluded that 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase catalyzes the formation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol from 2*C*-methyl-D-erythritol 4-phosphate and CTP (Fig 3.3.3). The cognate enzyme was named 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase.

2*C*-Methyl-D-erythritol 4-phosphate

4-Diphosphocytidyl-2*C*-methyl-D-erythritol
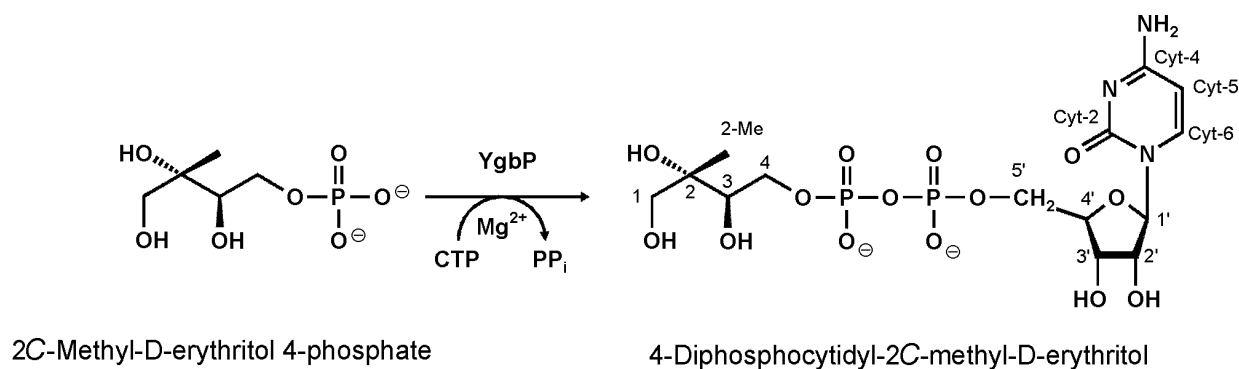
Figure 3.3.3 Reaction catalyzed by 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase (YgbP protein)

3.3.5 Characterization of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase

3.3.5.1 Native molecular mass of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase

The molecular mass of the native 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase was estimated by gel filtration on a Superdex 75 HR column (2.6 x 60 cm, Amersham Pharmacia Biotech) which had been equilibrated with 100 mM sodium chloride in buffer B (2.2.5) at a flow rate of 2 ml min^{-1}. The molecular mass was estimated by comparing with the elution of standard proteins. The elution volume of the enzyme was 150 ml corresponding to molecular mass of about 50 kDa. From Fig. 3.3.2, 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase migrated at a molecular mass of about 26 kDa on SDS-PAGE. The data suggested that the native protein is a homodimer.

3.3.5.2 Nucleoside triphosphate dependency

The nucleoside triphosphate dependency was reinvestigated with purified recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase (Table 3.3.3). Only CTP is utilized at a high rate. ATP, UTP and ITP could not be used as substrates. About 8% of activity was observed when CTP was replaced by GTP.

Table 3.3.3 Utilization of nucleoside triphosphate by recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase of *E. coli*

Nucleoside triphosphate	Relative activity (%)
CTP	100
UTP	0
GTP	8
ATP	0
ITP	0

3.3.5.3 pH Dependency

The enzyme activity was measured in different buffers (see below) as described under 2.4.2.6.4 B. The pH optimum was between 8.0-8.5 as shown in Fig. 3.3.4.

Buffers used :   50 mM Potassium phosphate, pH 6.3

                      50 mM Potassium phosphate, pH 7.0

                      50 mM Potassium phosphate, pH 7.4

                      50 mM Tris hydrochloride, pH 8.0

                      50 mM Tris hydrochloride, pH 8.5

                      50 mM Glycine-NaOH, pH 9.5

                      50 mM Glycine-NaOH, pH 10.0

                      50 mM Glycine-NaOH, pH 11.0
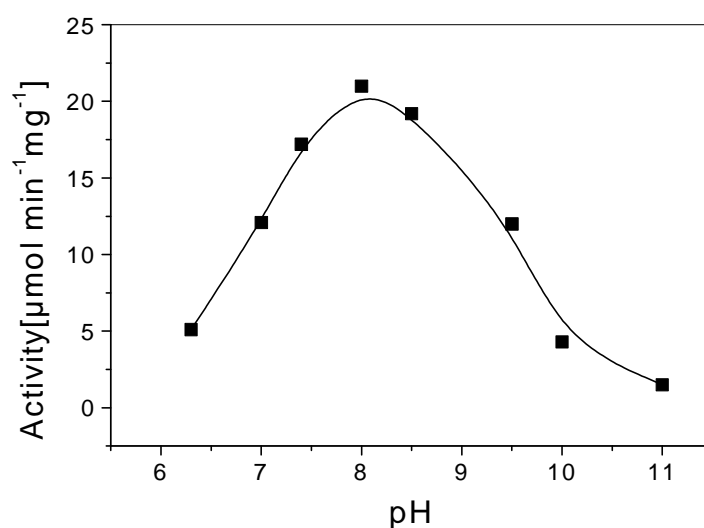
Figure 3.3.4 Effect of different pH values on 4-diphosphocytidyl-2C-methyl-D-erythritol synthase activity

3.3.5.4 Metal dependency

The enzyme activity was tested with various divalent cations in the assay mixtures (see methods 2.4.2.6.4 A) (Table 3.3.4). The enzyme was catalytically active in the presence of Co^{2+}, Mg^{2+}, Mn^{2+}, or Cu^{2+}, respectively. Other divalent cations such as Ni^{2+}, Ca^{2+}, Fe^{2+}, or Zn^{2+} could not serve as cofactors

Table 3.3.4   Effect of divalent cations on 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase activity

Cation at 5 mM	Relative activity (%)
Mg^{2+}	100[a]
Mn^{2+}	95
Co^{2+}	178
Cu^{2+}	28
Ca^{2+}	0
Zn^{2+}	0
Fe^{2+}	0

[a]The activity was referenced to 100% using Mg^{2+} as cofactor.

3.3.5.5   Substrate specificity

The substrate specificity of 4-diphosphocytidyl-2*C*-metyl-D-erythritol synthase was investigated with the sugar alcohols ribitol 5-phosphate and erythritol 4-phosphate (Table 3.3.5). The enzyme activities were measured as described under methods (2.4.2.6.4 B). The data showed that at concentration of 2 mM, erythritol 4-phosphate could serve as substrate albeit at low rate, whereas ribitol 5-phosphate could not serve as substrate.

Table 3.3.5   Substrate specificity of 4-diphosphocytidyl-2*C*-metyl-D-erythritol synthase

Compound	Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$)	Relative activity (%)
0.2 mM 2*C*-Methyl-D-erythritol 4-phosphate	17.2	100
2 mM Ribitol 5-phosphate	ND*	0
2 mM Erythritol 4-phosphate	3.2	19

*ND, not determined.

3.3.5.6 Inhibition of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase by erythritol 4-phosphate

4-Diphosphocytidyl-2*C*-methyl-D-erythritol synthase activity was determined in the presence of erythritol 4-phosphate in the standard assay (see methods 2.4.2.6.4 B). By increasing amounts of erythritol 4-phosphate in the assay mixtures an inhibition effect on enzyme activity was observed (Fig 3.3.6). A plot of relative enzyme activities versus erythritol 4-phosphate concentrations showed that erythritol 4-phosphate inhibits 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase with an IC_{50} of 1.38 mM (Fig. 3.3.6).
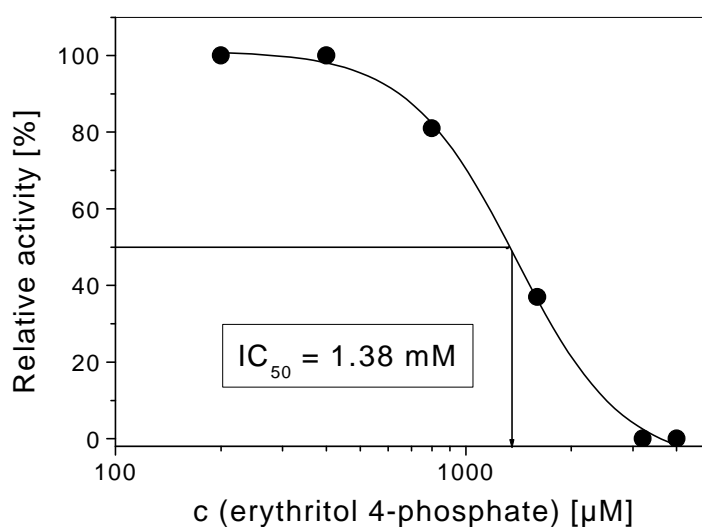
Figure 3.3.6 Inhibition of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase by erythritol 4-phosphate

3.3.5.7 Kinetic parameters of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase

The kinetic parameters of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase were determined by the direct HPLC assay (2.4.2.4.6 C). The Michaelis-Menten and Lineweaver-Burk plots are shown in Fig. 3.3.7. The reaction constant was determined by monitoring the forward reaction, namely the formation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol. Since the reaction attended to equilibrium, inorganic pyrophosphorylase was added in the assay reaction in order to hydrolyze the inorganic pyrophosphate which was formed in the reaction. The Michaelis constants (K_M) of 2*C*-methyl-D-erythritol 4-phosphate and CTP were 381 and 1200 µM, respectively with the maximum velocity (V_{max}) of 185 µmol min^{-1} mg^{-1}.
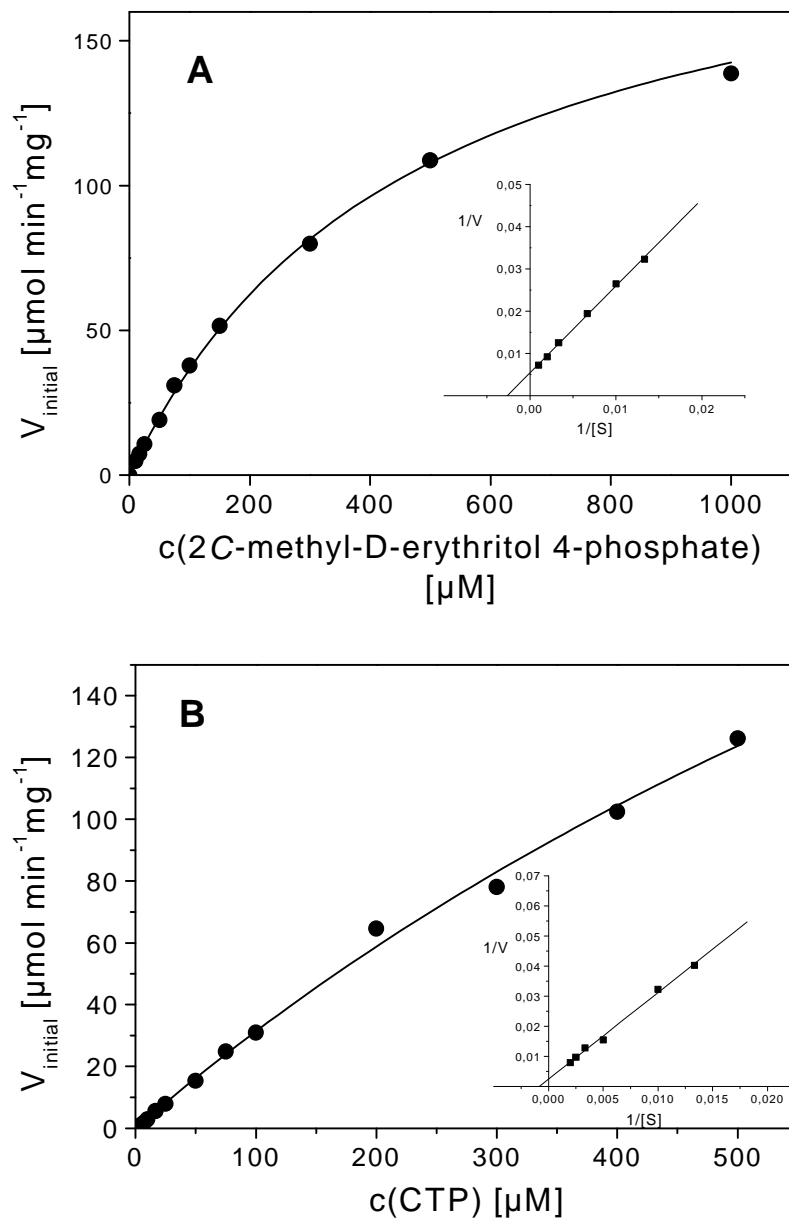
Figure 3.3.7 Michaelis-Menten plots of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase
(inset : Lineweaver-Burk plot); versus 2*C*-methyl-D-erythritol 4-phosphate
concentrations; B: versus CTP concentrations

3.4   4-DIPHOSPHOCYTIDYL-2*C*-METHYL-D-ERYTHRITOL KINASE

Genomes of organisms using the deoxyxylulose phosphate pathway for terpenoid biosynthesis must contain genes specifying all enzyme activities of that incompletely known pathway. Putative orthologs of *dxs*, *dxr* and *ygbP* genes specifying 1-deoxy-D-xylulose 5-phosphate synthase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase and 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase are found in eubacteria but not in archea. A comparative analysis of all published complete genomes retrieved the unannotated *ychB* gene of *E. coli* which followed the same distribution as the *dxs*, *dxr* and *ygbP* genes (Lüttgen et al. 2000). This evidence suggested that the hypothetical YchB protein might be involved in the deoxyxylulose phosphate pathway.

To prove this hypothesis, the *E. coli ychB* gene was cloned and hyperexpressed in a homologous host (see 3.4.1). Cell extracts of the recombinant *E. coli* strain were tested under different conditions using radiolabeled intermediates of the deoxyxylulose phosphate pathway as substrates. A radioactive product was observed when the reaction mixture contained [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol and ATP. This result indicated that the YchB protein converts 4-diphosphocytidyl-2*C*-methyl-D-erythritol in an ATP-dependent reaction into a new intermediate downstream from 4-diphosphocytidyl-2*C*-methyl-D-erythritol.

3.4.1   Overexpression of the *ychB* gene of *E. coli*

The *ychB* gene of *E. coli* was cloned into the pNCO113 plasmid under the control of a T5 promoter and a *lac* operator. The procedure of *ychB* gene expression is described in 2.4.2.1. The recombinant *E. coli* strain was found to produce a soluble polypeptide with an apparent mass of 31 kDa (about 5% of cytoplasmic protein) as judged by SDS-PAGE.

3.4.2 Purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase from *E. coli*

Enzyme activity tests were performed using [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol as substrate (2.4.2.6.5 A). Aliquots of the reaction mixtures were separated by thin layer chromatography (2.4.3.1) and the radiochromatograms were analyzed as described under 2.4.4. The *Rf* values of [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol and the reaction product were 0.36 and 0.20, respectively. The enzyme activity was determined as nmol of product formation per time unit. The purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase of *E. coli* is summarized in Table 3.4.1.

Cells of the recombinant *E. coli* strain XL1-pNCO-ychB (2 g) were suspended in 10 ml of buffer B (2.2.5). Cell extract was prepared as described under 2.4.2.2.2. The cell extract (192 mg) was applied to a column of Sepharose Q FF (2.0 x 10 cm, Amersham Pharmacia Biotech) which had been equilibrated with buffer B at a flow rate of 3 ml min^{-1}. The column was washed with 150 ml of buffer B and then developed by a linear gradient of 0-0.5 M NaCl in 150 ml. The YchB protein was eluted at 0.2-0.3 M NaCl (Fig. 3.4.1 A). Enzyme containing fractions were combined according to SDS-PAGE.

Saturated ammonium sulfate was added to the protein solution to a final concentration of 0.5 M. The protein solution (31 mg) was loaded on top of a Phenyl Sepharose 6FF column (high substance, 1.6 x 10 cm, Amersham Pharmacia Biotech) which had been equilibrated with 0.5 M ammonium sulfate in buffer B at a flow rate of 3 ml min^{-1}. The column was developed by a linear gradient of 0.5-0 M ammonium sulfate in buffer B (total volume, 100 ml). The YchB protein was eluted at 0.1-0 M ammonium sulfate as shown in Fig. 3.4.1 B. Fractions were combined and concentrated to 3 ml by ultrafiltration (Amicon UF-10, MWCO 10 kDa).

The protein solution (4.1 mg) was applied to a column of Superdex 75 HR (2.6 x 60 cm, Amersham Pharmacia Biotech). The column was developed with buffer E containing 100 mM NaCl at a flow rate of 3 ml min^{-1}. The YchB protein was eluted at volume of 170 ml (Fig 3.4.1 C). The purification of the YchB of *E. coli* resulted in 25% protein recovery with 40-fold purification. The homogeneity of the YchB protein is demonstrated by SDS-PAGE as shown Fig. 3.4.2.
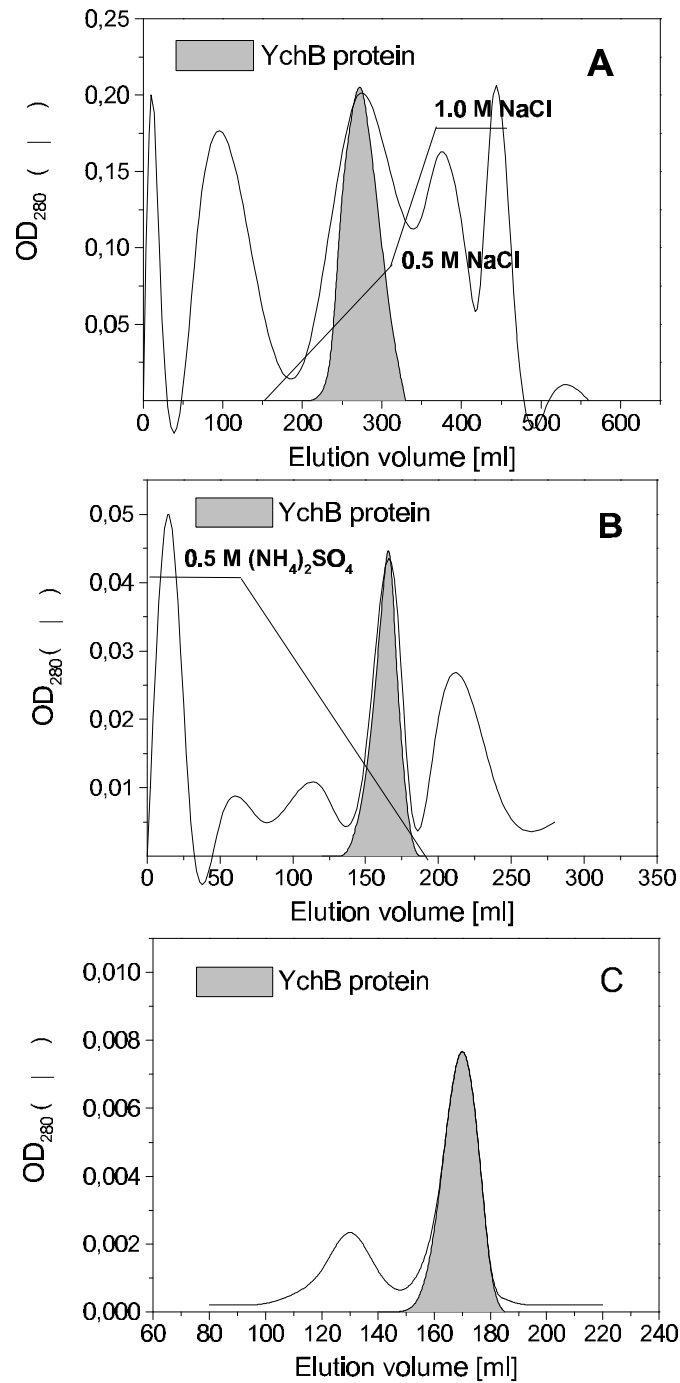
Figure 3.4.1 Chromatographic steps for the purification of recombinant 4-diphosphocytidyl-2C-methyl-D-erythritol kinase of *E. coli*; A: Sepharose Q FF; B: Phenyl Sepharose 6FF; C: Superdex 75 HR

Table 3.4.1   Purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase from *E. coli*.

Procedure	Total protein (mg)	Total activity (nmol min^{-1})	Specific activity (nmol min^{-1}mg^{-1})	Yield %	Purification factor
Cell extract	192	80	0.42	100	1
Sepharose Q FF	31	118	3.8	147	9
Phenyl Sepharose 6FF	4.1	74	18	92	43
Superdex 75 HR	1.2	20	17	25	40
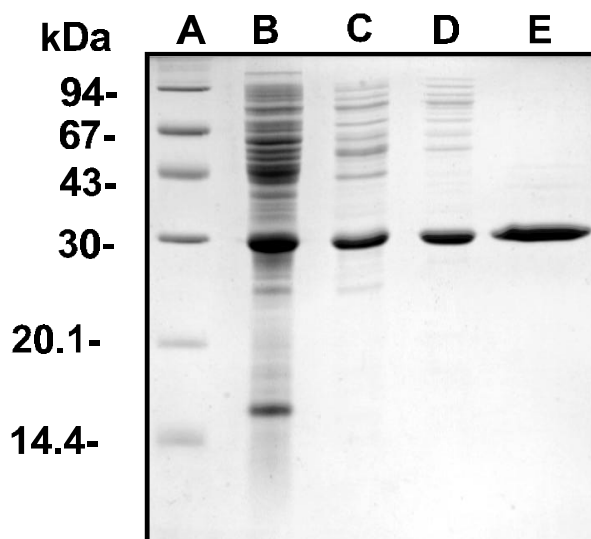
Figure 3.4.2   SDS-PAGE : purification of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase from *E. coli* ; A: molecular mass markers; B: cell free extract of the recombinant *E. coli* strain; C: after Sepharose Q FF; D: after Phenyl Sepharose 6FF; F: after Superdex 75 HR

3.4.3 Reaction product of 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase

For structure determination of the enzyme product, it was synthesized enzymatically, isolated chromatographically and analyzed by NMR spectroscopy. The synthesis mixture containing 100 mM Tris hydrochloride, pH 8.0, 5 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 5 mM [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol (117 µCi mmol^{-1}) and 100 µg of recombinant YchB protein of *E. coli* in a total volume of 4 ml was incubated at 37 $^{\circ}$C for 2 h. The reaction was terminated by ultrafiltration using a Centriprep-10 device (Amicon, MWCO 10 kDa). The reaction product was purified by HPLC using a column of Nucleosil 10 SB (Macherey Nagel) which was developed by a linear gradient of 0.1-1.0 M ammonium formate in 40% methanol (vol/vol) using the Elution No. 2 protocol as described under 2.4.3.3.2. The effluent was monitored with a radiomonitor and by measuring the UV absorbance at 270 nm. The elution volume of the enzyme product was 44 ml. Fractions were combined and lyophilized.

All NMR spectroscopic data of the YchB reaction product established 2*C*-methyl-D-erythritol and cytidine as structural motifs (Lüttgen et al. 2000). The ^{31}P NMR spectrum displayed two doublets at -7.28 ppm respectively -8.00 ppm reflecting the presence of a pyrophosphate motif (^{31}P^{31}P coupling constant, 20.8 Hz) and a double doublet at 0.49 ppm reflecting a phosphomonoester motif (^{31}P^{13}C coupling constants, 7.6 Hz and 1.7 Hz). The structure of the reaction product was assigned as 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate (Lüttgen et al. 2000).

These data clearly showed that the YchB protein catalyzes the ATP-dependent phosphorylation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol at C-2 hydroxy group yielding 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate. The enzyme was named 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase (Fig. 3.4.3).

4-Diphosphocytidyl-2*C*-methyl-D-erythritol

ATP

YchB

ADP

4-Diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate
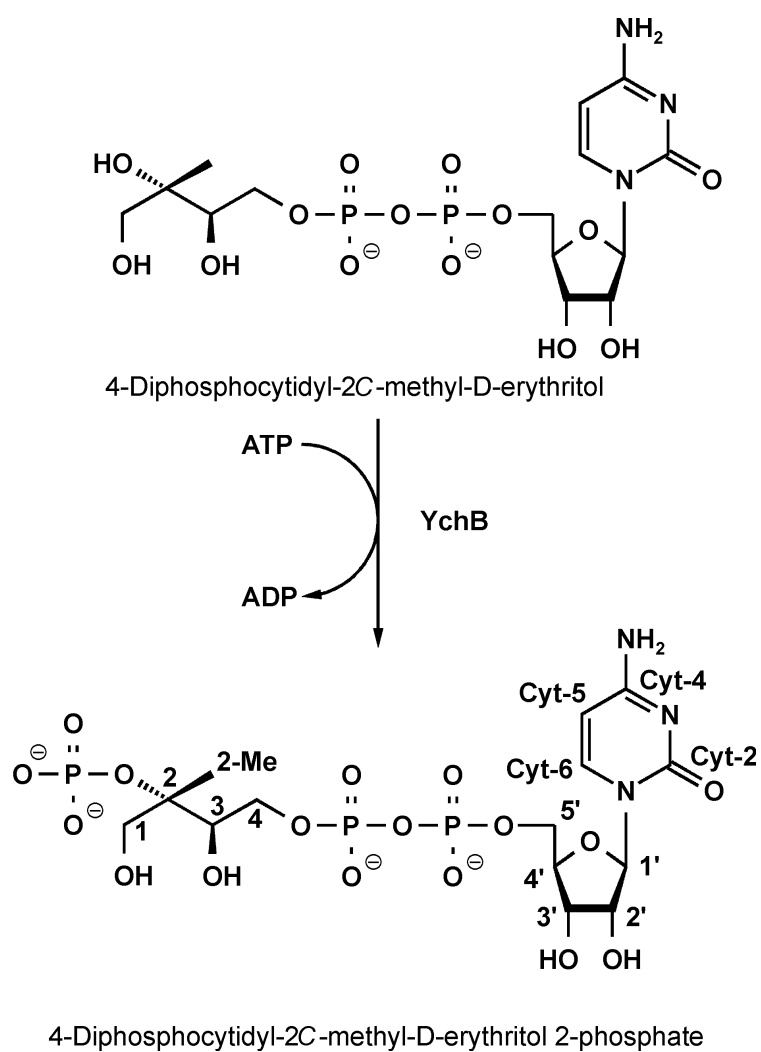
Figure 3.4.3   Reaction catalyzed by 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase (YchB protein)

3.4.4 Isopentenyl monophosphate is not a substrate of 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase

In contrast to the findings in this study (see 3.4.3), Croteau and Lange reported that the YchB proteins from *E. coli* and peppermint phosphorylate isopentenyl monophosphate yielding isopentenyl diphosphate (Lange and Croteau 1999b). In order to check this claim, [4-^{14}C] isopentenyl monophosphate was synthesized from [4-^{14}C]isopentenyl diphosphate using alkaline phosphatase (EC 3.1.3.1) (Rohdich et al. 2000). A HPLC assay was developed for direct detection of the reaction product. The rates for the phosphorylation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol respectively isopentenyl monophosphate were determined as described under 2.4.2.6.5 B and 2.4.2.6.6.

The phosphorylation rate of recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase was 34 µmol min^{-1} mg^{-1}, when 4-diphosphocytidyl-2*C*-methyl-D-erythritol was used as substrate, whereas no phosphorylation of isopentenyl monophosphate within the detection limit of this method (1.4 pmol min^{-1} mg^{-1}) was detected.

These data confirmed that 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase utilizes 4-diphosphocytidyl-2*C*-methyl-D-erythritol, but not isopentenyl monophosphate as physiological substrate.

3.5   2*C*-METHYL-D-ERYTHRITOL 2,4-CYCLODIPHOSPHATE SYNTHASE

The discovery of the *ygbP* gene of *E. coli* comprised also the discovery of the *ygbB* gene. In many microorganisms orthologs of the *E. coli ygbB* gene are tightly linked or fused to the *ygbP* gene, which was already shown to be involved in the deoxyxylulose phosphate pathway (Rohdich et al. 1999, Herz et al. 2000). These observations suggested that the YgbB protein is involved in the deoxyxylulose phosphate pathway. This hypothesis was supported further by the fact that putative orthologs of *dxs*, *dxr*, *ygbP*, *ychB* and *ygbB* are found in all completely sequenced genomes of microorganisms using the deoxyxylulose phosphate pathway (Rohdich et al. 1999, Lüttgen et al. 2000, Herz et al. 2000).

In order to determine the biological function of the *ygbB* gene, it was cloned and hyperexpressed (see 3.5.1). Cell extracts of the recombinant *ygbB E. coli* strain were incubated under different conditions with various radiolabeled substrates (2.4.2.6.7 A). Reaction mixtures were separated by thin layer chromatography (2.4.3.1) and the radiochromatograms were analyzed (2.4.4). New reaction products were observed in the presence of divalent cations such as Mg^{2+} or Mn^{2+} in reaction mixtures containing [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol and [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate, respectively. The YgbB protein was purified to homogeneity and used for preparation of the YgbB reaction products (see 3.5.3). The structures of these compounds were elucidated by NMR spectroscopy. The YgbB protein was characterized in some detail.

3.5.1   Overexpression of the *ygbB* gene in *E. coli*

The *ygbB* gene of *E. coli* was cloned into the pQE30 plasmid (Qiagen), which directs the synthesis of a modified YgbB protein with six consecutive histidine residues at the N-terminus. The procedure of *ygbB* gene expression is described under 2.4.2.1. Cells of the recombinant *E. coli* strain XL1-pQE-ygbB were prepared as described under 2.4.1.2. The recombinant strain produced large amounts of a 17 kDa polypeptide (about 30% of soluble protein) as judged by SDS-PAGE (Fig. 3.5.1).

3.5.2 Purification of recombinant 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase from *E. coli*

A HPLC assay was developed to determine the enzyme activity (2.4.2.6.7 B). The reaction mixture contained Mg^{2+} as cofactor. The enzyme activity was determined by measuring the absorbance at 270 nm. The enzyme activity was defined as µmol of CMP formation per time unit. The purification of recombinant 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase of *E. coli* is summarized in Table 3.5.1.

Cells of the recombinant *E. coli* strain XL1-pQE-ygbB (7.3 g) were suspended in 35 ml of buffer F (2.2.5) containing 20 mM imidazole. Cell extract was prepared as described under 2.4.2.2.2. The specific activity of the cell extract was 15 µmol $min^{-1}mg^{-1}$. The cell extract (806 mg) was loaded on top of a Ni^{2+}-Chelating Sepharose column (1.6 x 6.0 cm, Amersham Pharmacia Biotech) which had been equilibrated with 20 mM imidazole in buffer F at a flow rate of 3 ml min^{-1}. The column was washed with 100 ml of 20 mM imidazole in buffer F. The enzyme was eluted by a linear gradient of 20-500 mM imidazole in buffer F (total volume 300 ml). The enzyme was eluted at 100-200 mM imidazole. Fractions were combined according to SDS-PAGE and dialyzed against buffer G (2.2.5). The YgbB protein migrated as a single band at a molecular mass of about 17 kDa. The specific activity of the purified enzyme was 72 µmol $min^{-1}mg^{-1}$.

Table 3.5.1   Purification of recombinant 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase from *E. coli*

Procedure	Total protein (mg)	Total activity (µmol min^{-1})	Specific activity (µmol $min^{-1}mg^{-1}$)	Purification factor
Cell extract	806	11905	15	1
Ni^{2+}-Sepharose FF	273	19929	72	5
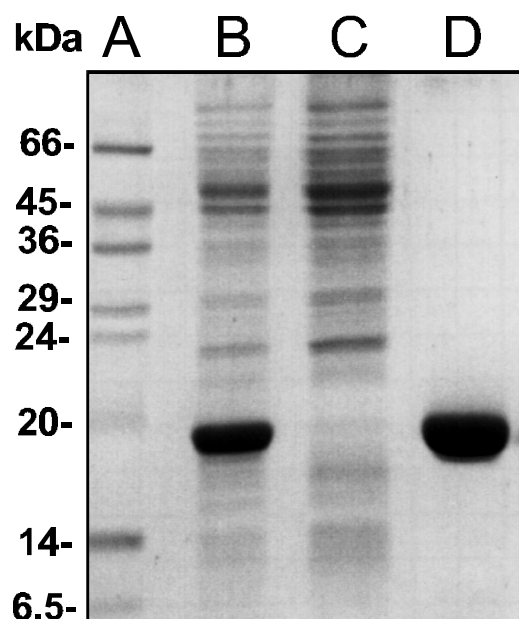
Figure 3.5.1 SDS-PAGE: purification of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase of *E. coli*; A: molecular mass markers; B: cell extract of the recombinant *E. coli* strain; C: flow through fractions after Ni^{2+}-Sepharose; D: after Ni^{2+}-Sepharose

3.5.3 Reaction products of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase

Incubation of purified recombinant YgbB protein with 4-diphosphocytidyl-2*C*-methyl-D-erythritol and its 2-phosphate afforded products with *Rf* values of 0.4 and 0.5, respectively when analyzed by thin layer chromatography (see 2.4.2.6.7 A). To determine the structure of the enzyme products, they were synthesized enzymatically, isolated chromatographically and analyzed by NMR spectroscopy.

A. Incubation with 4-diphosphocytidyl-2*C*-methyl-D-erythritol

A mixture containing 100 mM Tris hydrochloride, pH 8.0, 10 mM MnCl$_2$, 20 mM [2,2-methyl-^{13}C$_2$]4-diphosphocytidyl-2*C*-methyl-D-erythritol, 0.14 µCi of [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol (14.6µCi mmol^{-1}) and 0.3 mg of recombinant YgbB protein from *E. coli* in a total volume of 0.5 ml was incubated at 37 $^{\circ}$C for 2 h. The solution was passed through a Nanosep 10K membrane (Pall Filtron). The reaction product was purified by HPLC using a column of Nucleosil 10SB (4.6 x 250 mm) and 50 mM ammonium formate in 40% methanol (v/v) as eluent at a flow rate of 1 ml min^{-1} as described under 2.4.3.3.2. The effluent was

monitored with a radiomonitor and a UV monitor. The retention volume of the reaction product was 10 ml. Fractions were combined and lyophilized.

The ^{31}P NMR spectrum of the reaction product displayed a singlet at 21.7 ppm, suggesting a pentacyclic monophosphate structure (Herz et al. 2000). Analysis of the ^{1}H and ^{13}C NMR signature identified the reaction product as 2*C*-methyl-D-erythritol 3,4-cyclomonophosphate (Fig 3.5.2).

B. Incubation with 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate

In a first step, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate was prepared enzymatically using purified YchB protein (see 3.4). A mixture containing 100 mM Tris hydrochloride, pH 8.0, 5 mM MgCl$_2$, 5 mM ATP, 5 mM DTT, 2 µCi [2-^{14}C]4-diphosphocytidyl-2*C*-methyl-D-erythritol (117 µCi mmol^{-1}), 5 mM [2,2-methyl-^{13}C$_2$]4-diphosphocytidyl-2*C*-methyl-D-erythritol and 100 µg of recombinant YchB protein from *E. coli* in a total volume of 4 ml was incubated at 37 ^{o}C for 2 h. After the reaction was completed, 0.3 mg of recombinant YgbB protein from *E. coli* was added, and the mixture was further incubated at 37 ^{o}C for 1 h. Proteins were removed by ultrafiltration (Nanosep 10K membrane, Pall Filtron). The filtrate was applied to a column of Nucleosil 10SB and developed with 0.1 M ammonium formate in 40% methanol (v/v) with the Elution No.1 protocol as described under 2.4.3.3.2. The effluent was monitored with a radiomonitor and a UV detector. The retention volume of CMP and the reaction product were 14 ml and 24 ml, respectively. Fractions were collected and lyophilized.

The ^{31}P NMR spectrum of the enzyme product showed two ^{31}P NMR signals at –7.7 ppm and -11.7 ppm (^{31}P^{31}P coupling constant, 23.6 Hz), reflecting a diphosphate motif. ^{31}P^{13}C coupling of one phosphorous atom to the 2-methyl carbon and the absence of ^{31}P^{1}H coupling for the ^{31}P NMR signal at –11.66 ppm, in the conjunction with the ^{13}C NMR chemical shift for C-2 of the 2*C*-methyl-D-erythritol moiety (83.9 ppm) as compared with the respective chemical shift for C-2 of 4-diphosphocytidyl-2*C*-methyl-D-erythritol (73.8 ppm) indicated the connection of the diphosphate motif of C-2 (Herz et al. 2000). The spectroscopic NMR data confirmed the reaction product as 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (Fig 3.5.2).

From these data it was concluded that the YgbB protein converts under the release of CMP 4-diphosphocytidyl-2C-methyl-D-erythritol and its phosphate into 2*C*-methyl-D-erythritol 3,4-cyclomonophosphate and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, respectively (Fig 3.5.2). The catalytic rates for the formation of 2*C*-methyl-D-erythritol 3,4-cyclomonophosphate and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate were compared as described under 3.5.4.
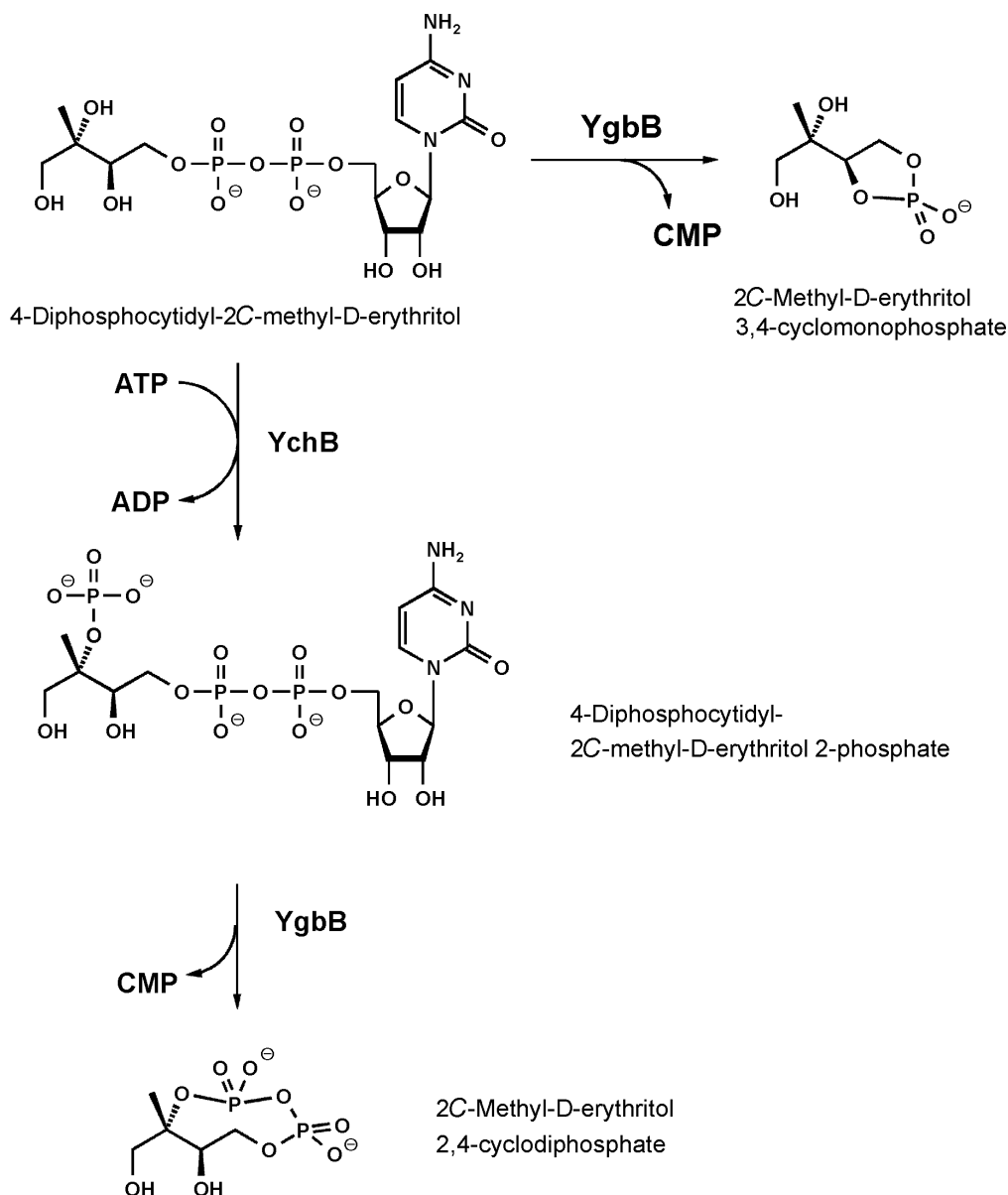
Figure 3.5.2 Reactions catalyzed by 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (YgbB protein)

3.5.4 Characterization of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase -from *E. coli*

3.5.4.1 Substrate specificity

The HPLC assay was used to determine the rate of product formation using 4-diphosphocytidyl-2*C*-methyl-D-erythritol and its phosphate as substrates, respectively (see 2.4.2.6.6 B). Both substrates exhibited different rates of CMP formation (Table 3.5.2). The enzyme showed high affinity to 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate with a specific activity of 72 µmol $min^{-1}mg^{-1}$. However, the enzyme activity was about 3600-fold (0.03%) lower when 4-diphosphocytidyl-2*C*-methyl-D-erythritol was used as substrate. These data suggested that the cyclization of 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate is the major reaction. Therefore, the YgbB protein was named 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase.

Table 3.5.2   Substrate specificity of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase

Substrate	Specific activity (µmol $min^{-1}mg^{-1}$)	Relative activity (%)
4-Diphosphocytidyl-2*C*-methyl-D-erythritol	0.02	0.03
4-Diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate	72	100

3.5.4.2 pH Dependency

The enzyme activity was measured in different buffers (see below) as described under 2.4.2.6.7 B. The pH optimum was 7 as shown in Fig 3.5.3.

Buffers used :   100 mM Sodium citrate, pH 6.0

                     100 mM Sodium potassium phosphate, pH 6.5

                     100 mM Potassium phosphate, pH 7.0

                     100 mM Tris hydrochloride, pH 7.6

                     100 mM Tris hydrochloride, pH 8.0

                     100 mM Glycine-NaOH, pH 9.4

                     100 mM Glycine-NaOH, pH 10.0
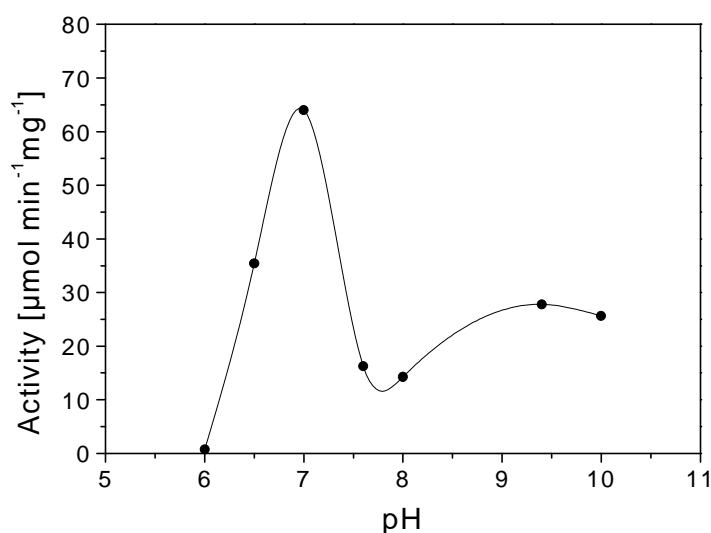
Figure 3.5.3 Effect of different pH values on 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase activity

3.5.4.3 Metal dependency

As already mentioned, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase requires divalent cations for its activity (3.5.2). The enzyme activity was tested with various divalent cations in the assay mixtures (Table 3.5.3). Maximum activity was observed in reactions containing Mn^{2+}, Mg^{2+}, or Zn^{2+}. In contrast, Co^{2+}, Ca^{2+}, Fe^{2+}, or Cu^{2+} could serve as cofactors albeit at lower rates.

Table 3.5.3 Effect of divalent cations on 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase activity

Cations at 2 mM	Specific activity (μmol min^{-1}mg^{-1})	Relative activity (%)
Plus 0.5 mM EDTA	ND*	0
Mg^{2+}	73	100
Mn^{2+}	74	102
Zn^{2+}	67	93
Co^{2+}	41	57
Ca^{2+}	16	22
Fe^{2+}	4	6
Cu^{2+}	1	2
Ni^{2+}	ND	0

*ND, not determined.

3.5.4.4 Kinetic parameters of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase

The kinetic parameters of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase were determined by the HPLC assay (2.4.2.6.7 B). The Michaelis-Menten and Lineweaver-Burk plots are shown in Fig. 3.5.4. The Michaelis constant (K_M) and maximum velocity (V_{max}) of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase were 37 µM and 76 µmol $min^{-1}mg^{-1}$, respectively.
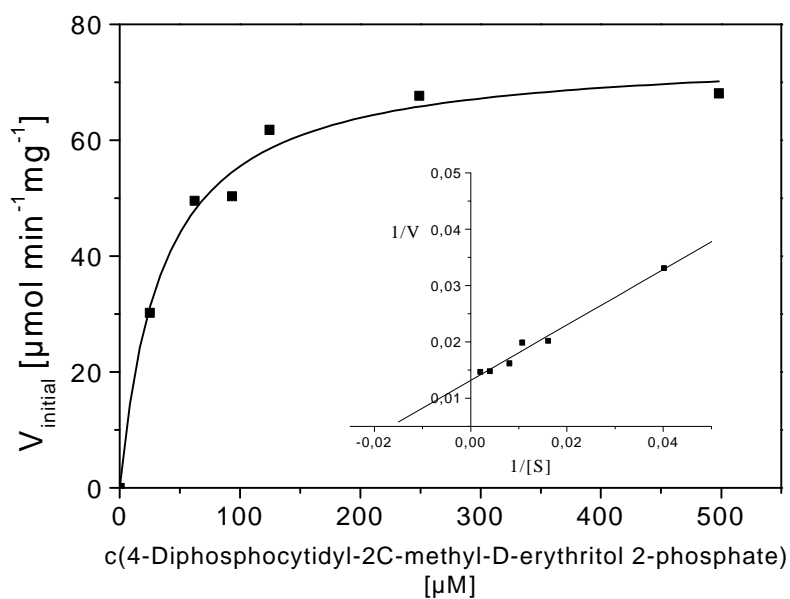
Figure 3.5.4 Michaelis-Menten plot of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (inset : Lineweaver-Burk plot)

4 DISCUSSION

In this study, enzymes catalyzing hitherto unknown steps of the deoxyxylulose phosphate pathway were isolated and characterized. The genes specifying these enzymes were found either by classical approach, for instance by purification of wild type protein, or by a bioinformatic approach using completely sequenced genomes. Such, the *E. coli xylB* and *dxr* genes coding for D-xylulokinase and 1-deoxy-D-xylulose 5-phosphate reductoisomerase were cloned after purification of the cognate wild-type protein (Wungsintaweekul et al. 2001, see result 3.2), whereas the *E. coli ispD*, *E* and *F* genes specifying 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase and 2*C*-methyl-D-erythritol 2,4-cyclodiphospate synthase were cloned bioinformatically comparing completely sequenced genomes (Rohdich et al. 1999, Lüttgen et al. 2000, Herz et al. 2000). The latter approach illustrates the role of biosynthetic studies and highlights the increasing role of molecular biology (Cane 2000).

4.1 D-Xylulokinase phosphorylates 1-deoxy-D-xylulose

1-Deoxy-D-xylulose was shown to be incorporated into ubiquinone by *E. coli* (Broers 1994, Giner et al. 1998, Putra et al. 1998) with a high rate. Similar incorporation rates were found into carotenoids and phytol by cell cultures of *Catharanthus roseus* (Arigoni et al. 1997) and into isoprene, phytol and monoterpenes by various higher plants and certain algae (Schwender et al. 1997). 1-Deoxy-D-xylulose was also shown to be transformed into 2*C*-methyl-D-erythritol by leaves of *Liriodendron tulipifera* (Sagner et al. 1998). Recent studies established that the early steps of the deoxyxylulose phosphate pathway proceed via phosphoric acid esters rather than unphosphorylated carbohydrates (Sprenger et al. 1997, Lois et al. 1998). From this evidence, the utilization of unphosphorylated 1-deoxy-D-xylulose in the deoxyxylulose phosphate pathway seemed to require a phosphorylation step.

An enzyme with 1-deoxy-D-xylulose phosphorylation activity was isolated from cell extracts of wild type *E. coli* by a sequence of 8 chromatographic steps. The protein was obtained in an approximately 3400-fold purification and was sequenced (3.1.1-3.1.2). Sequence similarity and molecular weight suggested that this protein was identical with D-xylulokinase (EC 2.7.1.17) specified by the *xylB* gene of *E. coli*. This enzyme catalyzes the ATP-dependent

4. DISCUSSION

phosphorylation of D-xylulose yielding D-xylulose 5-phosphate (Lawlis et al. 1984). The DNA and amino acid sequences of the *xylB* gene are shown in Fig 4.1. The DNA sequence predicted a protein of 484 amino acids with a molecular weight of 52.6 kDa. The N-terminus determined by Edman degradation of the purified wild type protein is indicated by a box.

```
ATGTATATCGGGATAGATCTTGGCACCTCGGGCGTAAAAGTTATTTTGCTCAACGAGCAG - 60
 M  Y  I  G  I  D  L  G  T  S  G  V  K  V  I  L  L  N  E  Q   :20
GGTGAGGTGGTTGCTGCGCAAACGGAAAAGCTGACCGTTTCGCGCCCGCATCCACTCTGG - 120
 G  E  V  V  A  A  Q  T  E  K  L  T  V  S  R  P  H  P  L  W   :40
TCGGAACAAGACCCGGAACAGTGGTGGCAGGCAACTGATCGCGCAATGAAAGCTCTGGGC - 180
 S  E  Q  D  P  E  Q  W  W  Q  A  T  D  R  A  M  K  A  L  G   :60
GATCAGCATTCTCTGCAGGACGTTAAAGCATTGGGTATTGCCGGCCAGATGCACGGAGCA - 240
 D  Q  H  S  L  Q  D  V  K  A  L  G  I  A  G  Q  M  H  G  A   :80
ACCTTGCTGGATGCTCAGCAACGGGTGTTACGCCCTGCCATTTTGTGGAACGACGGGCGC - 300
 T  L  L  D  A  Q  Q  R  V  L  R  P  A  I  L  W  N  D  G  R   :100
TGTGCGCAAGAGTGCACTTTGCTGGAAGCGCGAGTTCCGCAATCGCGGGTGATTACCGGC - 360
 C  A  Q  E  C  T  L  L  E  A  R  V  P  Q  S  R  V  I  T  G   :120
AACCTGATGATGCCCGGATTTACTGCGCCTAAATTGCTATGGGTTCAGCGGCATGAGCCG - 420
 N  L  M  M  P  G  F  T  A  P  K  L  L  W  V  Q  R  H  E  P   :140
GAGATATTCCGTCAAATCGACAAAGTATTATTACCGAAAGATTACTTGCGTCTGCGTATG - 480
 E  I  F  R  Q  I  D  K  V  L  L  P  K  D  Y  L  R  L  R  M   :160
ACGGGGGAGTTTGCCAGCGATATGTCTGACGCAGCTGGCACCATGTGGCTGGATGTCGCA - 540
 T  G  E  F  A  S  D  M  S  D  A  A  G  T  M  W  L  D  V  A   :180
AAGCGTGACTGGAGTGACGTCATGCTGCAGGCTTGCGACTTATCTCGTGACCAGATGCCC - 600
 K  R  D  W  S  D  V  M  L  Q  A  C  D  L  S  R  D  Q  M  P   :200
GCATTATACGAAGGCAGCGAAATTACTGGTGCTTTGTTACCTGAAGTTGCGAAAGCGTGG - 660
 A  L  Y  E  G  S  E  I  T  G  A  L  L  P  E  V  A  K  A  W   :220
GGTATGGCGACGGTGCCAGTTGTCGCAGGCGGTGGCGACAATGCAGCTGGTGCAGTTGGT - 720
 G  M  A  T  V  P  V  V  A  G  G  G  D  N  A  A  G  A  V  G   :240
GTGGGAATGGTTGATGCTAATCAGGCAATGTTATCGCTGGGGACGTCGGGGGTCTATTTT - 780
 V  G  M  V  D  A  N  Q  A  M  L  S  L  G  T  S  G  V  Y  F   :260
GCTGTCAGCGAAGGGTTCTTAAGCAAGCCAGAAAGCGCCGTACATAGCTTTTGCCATGCG - 840
 A  V  S  E  G  F  L  S  K  P  E  S  A  V  H  S  F  C  H  A   :280
CTACCGCAACGTTGGCATTTAATGTCTGTGATGCTGAGTGCAGCGTCGTGTCTGGATTGG - 900
 L  P  Q  R  W  H  L  M  S  V  M  L  S  A  A  S  C  L  D  W   :300
GCCGCGAAATTAACCGGCCTGAGCAATGTCCCAGCTTTAATCGCTGCAGCTCAACAGGCT - 960
 A  A  K  L  T  G  L  S  N  V  P  A  L  I  A  A  Q  Q  A   :320
GATGAAAGTGCCGAGCCAGTTTGGTTTCTGCCTTATCTTTCCGGCGAGCGTACGCCACAC - 1020
 D  E  S  A  E  P  V  W  F  L  P  Y  L  S  G  E  R  T  P  H   :340
AATAATCCCCAGGCGAAGGGGGTTTTCTTTGGTTTGACTCATCAACATGGCCCCAATGAA - 1080
 N  N  P  Q  A  K  G  V  F  F  G  L  T  H  Q  H  G  P  N  E   :360
CTGGCGCGAGCAGTGCTGGAAGGCGTGGGTTATGCGCTGGCAGATGGCATGGATGTCGTG - 1140
 L  A  R  A  V  L  E  G  V  G  Y  A  L  A  D  G  M  D  V  V   :380
CATGCCTGCGGTATTAAACCGCAAAGTGTTACGTTGATTGGGGGCGGGGCGCGTAGTGAG - 1200
 H  A  C  G  I  K  P  Q  S  V  T  L  I  G  G  A  R  S  E   :400
TACTGGCGTCAGATGCTGGCGGATATCAGCGGTCAGCAGCTCGATTACCGTACGGGGGGG - 1260
 Y  W  R  Q  M  L  A  D  I  S  G  Q  Q  L  D  Y  R  T  G  G   :420
GATGTGGGGCCAGCACTGGGCGCAGCAAGGCTGGCGCAGATCGCGGCGAATCCAGAGAAA - 1320
 D  V  G  P  A  L  G  A  A  R  L  A  Q  I  A  A  N  P  E  K   :440
TCGCTCATTGAATTGTTGCCGCAACTACCGTTAGAACAGTCGCATCTACCAGATGCGCAG - 1380
 S  L  I  E  L  L  P  Q  L  P  L  E  Q  S  H  L  P  D  A  Q   :460
CGTTATGCCGCTTATCAGCCACGACGAGAAACGTTCCGTCGCCTCTATCAGCAACTTCTG - 1440
 R  Y  A  A  Y  Q  P  R  R  E  T  F  R  R  L  Y  Q  Q  L  L   :480
CCATTAATGGCGTAA - 1455
 P  L  M  A  \\ :484
```

Figure 4.1 Nucleotide and amino acid sequences of D-xylulokinase of *E. coli*. The N-terminus peptide sequence determined by Edman degradation is indicated by a box.

90
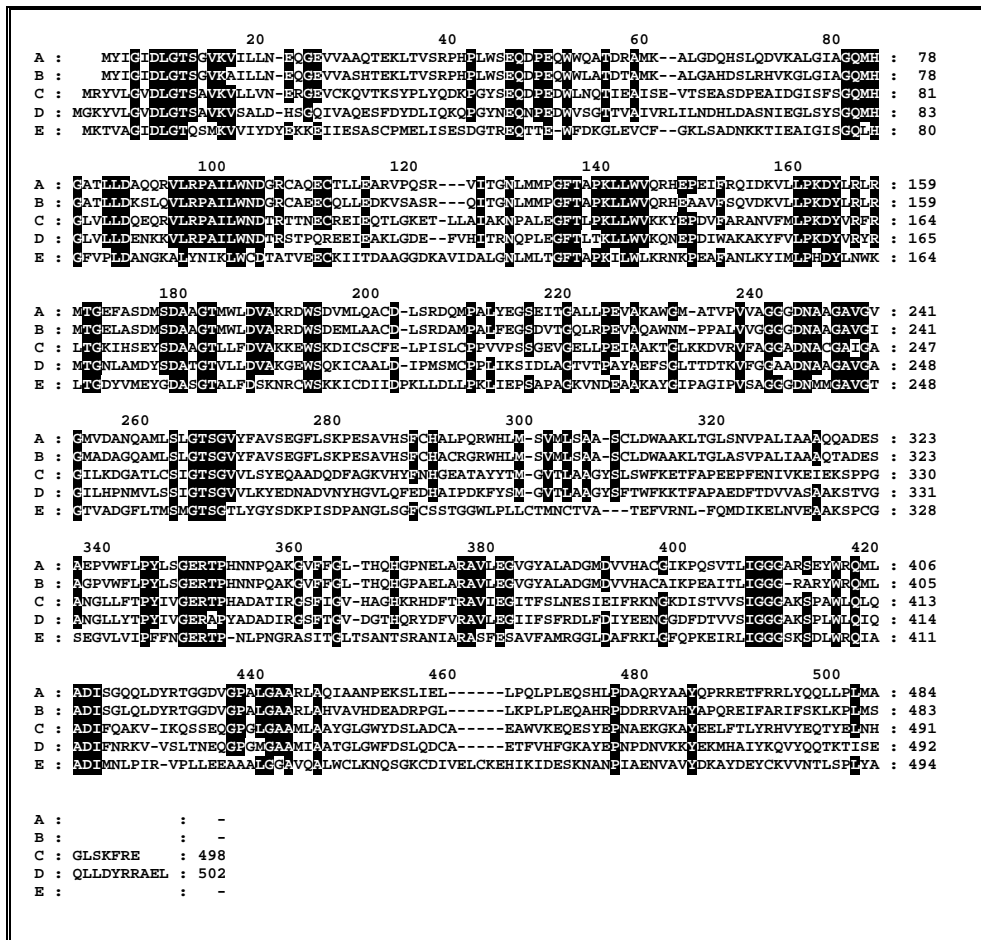
Figure 4.2   Amino acid sequence alignment of D-xylulokinase of *E. coli* and its orthologs. A, *Escherichia coli*; B, *Klebsiella pneumoniae*; C, *Bacillus halodurans*; D, *Lactococcus brevis*; E, *Piromyces sp.* Identical residues among four or five proteins are indicated in inverse contrast.

Enzymes with similar sequences are predicted by genes from some bacteria as well as from a fungus (*Piromyces sp.*) (Fig. 4.2). D-Xylulokinases belong to a FGGY family of carbohydrate kinases which includes fucokinase, gluconokinase and glycerokinase (Sofia et al. 1994).

As shown in results (3.1), the *xylB* gene had already been cloned and the recombinant protein was isolated by three chromatographic steps, resulting in 3-fold purification and 51% protein recovery. The enzyme prefers to utilize ATP as a phosphate group donor rather than CTP, UTP, ITP and GTP. Recombinant D-xylulokinase phosphorylates its physiological substrate D-xylulose at a rate of 51 µmol min^{-1}mg^{-1} (turnover number 45 s^{-1} per subunit) (3.1.6.1). It had been shown that D-xylulokinases from *Klebsiella aerogenes* and *Pichia stipitis* phosphorylate D-xylulose at rates of 150 and 21.4 µmol min^{-1}mg^{-1} (turnover number 150 and 25 s^{-1} per subunit, respectively) (Neuberger et al. 1981, Flanagan and Waites 1992). The K_M values of

D-xylulokinases of *K. aerogenes*, *P. stipitis* and bovine liver are 0.8, 0.52 and 0.14 mM, respectively (Neuberger et al. 1981, Flanagan and Waites 1992, Dill et al. 1994). As shown in this study, the *E. coli* enzyme also phosphorylates 1-deoxy-D-xylulose at the position 5 yielding 1-deoxy-D-xylulose 5-phosphate (see 3.1.5), but at a lower rate of 32 µmol min^{-1}mg^{-1} (turnover number 28 s^{-1} per subunit). The Michaelis constant (K_M) of D-xylulokinase of *E. coli* for 1-deoxy-D-xylulose is 1.4 mM (3.1.6.3). These data indicated that D-xylulokinase has a lower affinity for 1-deoxy-D-xylulose as compared to D-xylulose, but they demonstrate that D-xylulokinase of *E. coli* could enable the utilization of any available 1-deoxy-D-xylulose, providing a "salvage reaction" for the generation of 1-deoxy-D-xylulose 5-phosphate, the precursor for terpenoids as well as for the vitamins thiamin and pyridoxal (Fig 4.5).

4.2   1-Deoxy-D-xylulose 5-phosphate reductoisomerase catalyzes a C-C skeleton rearrangement and reduction in a single step

In this study, an enzyme activity was detected which converted 1-deoxy-D-xylulose 5-phosphate into 2*C*-methyl-D-erythritol 4-phosphate in a NADPH-dependent reaction. The putative enzyme from wild type *E. coli* was purified by a sequence of 8 chromatographic steps. The resulting pure enzyme was purified approximately 60-fold. The amino acid sequence of the purified protein was determined by mass spectrometry (Bieman 1989). The peptide masses of 11 fragments matched the sequence of 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli* which recently was identified by Kuzuyama et al. (1998). The matched peptides cover 22% of the whole protein sequence as shown in Fig. 4.3. The protein sequence consists of 398 amino acids with a molecular mass of 43.4 kDa.

```
ATGAAGCAACTCACCATTCTGGGCTCGACCGGCTCGATTGGTTGCAGCACGCTGGACGTG  -   60
  M   K   Q   L   T   I   L   G   S   T   G   S   I   G   C   S   T   L   D   V  :   20
GTGCGCCATAATCCCGAACACTTCCGCGTAGTTGCGCTGGTGGCAGGCAAAAATGTCACT  -  120
  V   R   H   N   P   E   H   F   R   V   V   A   L   V   A   G   K   N   V   T  :   40
CGCATGGTAGAACAGTGCCTGGAATTCTCTCCCCGCTATGCCGTAATGGACGATGAAGCG  -  180
  R   M   V   E   Q   C   L   E   F   S   P   R   Y   A   V   M   D   D   E   A  :   60
AGTGCGAAACTTCTTAAAACGATGCTACAGCAACAGGGTAGCCGCACCGAAGTCTTAAGT  -  240
  S   A   K   L   L   K   T   M   L   Q   Q   Q   G   S   R   T   E   V   L   S  :   80
GGGCAACAAGCCGCTTGCGATATGGCAGCGCTTGAGGATGTTGATCAGGTGATGGCAGCC  -  300
  G   Q   Q   A   A   C   D   M   A   A   L   E   D   V   D   Q   V   M   A   A  :  100
ATTGTTGGCGCTGCTGGGCTGTTACCTACGCTTGCTGCGATCCGCGCGGGTAAAACCATT  -  360
  I   V   G   A   A   G   L   L   P   T   L   A   A   I   R   A   G   K   T   I  :  120
TTGCTGGCCAATAAAGAATCACTGGTTACCTGCGGACGTCTGTTTATGGACGCCGTAAAG  -  420
  L   L   A   N   K   E   S   L   V   T   C   G   R   L   F   M   D   A   V   K  :  140
CAGAGCAAAGCGCAATTGTTACCGGTCGATAGCGAACATAACGCCATTTTTCAGAGTTTA  -  480
  Q   S   K   A   Q   L   L   P   V   D   S   E   H   N   A   I   F   Q   S   L  :  160
CCGCAACCTATCCAGCATAATCTGGGATACGCTGACCTTGAGCAAAATGGCGTGGTGTCC  -  540
  P   Q   P   I   Q   H   N   L   G   Y   A   D   L   E   Q   N   G   V   V   S  :  180
ATTTTACTTACCGGGTCTGGTGGCCCTTTCCGTGAGACGCCATTGCGCGATTTGGCAACA  -  600
  I   L   L   T   G   S   G   G   P   F   R   E   T   P   L   R   D   L   A   T  :  200
ATGACGCCGGATCAAGCCTGCCGTCATCCGAACTGGTCGATGGGGCGTAAAATTTCTGTC  -  660
  M   T   P   D   Q   A   C   R   H   P   N   W   S   M   G   R   K   I   S   V  :  220
GATTCGGCTACCATGATGAACAAAGGTCTGGAATACATTGAAGCGCGTTGGCTGTTTAAC  -  720
  D   S   A   T   M   M   N   K   G   L   E   Y   I   E   A   R   W   L   F   N  :  240
GCCAGCGCCAGCCAGATGGAAGTGCTGATTCACCCGCAGTCAGTGATTCACTCAATGGTG  -  780
  A   S   A   S   Q   M   E   V   L   I   H   P   Q   S   V   I   H   S   M   V  :  260
CGCTATCAGGACGGCAGTGTTCTGGCGCAGCTGGGGGAACCGGATATGCGTACGCCAATT  -  840
  R   Y   Q   D   G   S   V   L   A   Q   L   G   E   P   D   M   R   T   P   I  :  280
GCCCACACCATGGCATGGCCGAATCGCGTGAACTCTGGCGTGAAGCCGCTCGATTTTTGC  -  900
  A   H   T   M   A   W   P   N   R   V   N   S   G   V   K   P   L   D   F   C  :  300
AAACTAAGTGCGTTGACATTTGCCGCACCGGATTATGATCGTTATCCATGCCTGAAACTG  -  960
  K   L   S   A   L   T   F   A   A   P   D   Y   D   R   Y   P   C   L   K   L  :  320
GCGATGGAGGCGTTCGAACAAGGCCAGGCAGCGACGACAGCATTGAATGCCGCAAACGAA  - 1020
  A   M   E   A   F   E   Q   G   Q   A   A   T   T   A   L   N   A   A   N   E  :  340
ATCACCGTTGCTGCTTTTCTTGCGCAACAAATCCGCTTTACGGATATCGCTGCGTTGAAT  - 1080
  I   T   V   A   A   F   L   A   Q   Q   I   R   F   T   D   I   A   A   L   N  :  360
TTATCCGTACTGGAAAAAATGGATATGCGCGAACCACAATGTGTGGACGATGTGTTATCT  - 1140
  L   S   V   L   E   K   M   D   M   R   E   P   Q   C   V   D   D   V   L   S  :  380
GTTGATGCGAACGCGCGTGAAGTCGCCAGAAAAGAGGTGATGCGTCTCGCAAGCTGA      - 1197
  V   D   A   N   A   R   E   V   A   R   K   E   V   M   R   L   A   S  \\       :  398
```

Figure 4.3 Nucleotide and amino acid sequences of 1-deoxy-D-xylulose 5-phosphate reductoisomerase. The tryptic peptides identified by peptide sequencing are indicated by boxes.

An alignment of 1-deoxy-D-xylulose 5-phosphate reductoisomerase is shown in Fig 4.4. The amino acid sequences contain a conserved motif in the N-terminal region which is proposed to be the NADPH-binding site (Kuzuyama et al. 1998b, Grolle et al. 2000). Recently, Kuzuyama et al. reported that Glu[231] plays an important role in the conversion of 1-deoxy-D-xylulose 5-phosphate into 2C-methyl-D-erythritol 4-phosphate. Gly[14] was proposed to maintain the secondary or tertiary structure of the enzyme. The conserved His[153], His[209] and His[257] associate with 1-deoxy-D-xylulose 5-phosphate binding in the enzyme molecule (see Fig. 4.4, Kuzuyama et al. 2000c).
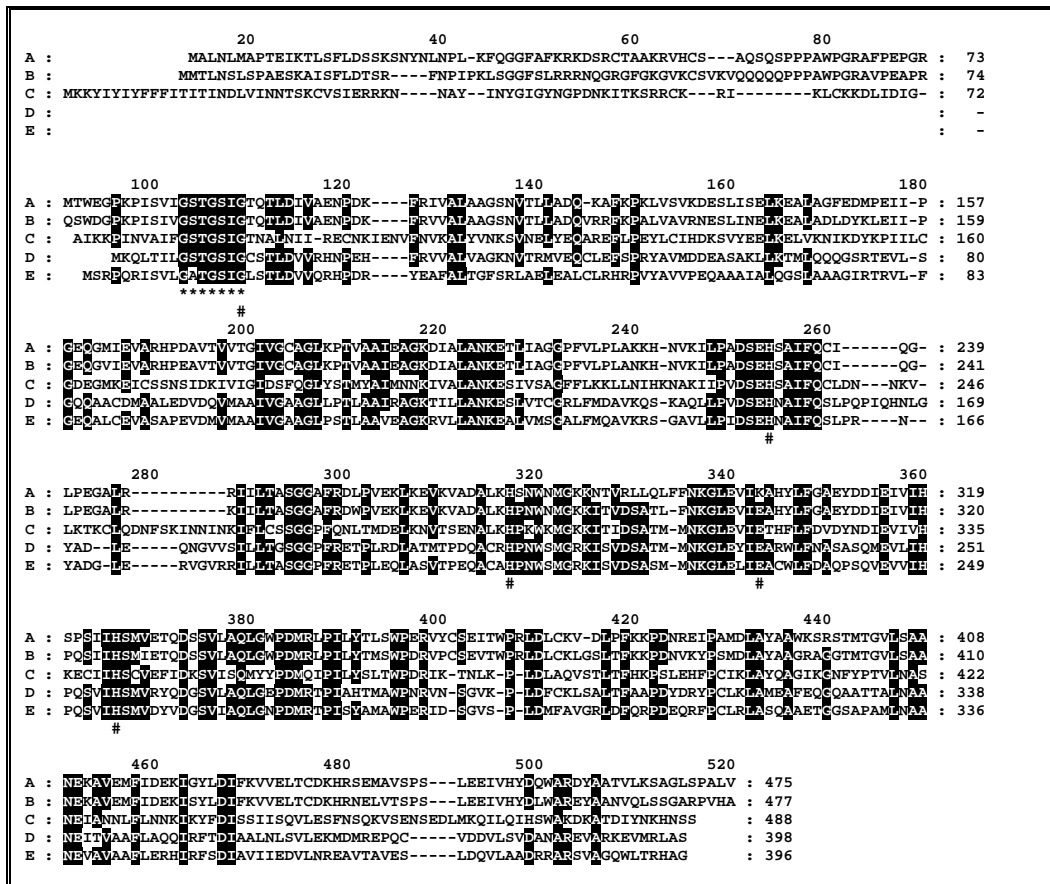
Figure 4.4   Amino acid sequence alignment of 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Escherichia coli* and its orthologs. A, *Mentha piperita*; B, *Arabidopsis thaliana*; C, *Plasmodium falciparum*; D, *E. coli*; E, *Pseudomonas aeruginosa*. Identical residues among four or five proteins are indicated in inverse contrast. Residues representing a putative NADPH-binding site are indicated by asterisks. Residues representing catalytic amino acid residues are indicated by #.

Interestingly, the amino acid sequences of the plant enzymes such as *Mentha piperita* and *Arabidopsis thaliana* carry plastid leader sequences (see Fig 4.4). This evidence is in line with the occurrence of the deoxyxylulose phosphate pathway in plastids (Lange and Croteau 1999a, Schwender et al. 1999). Moreover, the amino acid sequence of *Plasmodium falciparum* contains an endoplasmic reticulum signal peptide indicating that in *P. falciparum* the deoxyxylulose phosphate pathway is located in its apicoplasts (Fig. 4.4, Jomaa et al. 1999).

1-Deoxy-D-xylulose 5-phosphate reductoisomerase uses 1-deoxy-D-xylulose 5-phosphate and NADPH as substrates and requires divalent cations such as Mg^{2+}, Mn^{2+} or Co^{2+} as cofactors. The enzyme does not utilize 1-deoxy-D-xylulose. It should be noted that the successful incorporation of 1-deoxy-D-xylulose into terpenoids of various organisms required a

phosphorylation step catalyzed by the kinase identified in results (4.1). The enzyme prefers to use NADPH rather than NADH. It transfers the H_{si} from NADPH and is therefore belonging to the class B dehydrogenases (Arigoni et al. 1999b, Radykewicz et al. 2000, Proteau et al. 1999). The pH optimum of the enzyme is between 7.5-8.5 and the temperature optimum is between 30-40 ^{o}C. The enzyme is stable when stored at –70°C for at least 6 months. The K_M and V_{max} values are 171 µM and 18 µmol min^{-1}mg^{-1} (turnover number 13 s^{-1} per subunit). It utilizes NADPH as cofactor with a K_M of 25 µM.

Fosmidomycin [3-(N-formyl-N-hydroxyamino) propyl phosphate] inhibits 1-deoxy-D-xylulose 5-phosphate reductoisomerase with an IC$_{50}$ of 56 nM. In contrast, fosfomycin does not inhibit the enzyme (Fig 3.2.10-3.2.11). The inhibitory effect of fosmidomycin on 1-deoxy-D-xylulose 5-phosphate reductoisomerase can be explained by a structural similarity of fosmidomycin to 2C-methyl-D-erythrose 4-phosphate, a putative intermediate of the enzyme reaction. Fosmidomycin had been reported as a mixed-type inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli* with a K_I value of 38 nM (Kuzuyama et al. 1998a). For 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Zymomonas mobilis*, fosmidomycin served as a competitive inhibitor with a K_I value of 600 nM (Grolle et al. 2000). Grolle et al. investigated the inhibition effect of 1,2-dideoxy-D-threo-3-hexulose, a homolog of 1-deoxy-D-xylulose and 2-dimethylphosphinoyl-2-hydroxyacetic acid, a strong competitive inhibitor of the ketol acid reductoisomerase (EC 1.1.1.86) on 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *Z. mobilis*. The ketol acid reductoisomerase is an enzyme of the branched chain amino acid biosynthetic pathway which catalyzes an analogous rearrangement and reduction reaction to the 1-deoxy-D-xylulose 5-phosphate reductoisomerase. Their results showed that both compounds do not inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity (Grolle et al. 2000).

Fosmidomycin had been proven to be an efficient inhibitor of the plastidic and bacterial isoprenoid biosynthesis (Zeidler et al. 1998, Shigi 1989), and it specifically blocks the recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *E. coli*, *Plasmodium falciparum*, *Zymomonas mobilis*, *Pseudomonas aeruginosa*, *Streptomyces* sp. CL 190 and *Arabidopsis thaliana* (Kuzuyama et al. 1998a, Jomaa et al. 1999, Grolle et al. 2000, Altincicek et al. 2000, Takagi et al. 2000, Schwender et al. 1999). Thus fosmidomycin has potential as herbicide and antibiotic against pathogenic bacteria as well as antimalarial drug.

4.3   IspD, IspE and IspF are novel enzymes catalyzing the next steps consecutively

In search for enzymes catalyzing further steps of the deoxyxylulose phosphate pathway, a partially purified enzyme was shown to convert 2*C*-methyl-D-erythritol 4-phosphate into a new product in a CTP-dependent reaction. This finding led to the discovery of the *ygbP* gene of *E. coli* (Rohdich et al. 1999). The putative *ygbP* orthologs distribute in line with orthologs of *dxs* and *dxr* genes which were already shown to be involved in the deoxyxylulose phosphate pathway (Rohdich et al. 1999). The YgbP protein was therefore assumed tentatively as a candidate enzyme catalyzing the next step of the deoxyxylulose phosphate pathway. Therefore, it was cloned and overexpressed. The recombinant protein was purified by a sequence of three chromatographic steps which gave 44% yield of protein and 11-fold purification. The recombinant enzyme was shown to catalyze the conversion of 2*C*-methyl-D-erythritol 4-phosphate into 4-diphosphocytidyl-2*C*-methyl-D-erythritol by reaction with CTP. The catalytic reaction of the enzyme is a transfer of a cytidylphosphate to 2*C*-methyl-D-erythritol 4-phosphate, producing inorganic pyrophosphate, indicating that the enzyme belongs to the wide spread class of „nucleotidylyl transferases" or „nucleoside diphosphate pyrophosphorylases". Based on its catalytic reaction, the YgbP protein was named 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase (Rohdich et al 1999, Kuzuyama et al 2000a).

The protein is a homodimer with a subunit size of 26 kDa. The purified recombinant protein utilizes CTP as cosubstrate; only 8% of activity was observed in the case of GTP. The purified protein has a optimum pH between 8.0-8.5. It requires Mg^{2+}, Mn^{2+} or Co^{2+} for activity. The K_M values for 2*C*-methyl-D-erythritol 4-phosphate and CTP are 381 and 1200 µM, respectively with the maximum velocity (V_{max}) of 185 µmol $min^{-1}mg^{-1}$ or turnover number of 80 s^{-1} per subunit. The enzyme is specific for 2*C*-methyl-D-erythritol 4-phosphate; erythritol 4-phosphate is able to serve as substrate at only about 20% of total activity. Ribitol 5-phosphate does not serve as substrate (Table 3.3.5). Erythritol 4-phosphate inhibits the enzyme with an IC_{50} of 1.38 mM (Fig 3.3.6).

As well as the *ygbP* gene, the unannotated *ychB* gene was found by genomic analysis. Putative orthologs of the unannotated *ychB* gene of *E. coli* follow the distribution of *dxs*, *dxr* and *ygbP* genes (Lüttgen et al. 2000), which have been shown to specify enzymes of the deoxyxylulose phosphate pathway of terpenoid biosynthesis. The recombinant *E. coli* ychB strain was found

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to produce a soluble polypeptide with an apparent mass of 31 kDa under denaturating condition. The recombinant protein was purified to homogeneity by three chromatographic steps. The procedure resulted in an approximately 40-fold purification, yielding 25% protein recovery. The recombinant enzyme was shown to catalyze phosphorylation at the position 2 hydroxy group of 4-diphosphocytidyl-2*C*-methyl-D-erythritol in an ATP dependent reaction. Thus, it was named 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase. Deduced amino acid sequences of putative *ychB* orthologs show a glycine rich conserved motif (residue 90-107) of *E. coli* assigning this region as an ATP-binding site (Post et al. 1993). The amino acid sequence of the YchB protein shares also some similarity with the GHMP family of kinases, which includes galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase (Lüttgen et al. 2000, Kuzuyama et al. 2000d).

As Lange and Croteau claimed that the YchB protein catalyzes the last step in the deoxyxylulose phosphate pathway from isopentenyl monophosphate to isopentenyl diphosphate (Lange and Croteau 1999b), the catalytic rate for the substrates isopentenyl monophosphate and 4-diphosphocytidyl-2*C*-methyl-D-erythritol were compared. Even at very high concentrations of the recombinant enzyme, no isopentenyl monophosphate kinase activity was detected. The data show that 4-diphosphocytidyl-2*C*-methyl-D-erythritol and not isopentenyl monophosphate is the true substrate for 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase of *E. coli* (see 3.4.4).

The discovery of the *E. coli ygbP* gene revealed also the unannotated *E. coli ygbB* gene which is fused to the *ygbP* gene (Rohdich et al. 1999). Moreover, it distributes in the same set of microorganisms. Therefore, the *ygbB* gene was assumed to be a putative gene downstream in this pathway. Cell extracts of recombinant *ygbB E. coli* cells contained large amounts of a 17 kDa polypeptide, which was isolated to apparent homogeneity by nickel-chelating chromatography with 5-fold purification (see 3.5.2). The data showed that the purified recombinant YgbB protein converts 4-diphosphocytidyl-2*C*-methyl-D-erythritol and its phosphate into 2*C*-methyl-D-erythritol 3,4-cyclomonophosphate and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, respectively (see 3.5.3). The catalytic activity of the enzyme was proposed to cyclize the substrate under release of CMP. Comparison of the catalytic rates with both substrates showed that the YgbB protein utilizes 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate 3600-fold better than 4-diphosphocytidyl-2*C*-methyl-D-erythritol as substrate (see 3.5.4.1). Thus, the cyclization of 4-diphosphocytidyl-2*C*-methyl-D-erythritol

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2-phosphate into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate is the major reaction (3.5.4.1). The YgbB protein was named 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (Herz et al. 2000, Takagi et al. 2000).

The YgbB protein has a pH-optimum at 7.0 and requires Mn^{2+}, Mg^{2+} or Zn^{2+} for activity. The Michaelis constant (K_M) and the maximum velocity (V_{max}) of the enzyme are 37 µM and 76 µmol $min^{-1}mg^{-1}$, respectively. The protein has a high affinity to the substrate with a turnover rate of 22 s^{-1} per subunit.

From *in vitro* studies, 4-diphosphocytidyl-2*C*-methyl-D-erythritol, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate were shown to be intermediates in the deoxyxylulose phosphate pathway in *E. coli* (Rohdich et al. 1999, Kuzuyama et al. 2000a, Lüttgen et al. 2000, Kuzuyama et al. 2000d, Herz et al. 2000, Takagi et al. 2000) (Fig. 4.5). In feeding experiments with intact chromoplasts of *Capsicum annuum* using those radiolabeled intermediates, the incorporation rates into the carotenoid fraction were 40%, 30% and 55% of the proffered radioactivity, respectively. In contrast, radiolabeled 2*C*-methyl-D-erythritol 3,4-cyclomonophosphate was not incorporated into carotenoids (Herz et al. 2000). This evidence confirmed that 4-diphosphocytidyl-2*C*-methyl-D-erythritol, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate are intermediates of the deoxyxylulose phosphate pathway in bacteria and plants.
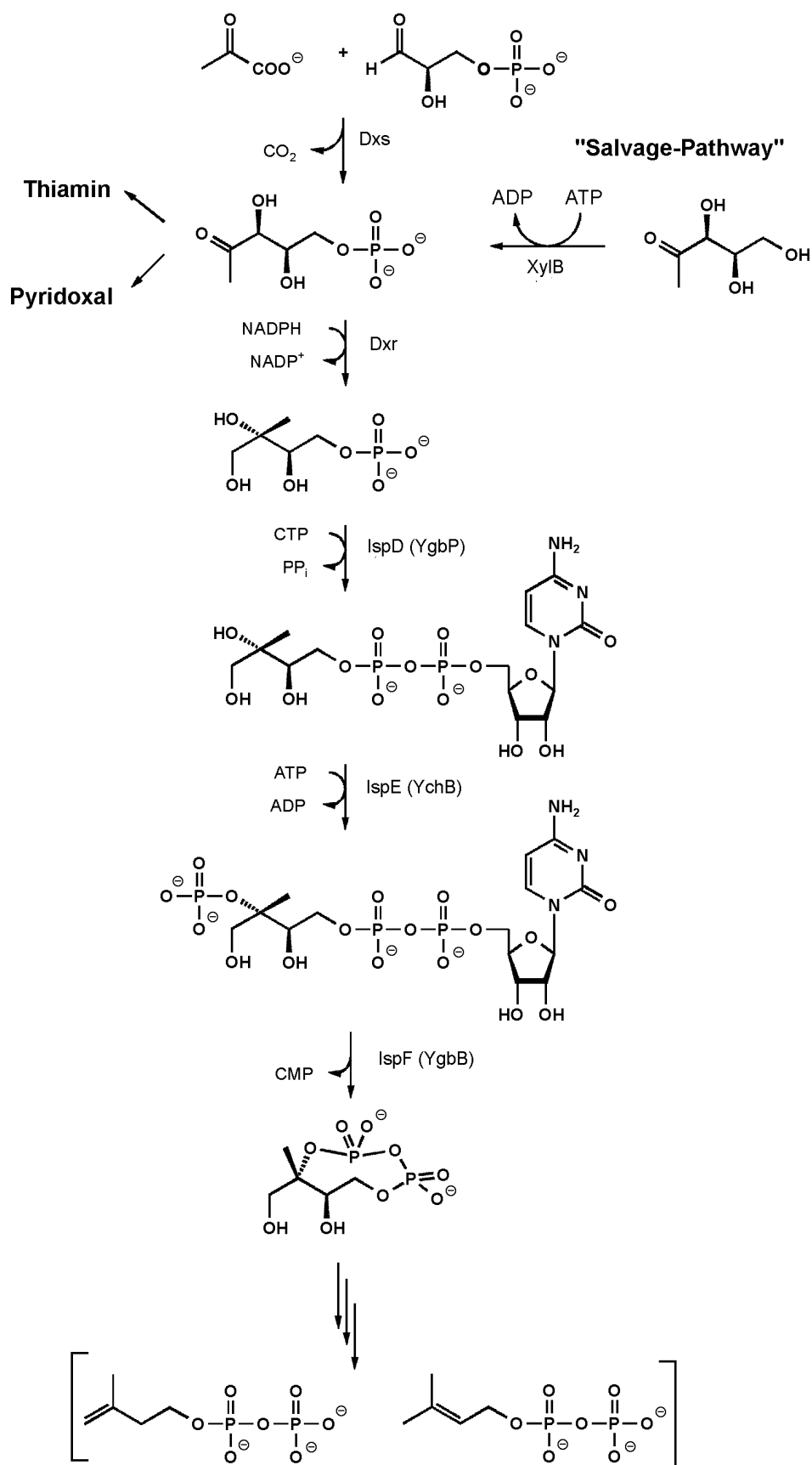
Figure 4.5   The deoxyxylulose phosphate pathway

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Recently, the *ygbP*, *ychB* and *ygbB* genes specifying 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase were renamed properly to *ispD*, *ispE* and *ispF*, respectively.

Further steps from 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate to isopentenyl diphosphate remain unknown. From a structural point of view, it can be proposed that the ring of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate is opened followed by a loss of two molecules of H_2O and two reductions yielding isopentenyl diphosphate and its isomer dimethylallyl diphosphate. These enzymatic reactions require two dehydratases and two reductases.

The deoxyxylulose phosphate pathway occurs in eubacteria but not in mammal (see reviews Eisenreich et al. 1998, Rohmer et al. 1999, Lichtenthaler et al. 2000). Therefore, inhibitors of this pathway can not directly affect the isoprenoid metabolism of humans. Altincicek et al. reported that fluoropyruvate could inhibit 1-deoxy-D-xylulose 5-phosphate synthase of *E. coli* and *Pseudomonas aeruginosa* (Altincicek et al. 2000). Fosmidomycin, an inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, is a compound which has a potential to be herbicide, antibacterial, or antiparasite (Fellermeier et al. 1999, Kuzuyama et al 1998a, Jomaa et al. 1999, Ridley 1999). As shown here, erythritol 4-phosphate is shown to be a putative inhibitor of 4-diphosphosphocytidyl-2*C*-methyl-D-erythritol synthase. Further targets for drug discovery are 4-diphosphosphocytidyl-2*C*-methyl-D-erythritol kinase and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase as well as the other unknown enzymes. Understanding the enzymatic reactions and mechanisms obtained by this study will be useful for the development of new enzyme inhibitors for the purpose of medicinal and plant biotechnology.

5 SUMMARY

Terpenes are one of the largest groups of natural products with important representatives in all taxonomic groups. The more than 30,000 naturally occurring terpenes comprise a wide variety of biological functions and medicinal applications. Studies performed independently in the research groups of Arigoni and Rohmer established the existence of the deoxyxylulose pathway for the biosynthesis of isoprenoid precursors in certain bacteria, protozoa and plants. This study reports about enzymes of *Escherichia coli* which are involved in the deoxyxylulose phosphate pathway. Enzyme activity determinations were developed and established. The proteins were purified to homogeneity by column chromatography and were characterized. The reaction products of the enzymatic reactions were confirmed.

1. Enzyme assays were performed by radiochemical, photometric and HPLC assays. They were modified and validated based on their sensitivity, accuracy and convenience. Radiochemical assays were used for screening of enzyme activity from wild type *E. coli*. In contrast, the photometric and especially HPLC assays were used for enzyme characterization and determination of the kinetic parameters.

2. Proteins from wild type and recombinant *E. coli* strains were purified by a combination of different kinds of column chromatography. The purification procedures were optimized according to the amount of protein recovery.

3. 1-Deoxy-D-xylulose is phosphorylated at position 5 to 1-deoxy-D-xylulose 5-phosphate by D-xylulokinase with a maximum velocity of 32 μmol min^{-1}mg^{-1}. The phosphorylation activity of this enzyme provides a salvage reaction for 1-deoxy-D-xylulose 5-phosphate the precursor for isopentenyl diphosphate as well as for the vitamins thiamin and pyridoxal.

4. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase catalyzes the intramolecular rearrangement and reduction of 1-deoxy-D-xylulose 5-phosphate yielding the branched polyol, 2*C*-methyl-D-erythritol 4-phosphate, in a single step. The enzyme utilizes NADPH as cosubstrate and Mg^{2+}, Mn^{2+} or Co^{2+} as cofactor. Fosmidomycin inhibits the enzyme with an IC$_{50}$ of 56 nM. In the next step, 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase (IspD) converts 2*C*-methyl-D-erythritol 4-phosphate into 4-diphosphocytidyl-2*C*-methyl-

D-erythritol by reaction with CTP. The K_M values for 2C-methyl-D-erythritol 4-phosphate and CTP are 381 and 1200 µM, respectively with the maximum velocity of 185 µmol $min^{-1}mg^{-1}$. Erythritol 4-phosphate inhibits the enzyme with an IC_{50} of 1.38 mM. 4-Diphosphocytidyl-2C-methyl-D-erythritol is then phosphorylated specifically at the position 2 by 4-diphosphocytidyl-2C-methyl-D-erythritol kinase (IspE) yielding 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate. 4-Diphosphocytidyl-2C-methyl-D-erythritol kinase could not catalyze the phosphorylation of isopentenyl monophosphate. In a further step in the deoxyxylulose phosphate pathway, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF) converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate into 2C-methyl-D-erythritol 2,4-cyclodiphosphate and CMP. 2C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase also converts 4-diphosphocytidyl-2C-methyl-D-erythritol into 2C-methyl-D-erythritol 3,4-cyclomonophosphate. The latter compound is shown to be an *in vitro* product without metabolic relevance.

5. The recombinant proteins are used as tools for the preparation of large amounts of intermediates in order to elucidate next steps leading to isopentenyl diphosphate.

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Appendix

Table 1   Chemicals used in this study.

Chemical	Source
1-Propanol	Merck, Darmstadt, Germany
Acrylamide	Roth, Karlsruhe, Germany
Ammonium formate	Fluka, Neu-Ulm, Germany
Ammonium persulfate	Sigma, Deisenhofen, Germany
Ammonium sulfate	Merck, Darmstadt, Germany
Casein hydrolysate	Gibco-BRL, Eggenstein, Germany
Dithioerythritol	Biomol, Hamburg, Germany
Dithiothreitol	Biomol, Hamburg, Germany
EDTA disodium	Merck, Darmstadt, Germany
Hydrochloric Acid	Merck, Darmstadt, Germany
Imidazole	Sigma-Aldrich, Steinheim, Germany
IPTG (Isopropyl-1-thio-ß-D-galactopyranoside)	Eurogentec, Cambridge, England
Magnesium chloride	Fluka, Neu-Ulm, Germany
Ortho-phosphoric acid 85%	Fluka, Neu-Ulm, Germany
Permablend®	Packard, Zürich, Switzerland
Phenethylamine	Fluka, Neu-Ulm, Germany
Phenylmethanesulfonyl fluoride	Merck, Darmstadt, Germany
Potassium chloride	Fluka, Neu-Ulm, Germany
Propylene glycol	Sigma, Deisenhofen, Germany
Serva Blue G (Coomassie Brilliant Blue G-250)	Serva, Heidelberg, Germany
Sodium azide	Fluka, Neu-Ulm, Germany
Sodium chloride	Fluka, Neu-Ulm, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
TEMED	Roth, Karlsruhe, Germany
Tetrabutylammonium hydrogen-sulfate	Fluka, Neu-Ulm, Germany
Tris	Biomol, Hamburg, Germany
Yeast extract	Gibco-BRL, Eggenstein, Germany

Appendix

Table 2   Substrates and cofactors used for enzyme activity tests.

Substrate/Cofactor	Source
[1,2-^{14}C]1-Deoxy-D-xylulose	Dr. S. Sagner, LMU, München
[1,2-^{14}C]1-Deoxy-D-xylulose 5-phosphate	Dr. S. Sagner, LMU, München
[2-^{13}C$_1$]1-Deoxy-D-xylulose	Dr. C. Latzel, LMU, München
1-Deoxy-D-xylulose	Dr. C. Latzel, LMU, München
2*C*-Methyl-D-erythritol 4-phosphate	Dr. K. Kis, TU, München
2*C*-Methyl-D-erythritol	Dr. K. Kis, TU, München
Ribitol 5-phosphate	Dr. K. Kis, TU, München
Erythritol 4-phosphate	Dr. K. Kis, TU, München
[3,4,5-^{13}C$_3$]1-Deoxy-D-xylulose 5-phosphate	S. Hecht, TU, München
[2,2´-^{13}C$_2$]2*C*-Methyl –D-erythritol 4-phosphate	S. Hecht, TU, München
[2,2´-^{13}C$_2$]4-diphosphocytidyl-2*C*-methyl-D-erythritol	C. A. Schuhr, TU, München
[1,2,2-methyl,3,4-^{13}C$_5$]4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate	S. Hecht, TU, München
[2-^{14}C]-Pyruvate	Dupont/NEN, Boston, MA, USA
[4-^{14}C]-Isopentenyl pyrophosphate	Dupont/NEN, Boston, MA, USA
ATP	Sigma, Deisenhofen, Germany
CMP	Sigma, Deisenhofen, Germany
CTP	Sigma, Deisenhofen, Germany
DL-Glyceraldehyde 3-phosphate	Sigma, Deisenhofen, Germany
D-Ribulose	Fluka, Neu-Ulm, Germany
D-Xylulose	Fluka, Neu-Ulm, Germany
Fosfomycin	Prof. Dr M.H. Zenk, Biozentrum-Pharmazie, Universität Halle, Halle
Fosmidomycin	Prof. Dr M.H. Zenk, Biozentrum-Pharmazie, Universität Halle, Halle
Glutathione reduced form	Sigma, Deisenhofen, Germany
GTP	Sigma, Deisenhofen, Germany
ITP	Sigma, Deisenhofen, Germany
NAD	Boehringer-Mannheim, Mannheim, Germany
NADH	Biomol, Hamburg, Germany
NADP	Boehringer-Mannheim, Mannheim, Germany
NADPH	Boehringer-Mannheim, Mannheim, Germany
Phosphoenolpyruvate	Boehringer-Mannheim, Mannheim, Germany
Pyridoxal 5-phosphate	Sigma, Deisenhofen, Germany
Thiamine pyrophosphate	Sigma, Deisenhofen, Germany
Uridine 5´-diphosphoglucose (UDPG)	Sigma, Deisenhofen, Germany
UTP	Sigma, Deisenhofen, Germany
α-D-Glucose-1,6-bisphosphate	Sigma, Deisenhofen, Germany

Appendix

Table 3   Enzymes used in this study

Enzyme	Source
1-Deoxy-D-xylulose 5-phosphate reductoisomerase	This study
2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	This study
4-Diphosphocytidyl-2*C*-methyl-D-erythritol kinase	This study
4-Diphosphocytidyl-2*C*-methyl-D-erythritol synthase	This study
Alkaline phosphatase (EC 3.1.3.1) type I-S	Sigma, Deisenhofen, Germany
DNase I	Boehringer-Mannheim, Mannheim, Germany
D-Xylulokinase (EC 2.7.1.17)	This study
Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) Type V : From Baker Yeast	Sigma, Deisenhofen, Germany
Inorganic pyrophosphatase (EC 3.6.1.1) From *E. coli*	Sigma, Deisenhofen, Germany
Lactate dehydrogenase (EC 1.1.1.27)	Boehringer-Mannheim, Mannheim, Germany
Lysozyme (EC 3.2.1.17) From Chicken Egg White	Sigma, Deisenhofen, Germany
Phosphoglucomutase (EC 5.4.2.2) Rabbit Muscle	Sigma, Deisenhofen, Germany
Pyruvate kinase (EC 2.7.1.40)	Sigma, Deisenhofen, Germany
UDP-Glucose pyrophosphorylase (EC 2.7.7.9)	Sigma, Deisenhofen, Germany