Biosynthesis of terpenoids: YgbB protein converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate to 2C-methyl-D-erythritol 2,4-cyclodiphosphate

Stefan Herz*, Juraithip Wungsintaweekul†, Christoph A. Schuhr*, Stefan Hecht*, Holger Lüttgen*, Sylvia Sagner†, Monika Fellermeier†, Wolfgang Eisenreich*, Meinhart H. Zenk†, Adelbert Bacher*, and Felix Rohdich*‡

*Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Germany; and †Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstrasse 29, D-80333 Munich, Germany

Contributed by Meinhart H. Zenk, December 17, 1999

In many microorganisms, the putative orthologs of the *Escherichia coli ygbB* gene are tightly linked or fused to putative orthologs of *ygbP*, which has been shown earlier to be involved in terpenoid biosynthesis. The *ygbB* gene of *E. coli* was expressed in a recombinant *E. coli* strain and was shown to direct the synthesis of a soluble, 17-kDa polypeptide. The recombinant protein was found to convert 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate into 2C-methyl-D-erythritol 2,4-cyclodiphosphate and CMP. The structure of the reaction product was established by NMR spectroscopy using ^{13}C-labeled substrate samples. The enzyme-catalyzed reaction requires Mn^{2+} or Mg^{2+} but no other cofactors. Radioactivity from [2-^{14}C]2C-methyl-D-erythritol 2,4-cyclodiphosphate was diverted efficiently to carotenoids by isolated chromoplasts from *Capsicum annuum* and, thus, was established as an intermediate in the deoxyxylulose phosphate pathway of isoprenoid biosynthesis. YgbB protein also was found to convert 4-diphosphocytidyl-2C-methyl-D-erythritol into 2C-methyl-D-erythritol 3,4-cyclophosphate. This compound does not serve as substrate for the formation of carotenoids by isolated chromoplasts and is assumed to be an *in vitro* product without metabolic relevance.

Seminal studies performed independently in the research groups of Arigoni and Rohmer established the existence of a nonmevalonate pathway for the biosynthesis of isoprenoid precursors in certain bacteria and in protozoa and plants (for review, see refs. 1 and 2). 1-Deoxy-D-xylulose serves as an efficient precursor of terpenoids via this alternative pathway (3–8), and its 5-phosphate (**3**) was shown to be formed from glyceraldehyde 3-phosphate (**1**) and pyruvate (**2**) (3, 9, 10) by an enzyme specified by the *dxs* gene of *Escherichia coli* (11, 12) and its plant orthologs (13, 14) (Fig. 1). 1-Deoxy-D-xylulose 5-phosphate was shown to be converted into the branched chain polyol 2C-methyl-D-erythritol 4-phosphate (**4**) by reductoisomerases specified by *dxr* genes of *E. coli*, *Mentha piperita, Arabidopsis thaliana,* and *Plasmodium falciparum* (17–20). More recently, we found that 2C-methyl-D-erythritol 4-phosphate can be converted into 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (**6**) via 4-diphosphocytidyl-2C-methyl-D-erythritol by the sequential action of enzymes specified by the *ygbP* and *ychB* genes of *E. coli* (15, 16). This paper shows that YgbB protein converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate into the cyclic 2,4-diphosphate of 2C-methyl-D-erythritol.

Experimental Procedures

Materials. Oligonucleotides were custom-synthesized by MWG Biotec, Ebersberg, Germany. The preparation of ^{13}C- and ^{14}C-labeled samples of 4-diphosphocytidyl-2C-methyl-D-erythritol, 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate, and 2C-methyl-D-erythritol 4-phosphate will be described elsewhere.

Enzymes. The preparation of recombinant YchB protein of *E. coli* has been described earlier (16).

Construction of a Hyperexpression Strain. The *ygbB* gene of *E. coli* was amplified by PCR, using the oligonucleotides shown in Table 1 as primers and chromosomal *E. coli* DNA as template. The amplificate was digested with *Bam*HI and *Pst*I and then was ligated into the pQE30 plasmid (Qiagen), which had been treated with the same enzymes. The reaction mixture was electrotransformed into the *E. coli* strain XL1-Blue (21). The resulting *E. coli* strain is designated XL1-pQEygbB.

Purification of Recombinant YgbB Protein. *E. coli* XL1-pQEygbB cells (2 g) were suspended in 30 ml of 100 mM Tris hydrochloride (pH 8.0) containing 20 mM imidazole hydrochloride and 500 mM NaCl (standard buffer). Cell extract was prepared as described earlier (16) and was applied to a column of Ni^{2+}-chelating Sepharose FF (2 × 8 cm; flow rate, 3 ml/min) that had been equilibrated with standard buffer. The column was washed with 100 ml of standard buffer and developed with a linear gradient of 20–500 mM imidazole in standard buffer (total volume, 300 ml). Fractions were combined and dialyzed overnight against 100 mM Tris hydrochloride (pH 8.0). The solution was concentrated by ultrafiltration and applied to a Superdex 75 HR 26/60 column (flow rate, 3 ml/min) that had been equilibrated with 100 mM NaCl in standard buffer. YgbB protein was eluted at 150 ml.

Assay of YgbB Protein. Assay mixtures containing 100 mM Tris hydrochloride (pH 8.0), 5 mM $MnCl_2$, 5 mM DTT, 2 μg of protein, and 11.4 μM [2-^{14}C]diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (17.5 μCi/μmol) or 11.4 μM [2-^{14}C]diphosphocytidyl-2C-methyl-D-erythritol (17.5 μCi/μmol) in a total volume of 50 μl were incubated at 37°C for 10 min. Aliquots (40 μl) were applied to TLC plates (Polygram Sil N-HR; Macherey & Nagel) that were developed in *n*-propanol/ethyl acetate/H_2O (6:1:3, vol/vol). Radioactivity was monitored by a PhosphorImager (Storm 860; Molecular Dynamics). The R_f values of 2C-methyl-D-erythritol 2,4-cyclodiphosphate and 2C-methyl-D-erythritol 3,4-cyclophosphate were 0.4 and 0.5, respectively.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF230738).

‡To whom reprint requests should be addressed. E-mail: felix.rohdich@ch.tum.de.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.040554697. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.040554697

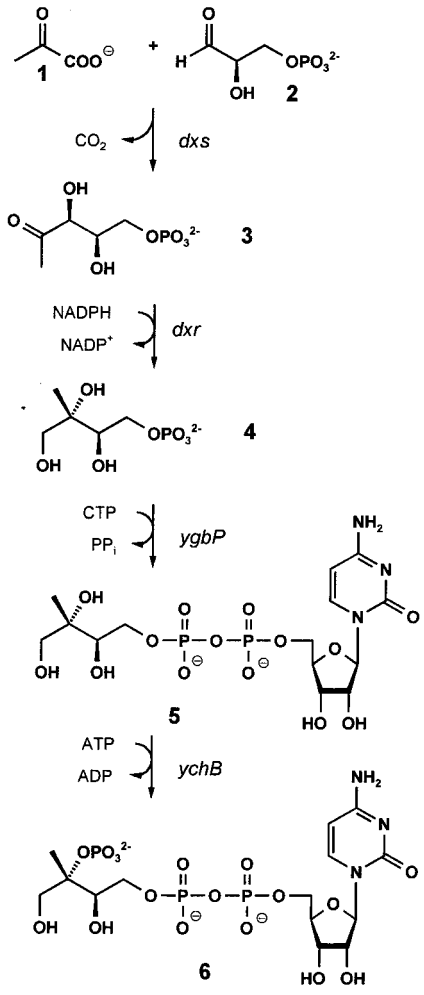

Fig. 1. The deoxyxylulose phosphate pathway of isoprenoid biosynthesis (1, 2, 15, 16).

Enzymatic Formation of 2C-Methyl-D-Erythritol 2,4-Cyclodiphosphate. 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-2C-methylerythritol (117 μCi/mmol), 5 mM 4-diphosphocytidyl-2C-methylerythritol labeled with ^{13}C as indicated, and 0.1 mg of recombinant YchB protein from *E. coli* (16) in a total volume of 4 ml was incubated at 37°C. The reaction was monitored by ^{31}P NMR. After 2 h, 0.3 mg of recombinant YgbB protein from *E. coli* was added, and the mixture was incubated at 37°C for 1 h. The solution was passed through a Nanosep 10K membrane (Pall Gelman, Rondorf, Germany) and applied to an HPLC column of Nucleosil 10SB (4.6 × 250 mm; Macherey & Nagel). The column was developed with 0.1 M ammonium formate in 40% methanol (vol/vol). The effluent was monitored by refractometry and scintillation counting. The retention volumes of CMP and 2C-methyl-D-erythritol 2,4-cyclodiphosphate were 14 ml and 34 ml, respectively. Fractions were collected and lyophilized.

Table 1. Oligonucleotides used in this study

Primer	Sequence
ygbBvo	5′-GAGAAGGATCCATGCGAATTGGACACGGTTTTGACG-3′
ygbBhi	5′-TATTATCTGCAGCCTTGCGGTTTACCGTGGAGG-3′

Table 2. Accession or contig numbers of *ygbP* and *ygbB* in various organisms

Bacteria	Accession no.*	
With bifunctional genes		
Campylobacter jejuni	Cj.seq	
Caulobacter crescentus	gcc_1641, gcc_574	
Helicobacter pylori	gb AE001474	
Rhodobacter capsulatus	emb X72382	
Treponema pallidum	gb AE001227	
Zymomonas mobilis	gb AF176314	
With adjacent genes		
Actinobacillus actinomycetemcomitans	Contig704	
Bacillus subtilis	emb Z99101	
Bordetella pertussis	Contig657	
Clostridium acetobutylicum	AE001437	
Clostridium difficile	Contig392	
E. coli	gb AE000358	
Haemophilus influenzae	gb U32750	
Klebsiella pneumoniae	Contig1719	
Mycobacterium avium	5759	
Mycobacterium bovis	Contig950	
Mycobacterium tuberculosis	emb Z92774	
Neisseria gonorrhoeae	Contig181	
Neisseria meningitidis	Contig3	
Pasteurella multocida	Contig556	
Pseudomonas aeruginosa	Contig52	
Salmonella paratyphi	SPA.0.2916	
Salmonella typhi	Contig404	
Salmonella typhimurium	Contig997	
Shewanella putrefaciens	gsp_845	
Thiobacillus ferrooxidans	949	
Vibrio cholerae	asm938	
Yersinia pestis	Contig730	
With unlinked genes	*ygbP*	*ygbB*
Aquifex aeolicus	gb AE000734	gb AE000715
Chlamydia pneumoniae	gb AE001642	gb AE001639
Chlamydia trachomatis	gb AE001320	gb AE001317
Chlorobium tepidum	gct_38	gct_41
Corynebacterium diphtheriae	Contig519	Contig402
Deinococcus radiodurans	8896	8835
Enterococcus faecalis	gef_6311	gef_6177
Porphyromonas gingivalis	1209	1207
Synechocystis sp.	dbj D990914	dbj D90906
Thermotoga maritima	gb AE001792	gb AE001738
Eukaryotes with unlinked genes		
A. thaliana	gb AC004136	
P. falciparum	1D_M9Fe7.plt	gb AE001394

*Accession and contig numbers as of December 6, 1999.

Enzymatic Formation of 2C-Methyl-D-Erythritol 3,4-Cyclophosphate. A mixture containing 100 mM Tris hydrochloride (pH 8.0), 10 mM $MnCl_2$, 20 mM [2,2-methyl-$^{13}C_2$]4-diphosphocytidyl-2C-methyl-D-erythritol, 0.14 μCi of [2-^{14}C]4-diphosphocytidyl-2C-methyl-D-erythritol (14.6 μCi/mmol), and 0.3 mg of purified recombinant YgbB protein from *E. coli* in a total volume of 0.5 ml was incubated for 2 h. The solution was passed through a Nanosep 10K membrane. The enzyme product was purified by HPLC, using a column of Nucleosil 10SB (4.6 × 250 mm) and 50 mM ammonium formate in 40% methanol (vol/vol) as eluent (flow rate, 1 ml/min). The effluent was monitored by refractometry and by scintillation counting. Fractions (retention volume, 10 ml) were collected and lyophilized.

NMR Spectroscopy. NMR spectra were recorded by using an AVANCE DRX500 or AC 250 spectrometer from Bruker

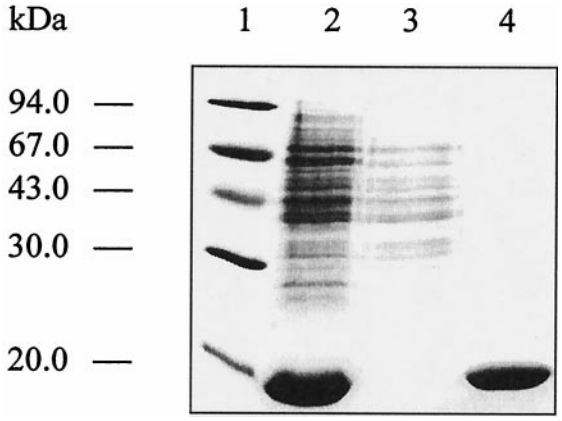

Fig. 2. SDS/PAGE. Lanes: 1, molecular mass markers; 2, crude cell extract of the recombinant *E. coli* strain XL1-pQEygbB; 3, flow-through fraction of Ni^{2+}-chelating Sepharose chromatography; 4, eluted fraction of YgbB after Ni^{2+}-chelating Sepharose chromatography.

(Billerica, MA). CMP, 2C-methyl-D-erythritol 2,4-cyclodiphosphate, and 2C-methyl-D-erythritol 3,4-cyclophosphate were measured in D_2O as solvent.

Preparation of Chromoplasts and Incorporation Assays. Chromoplasts of *C. annuum* were isolated as described previously (16). Incorporation experiments with [2-^{14}C]2C-methyl-D-erythritol 2,4-cyclodiphosphate and [2-^{14}C]2C-methyl-D-erythritol 3,4-cyclophosphate into carotenoids were performed as described previously (16).

Results

The *ygbP* gene specifying 4-diphosphocytidyl-2C-methyl-D-erythritol synthase is tightly linked to the unannotated reading frame *ygbB* on the *E. coli* chromosome (15, 22), and the two genes are likely to be cotranscribed. Putative orthologs of *ygbP* and *ygbB* also were found in the database in adjacent positions on the chromosomes of 21 other eubacteria (Table 2), and 6 eubacteria were found to contain genes assumed to specify bifunctional proteins with an N-terminal *ygbP* domain and a C-terminal *ygbB* domain (Table 2). These observations suggested that YgbB protein is involved in the deoxyxylulose phosphate pathway of terpenoid biosynthesis. This hypothesis was supported further by the fact that putative orthologs of *dxr*, *dxs*, *ygbP*, *ychB*, and *ygbB* are found in all completely sequenced genomes of microorganisms using the nonmevalonate pathway and are absent in all completely sequenced genomes of microorganisms using the mevalonate pathway with the exception of *Pyrococcus horikoshii* genome, which contains a putative ortholog of *ygbP* (15, 16).

The *ygbB* gene of *E. coli* was expressed in a recombinant *E. coli* host harboring the plasmid pQEygbB, which directs the synthesis of a modified YgbB protein with six consecutive histidine residues at the N terminus. Cell extracts of the recombinant *E. coli* cells contained large amounts of a 17-kDa polypeptide (about 30% of soluble protein), which was purified to apparent homogeneity by nickel-chelate chromatography (Fig. 2). N-terminal Edman degradation confirmed the predicted amino acid sequence (data not shown).

Incubation of purified recombinant YgbB protein with 4-diphosphocytidyl-2C-methyl-D-erythritol (**5**, Fig. 1) and its 2-phosphate (**6**) in the presence of Mn^{2+} or Mg^{2+} afforded products with R_f values of 0.4 and 0.5, respectively, when analyzed by TLC as described in *Experimental Procedures*. To determine the structure of the enzyme products, we prepared [1,3,4-^{13}C]-, [2,2-methyl-$^{13}C_2$]-, and [1,2,2-methyl,3,4-$^{13}C_5$]-labeled samples of 4-diphosphocytidyl-2C-methyl-D-erythritol (**5**) and its respective 2-phosphate (**6**) by using recombinant enzymes of the deoxyxylulose phosphate pathway as catalysts. The ^{13}C labels of these samples afforded enhanced sensitivity and selectivity in enzyme assays monitored by ^{13}C NMR (Fig. 3).

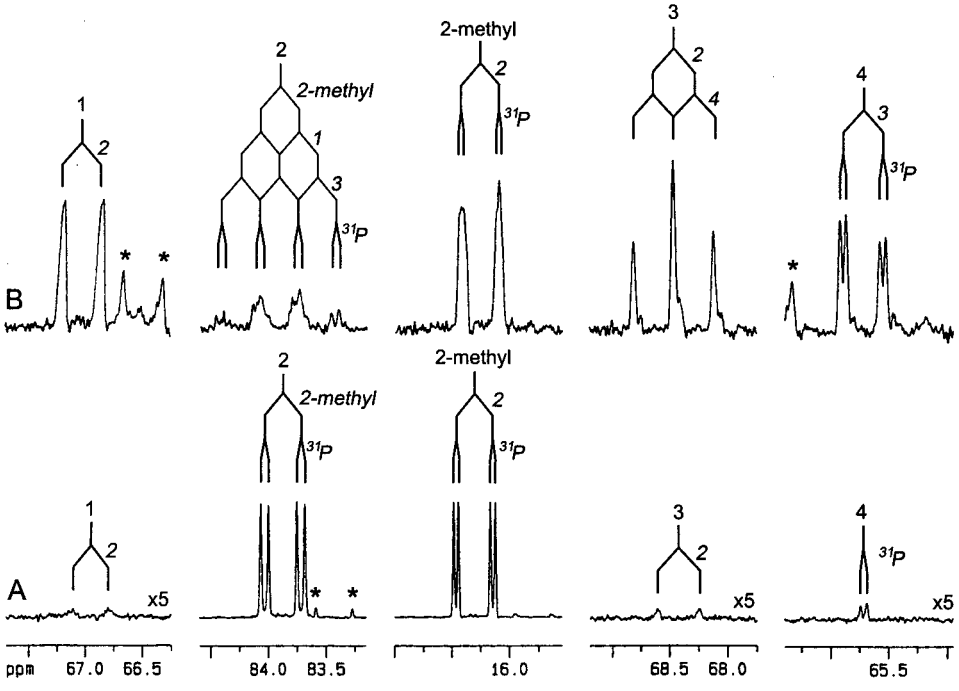

Fig. 3. ^{13}C NMR signals of 2C-methyl-D-erythritol 2,4-cyclodiphosphate obtained from the reaction of recombinant YgbB protein with [2,2-methyl-$^{13}C_2$]4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (*A*) or [1,2,2-methyl,3,4-$^{13}C_5$]4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (*B*). $^{13}C^{13}C$ couplings and $^{13}C^{31}P$ couplings are indicated. *, Impurities.

Table 3. NMR data of products from reaction with YgbB protein

Position	Chemical shifts, ppm				Coupling constants, Hz		
	^{1}H*	^{13}C†	^{31}P‡	J_{HH}	J_{PC}	J_{PP}	J_{CC}
				2C-methyl-D-erythritol 2,4-cyclodiphosphate (**8**)			
1	3.51 (d, 1)	66.95		12.2 (/1§)			41.8 (2)¶‖
1§	3.66 (d, 1)			12.2 (1)			
2		83.87			8.4 (P-2)‖		39.8 (2-Me)¶‖
2-Me	1.31 (s, 3)	16.30			5.3 (P-2)‖		39.8 (2)¶‖
3	4.01 (m, 1)	68.42		ND	ND		46.0 (2)¶
4	4.02 (m, 1)	65.72		ND	6.6 (P-4)**		42.7 (3)¶
4§	4.07 (m, 1)			ND			
P-4			−7.65			23.6 (P-2)	
P-2			−11.66		8.5 (2)‖, 5.3 (2-Me)‖	23.6 (P-4)	
				2C-methyl-D-erythritol 3,4-cyclomonophosphate (**7**)			
1	3.38 (d, 1)	65.64		12.0 (1§)			
1§	3.47 (d, 1)			12.0 (1)			
2		73.02			6.5‖		
2-Me	1.09 (s, 3)	17.73					
3	4.15 (m, 1)	77.61			1.7‖		
4	4.18 (m, 1)	64.96			1.1‖		
4§	4.34 (ddd, 1)			11.2 (4), 3.8, 7.2 (3)			
P			21.67 (s)				

*Referenced to external trimethylsilylpropane sulfonate. The multiplicities and the relative integral values of ^{1}H NMR signals of an unlabeled sample are indicated in parentheses.
†Referenced to external trimethylsilylpropane sulfonate.
‡Referenced to external phosphoric acid (85%, vol/vol).
§Indicates one of diastereotopic hydrogens bonded to the respective index carbon.
¶From the reaction with [1,2,2-methyl,3,4-$^{13}C_5$] 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate.
‖From the reaction with [2,2-methyl-$^{13}C_2$]4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate.
**From the reaction with [1,3,4-$^{13}C_1$]4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate.

The sensitivity enhancement is illustrated in Fig. 3*A* for the ^{13}C NMR signals of the product obtained from [2,2-methyl-$^{13}C_2$]4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate. The signals with low intensity reflect carbon atoms with natural ^{13}C abundance. The detected signal splittings (Fig. 3) were due to $^{13}C^{13}C$ and $^{13}C^{31}P$ couplings, as indicated. The ^{13}C-labeling approach also facilitated the signal assignment via analysis of the $^{13}C^{13}C$ and $^{13}C^{1}H$ spin networks by using two-dimensional homo- and heterocorrelation NMR experiments.

NMR data of the product obtained from the reaction of YgbB protein with ^{13}C-labeled 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (**6**) are summarized in Table 3. The $^{13}C^{13}C$ and $^{13}C^{1}H$ networks were gleaned by INADEQUATE and heteronuclear multiple quantum correlation spectroscopy, thus establishing a methylerythritol motif. ^{31}P NMR spectra showed two ^{31}P NMR signals at −7.7 ppm and −11.7 ppm (Fig. 4). Each of the signals was characterized by $^{31}P^{31}P$ coupling (coupling constant, 23.6 Hz), reflecting a diphosphate motif. $^{31}P^{13}C$ coupling of one phosphorous atom to the 2-methyl carbon

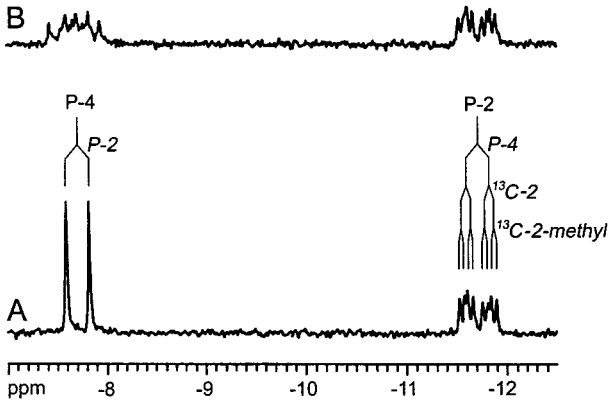

Fig. 4. ^{31}P NMR spectrum of [2,2-methyl-$^{13}C_2$]2C-methyl-D-erythritol 2,4-cyclodiphosphate. (*A*) ^{1}H-decoupled. (*B*) Without ^{1}H-decoupling.

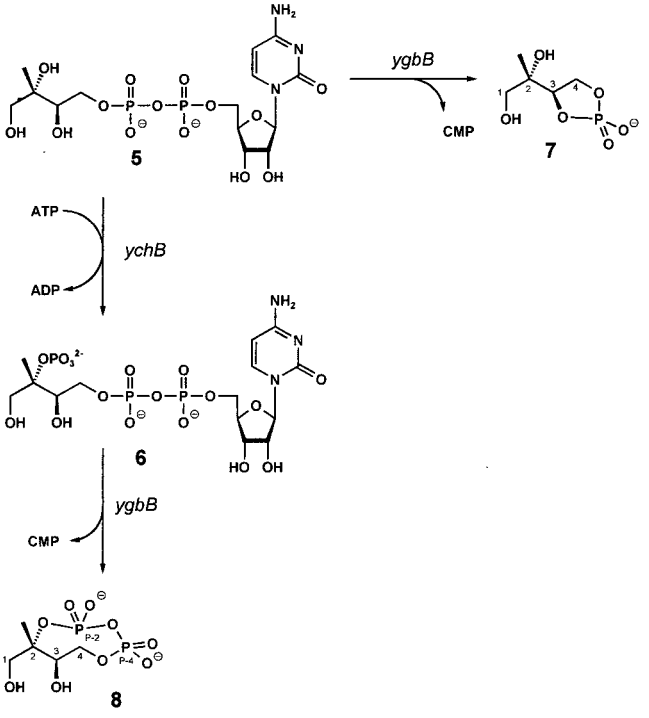

Fig. 5. Reactions catalyzed by YgbB protein.

BIOCHEMISTRY

and the absence of $^{31}P^{1}H$ coupling for the ^{31}P NMR signal at −11.66 ppm (Figs. 3 and 4), in conjunction with the ^{13}C NMR chemical shift for C-2 of the 2C-methyl-D-erythritol moiety (83.9 ppm) as compared with the respective chemical shift for C-2 of 4-diphosphocytidyl-2C-methyl-D-erythritol (73.8 ppm) (15) clearly indicated the connection of the diphosphate motif to C-2 (Table 3). These data established the structure as 2C-methyl-D-erythritol 2,4-cyclodiphosphate (**8**, Fig. 5), which is supported further by the coupling pattern of C-1, C-3, and C-4 (Fig. 3 and Table 3). This compound had been identified earlier by NMR spectroscopy as a metabolite in some bacteria (23–25). Our NMR data are in excellent agreement with those published in the literature.

The second reaction product obtained from 6 and YgbB protein was characterized as CMP by HPLC and by ^{31}P and ^{1}H NMR spectroscopy (data not shown). The ^{31}P NMR spectrum of the reaction product obtained by treatment of 4-diphosphocytidyl-2C-methyl-D-erythritol (**5**) with YgbB protein displayed a singlet signal at 21.7 ppm, suggesting a pentacyclic monophosphate structure (Table 3). Analysis of the ^{1}H and ^{13}C NMR signature (Table 3) identified the compound as 2C-methyl-D-erythritol 3,4-cyclophosphate (**7**, Fig. 5).

Feeding experiments in intact chromoplasts of *C. annuum* using [2-^{14}C]2C-methyl-D-erythritol 2,4-cyclodiphosphate (specific activity, 17.5 μCi/mmol) as substrate resulted in incorporation of 55% of the proffered radioactivity into the carotenoid fraction (data not shown). The addition of 2C-methyl-D-erythritol 4-phosphate did not diminish the incorporation of radioactivity. On the other hand, [2-^{14}C]2C-methyl-D-erythritol 3,4-cyclophosphate (specific activity, 17.5 μCi/mmol) was not incorporated into terpenoids by chromoplasts of *C. annuum*.

Separate application of [2-^{14}C]1-deoxy-D-xylulose 5-phosphate, [2-^{14}C]2C-methyl-D-erythritol 4-phosphate, [2-^{14}C]4-diphosphocytidyl-2C-methyl-D-erythritol, and [2-^{14}C]4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate to stroma of *C. annuum* chromoplasts gave incorporation into a compound that was identified unequivocally as 2C-methyl-D-erythritol 2,4-cyclodiphosphate by NMR analysis (data not shown); the incorporation rates achieved for the above-mentioned precursors were 50%, 55%, 100%, and 100%, respectively.

Discussion

YgbB protein of *E. coli* can use the respective products of YgbP protein (**5**) or YchB protein (**6**) as substrates affording the cyclic monophosphate **7** and the cyclic diphosphate **8**, respectively. Studies with plant chromoplasts showed that only the latter compound can be converted into carotenoids. We conclude that YgbP, YchB, and YgbB proteins act sequentially in the nonmevalonate terpenoid biosynthetic pathway both in bacteria and higher plants.

The cyclic diphosphate **8** has been isolated earlier from cultures of bacteria exposed to oxidative stress (23–25) and had been interpreted tentatively as a dead-end product derived from the deoxyxylulose phosphate pathway. In contrast to this hypothesis, our findings indicate that 2C-methyl-D-erythritol 2,4-cyclophosphate is a genuine intermediate of the deoxyxylulose phosphate pathway.

Putative *ygbP* and *ygbB* orthologs are closely linked or even fused in many microorganisms that use the deoxyxylulose phosphate pathway (Table 2) (15). In each case, the *ygbP* gene or domain is located upstream from the *ygbB* gene or domain.

Plasmodium falciparum has unlinked putative orthologs of *ygbP* and *ygbB*. A putative ortholog of *ygbP* was also found in *A. thaliana* (Table 2).

2C-methyl-D-erythritol 3,4-cyclomonophosphate (**7**) produced by YgbB protein from 4-diphosphocytidyl-2C-methyl-D-erythritol appears as an *in vitro* product without biosynthetic relevance. The compound cannot serve as substrate for any other known enzyme of the deoxyxylulose phosphate pathway and is not utilized by isolated chromoplasts.

We thank A. Werner and F. Wendling for help with the preparation of the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 369), the Fonds der Chemischen Industrie, and the Hans-Fischer-Gesellschaft.

1. Eisenreich, W., Schwarz, M., Cartayrade, A., Arigoni, D., Zenk, M. & Bacher, A. (1998) *Chem. Biol.* **5,** R221–R233.
2. Rohmer, M. (1999) *Comprehensive Natural Products Chemistry*, eds. Barton, D. & Nakanishi, K. (Pergamon, Oxford), Vol. 2, pp. 45–68.
3. Broers, S. T. J. (1994) Ph.D. thesis (Eidgenössische Technische Hochschule Zürich).
4. Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A. & Zenk, M. H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10600–10605.
5. Schwender, J., Zeidler, J., Gröner, R., Müller, C., Focke, M., Braun, S., Lichtenthaler, F. W. & Lichtenthaler, H. K. (1997) *FEBS Lett.* **414,** 129–134.
6. Piel, J., Donath, J., Bandemer, K. & Boland, W. (1998) *Angew. Chem. Int. Ed. Engl.* **37,** 2478–2481.
7. Rosa Putra, S., Lois, L.-M., Campos, N., Boronat, A. & Rohmer, M. (1998) *Tetrahedron Lett.* **29,** 23–26.
8. Giner, J.-L., Jaun, B. & Arigoni, D. (1998) *J. Chem. Soc. Chem. Commun.* 1857–1858.
9. Schwarz, M. K. (1994) Ph.D. thesis (Eidgenössische Technische Hochschule Zürich).
10. Rohmer, H., Seemann, M., Horbach, S., Bringer-Meyer, S. & Sahm, H. (1996) *J. Am. Chem. Soc.* **118,** 2564–2566.
11. Sprenger, G. A., Schörken, U., Wiegert, T., Grolle, S., deGraaf, A. A., Taylor, S. V., Begley, T. P., Bringer-Meyer, S. & Sahm, H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12857–12862.
12. Lois, L. M., Campos, N., Putra, S. R., Danielsen, K., Rohmer, M. & Boronat, A. (1997) *Proc. Natl. Acad. Sci. USA* **95,** 2105–2110.
13. Lange, B. M., Wildung, M. R., McCaskill, D. & Croteau, R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2100–2104.
14. Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R. A. & Camara, B. (1998) *Plant Physiol.* **117,** 1423–1431.
15. Rohdich, F., Wungsintaweekul, J., Fellermeier, M., Sagner, S., Herz, S., Kis, K., Eisenreich, W., Bacher, A. & Zenk, M. H. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 11758–11763.
16. Lüttgen, H., Rohdich, F., Herz, S., Wungsintaweekul, J., Hecht, S., Schuhr, C. A., Fellermeier, M., Sagner, S., Zenk, M. H., Bacher, A., *et al.* (2000) *Proc. Natl. Acad. Sci USA* **97,** 1062–1067.
17. Takahashi, S., Kuzuyama, T., Watanabe, H. & Seto, H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 9879–9884.
18. Lange, B. M. & Croteau, R. (1999) *Arch. Biochem. Biophys.* **365,** 170–174.
19. Schwender, J., Müller, C., Zeidler, J. & Lichtenthaler, H. K. (1999) *FEBS Lett.* **455,** 140–144.
20. Jomaa, H., Wiesner, J., Sanderbrand, S., Altinicicek, B., Weidemeyer, C., Hintz, M., Türbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H. K., *et al.* (1999) *Science* **285,** 1573–1576.
21. Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987) *BioTechniques* **5,** 376–379.
22. Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., *et al.* (1997) *Science* **277,** 1453–1474.
23. Turner, D., Santos, H., Fareleira, P., Pacheco, I., LeGall, Y. & Xavier, A. V. (1992) *Biochem. J.* **285,** 387–390.
24. Ostrovsky, D., Kharatian, E., Dubrovsky, T., Ogrel, O., Shipanova, I. & Sibeldina, L. (1992) *Biofactors* **4,** 63–68.
25. Ostrovsky, D., Diomina, G., Lysak, E., Matveeva, E., Ogrel, O. & Trutko, S. (1998) *Arch. Microbiol.* **171,** 69–72.