Studies on the nonmevalonate pathway to terpenes: The role of the GcpE (IspG) protein

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Recombinant *Escherichia coli* cells engineered for the expression of the *xylB* gene in conjunction with genes of the nonmevalonate pathway were supplied with ^{13}C-labeled 1-deoxy-D-xylulose. Cell extracts were analyzed directly by NMR spectroscopy. ^{13}C-labeled 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate was detected at high levels in cells expressing *xylB*, *ispC*, *ispD*, *ispE*, and *ispF*. The additional expression of the *gcpE* gene afforded 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate as an intermediate of the nonmevalonate pathway. Hypothetical mechanisms involving conserved cysteine residues are proposed for the enzymatic conversion of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate catalyzed by the GcpE protein.

isoprenoid biosynthesis | 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate

Terpenoids are one of the largest groups of natural products, with more than 30,000 known representatives (1). They are all assembled from the universal precursors dimethylallyl diphosphate and isopentenyl diphosphate, whose biosynthesis via the mevalonate pathway has been studied in considerable detail in animal cells and yeast (reviewed in refs. 2–5). An alternative biosynthetic route conducive to these universal terpene precursors via 1-deoxy-D-xylulose 5-phosphate and 2*C*-methyl-D-erythritol 4-phosphate has been discovered only recently in certain bacteria (6, 7) and plants (8) (reviewed in refs. 9–12). The available evidence requires a branching point between 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate and isopentenyl diphosphate (respectively, dimethylallyl diphosphate) (Fig. 1) (13–16).

Clusters of orthologous groups of genes involved in the mevalonate or nonmevalonate pathway show a mutually exclusive distribution in the genomes of completely sequenced microorganisms (12, 17–19). Orthologs of the *lytB* and *gcpE* genes follow the distribution of the established genes of the nonmevalonate pathway (20). From this fact and from the established metabolic function of *dxs*, *ispC*, *ispD*, *ispE*, and *ispF* for the conversion of pyruvate and D-glyceraldehyde 3-phosphate into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, it was deduced that *gcpE* and *lytB* specify enzymes for the conversion of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate into isopentenyl diphosphate and dimethylallyl diphosphate (20). Subsequently, the involvement of these genes in the nonmevalonate pathway has been confirmed by gene-targeting studies (21–23), but their metabolic roles are still unknown.

The incorporation rates of free 1-deoxy-D-xylulose into growing cultures of *Escherichia coli* were shown to be extraordinarily high (7, 13). More recently, we have shown that D-xylulokinase of *E. coli* specified by the *xylB* gene phosphorylates the 5-hydroxy group of 1-deoxy-D-xylulose at a high rate (24). In this article we report on recombinant *E. coli* strains engineered for overexpression of the *xylB* gene in conjunction with *ispCDEF* and *gcpE* or *lytB* genes. Incorporation experiments with ^{13}C-labeled 1-deoxy-D-xylulose have now enabled us to identify 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, an intermediate of the nonmevalonate pathway.

Experimental Procedures

Materials. [U-$^{13}C_5$] and [3,4,5-$^{13}C_3$]1-deoxy-D-xylulose were obtained as described (24, 25). Oligonucleotides were custom synthesized by MWG Biotec (Ebersberg, Germany).

Bacterial Strains and Plasmids. Bacterial strains and plasmids used in this study are summarized in Table 1.

DNA Sequence Determination. DNA was sequenced by the automated dideoxynucleotide method (26) with a 377 Prism sequencer from Perkin–Elmer.

Construction of Vectors with Synthetic Operons Comprising the *xylB*, *ispC*, *ispD*, *ispE*, *ispF*, and *ispG* Genes of *E. coli*. Vector constructs for the *in vivo* utilization of 1-deoxy-D-xylulose into recombinant *E. coli* strains were synthesized in consecutive cloning steps as follows. The *E. coli xylB* gene (GenBank accession no. AE000433) was amplified from bp position 8596 to 10144 by PCR with the use of chromosomal *E. coli* DNA as template and the oligonucleotides xylB*Eco*RI and xylB*Hin*dIII as primers (Table 2). The amplificate was purified, treated with *Eco*RI and *Hin*dIII, and ligated into the plasmid vector pBluescript SKII$^-$ (Stratagene), which had been treated with the same restriction enzymes. The resulting plasmid, pBSxylB, was electrotransformed into *E. coli* strain XL1-Blue (Stratagene) (27), yielding the recombinant strain XL1-pBSxylB.

The *E. coli ispC* gene (GenBank accession no. AE000126) was amplified from bp position 9887 to 11083 by PCR with the use of chromosomal *E. coli* DNA as template and the oligonucleotides dxr*Hin*dIII and dxr*Sal*I as primers (Table 2). The amplificate was purified, treated with *Hin*dIII and *Sal*I, and ligated into the plasmid vector pBSxylB, which had been treated with the same restriction enzymes. The resulting plasmid, pBSxylBispC, was electrotransformed into *E. coli* strain XL1-Blue, yielding the recombinant strain XL1-pBSxylBispC.

The *E. coli* ispDF operon (GenBank accession no. AE000358) was amplified from bp position 6275 to 7464 by PCR with the use of chromosomal *E. coli* DNA as template and the oligonucleotides ygbPBSalI and ygbPBXhoI as primers (Table 2). The amplificate was purified, treated with *Sal*I and *Xho*I, and ligated into the plasmid vector pBSxylBispC, which had been treated with the same restriction enzymes. The resulting plasmid pBSxylBCDF was electrotransformed into *E. coli* strain XL1-Blue, yielding the recombinant strain XL1-pBSxylBispCDF.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY033515).

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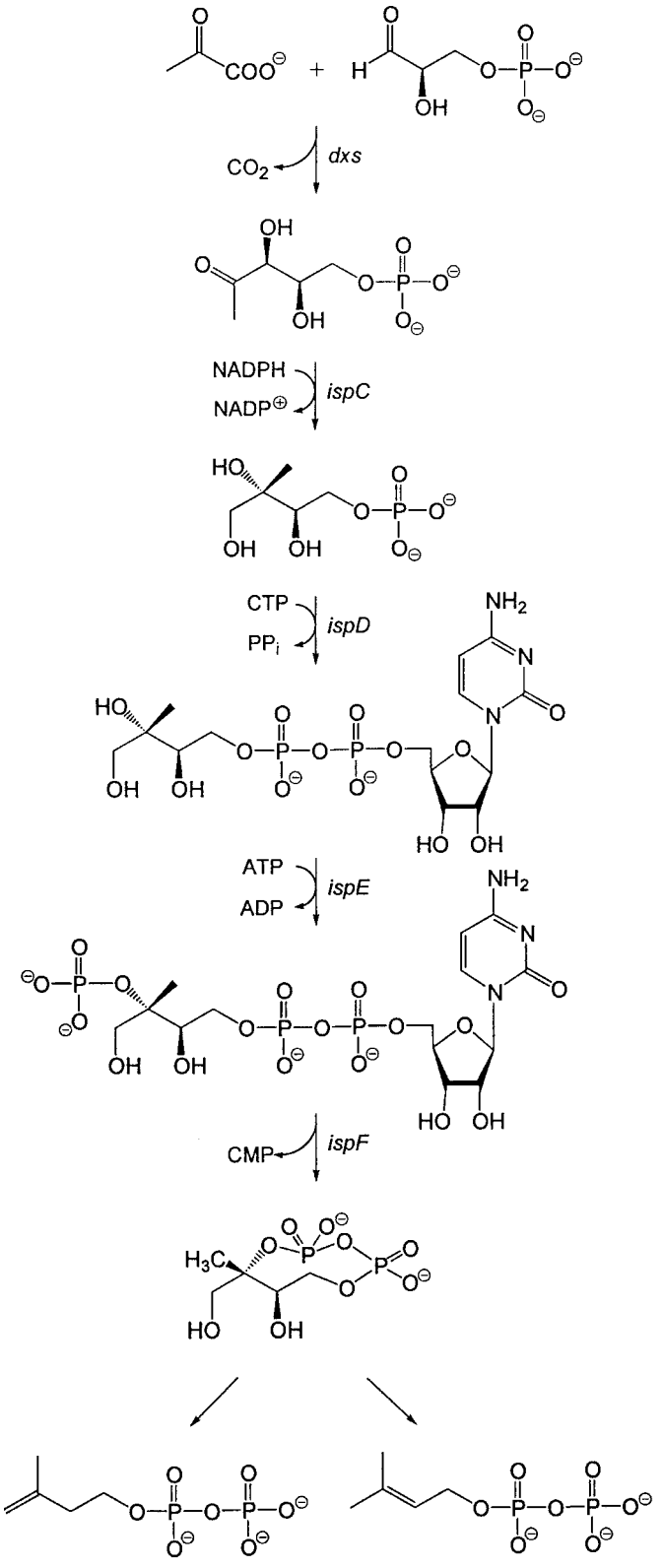

Fig. 1. The nonmevalonate pathway of isoprenoid biosynthesis.

The *E. coli ispE* gene (GenBank accession no. AE000219) was amplified from bp position 5720 to 6571 by PCR with the use of chromosomal *E. coli* DNA as template and the oligonucleotides ychB*Xho*I and ychB*Kpn*I as primers (Table 2). The amplificate was purified, treated with *Xho*I and *Kpn*I, and ligated into the plasmid vector pBSxylBispCDF, which had been treated with the same restriction enzymes. The resulting plasmid pBScyclo was electrotransformed into *E. coli* strain XL1-Blue, yielding the recombinant strain XL1-pBScyclo.

The *E. coli ispG* (formerly *gcpE*) gene (GenBank accession no. AE000338) was amplified from bp position 372 to 1204 by PCR with the use of chromosomal *E. coli* DNA as template and the oligonucleotides gcpE*Sac*II and gcpE*Not*I as primers (Table 2). The amplificate was purified, treated with *Sac*II and *Not*I, and ligated into the plasmid vector pBScyclo, which had been treated with the same restriction enzymes. The resulting plasmid pBSxispC-G was electrotransformed into *E. coli* strain XL1-Blue, yielding the recombinant strain XL1-pBSxispC-G.

Feeding of ^{13}C-Labeled 1-Deoxy-D-Xylulose to Recombinant Cells of *E. coli* Overexpressing the *xylB* Gene in Conjunction with the Genes of the Nonmevalonate Pathway. Terrific Broth medium (0.2 liter containing 36 mg of ampicillin) (28) was inoculated with recombinant *E. coli* XL1-Blue cells carrying plasmid pBSxylB or pBScyclo or pBSxispC-G. The cells were grown with shaking at 37°C overnight. At an optical density (600 nm) of 5.0, 30 mg (0.1 mmol) of cytidine and, in the case of plasmid XL1-pBSxispC-G, 25 mmol of lithium lactate in 20 ml of 100 mM Tris hydrochloride (pH 7.2), were added. After 30 min, 0.1 mmol of [U-$^{13}C_5$]- or [3,4,5-$^{13}C_3$]1-deoxy-D-xylulose was added. Aliquots of 40 ml were retrieved in intervals of 1 h. The cells were harvested, centrifuged for 10 min at 5,000 rpm and 4°C, washed with 0.9% NaCl, and centrifuged. The cells were resuspended in 600 μl of 10 mM NaF/D_2O in 50% (vol/vol) methanol-d_4, cooled on ice, and subjected to ultrasonic treatment. The suspension was centrifuged at 15,000 rpm for 15 min. To avoid degradation during work-up, the supernatants were subjected to NMR analysis without further purification.

NMR Spectroscopy. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with an AVANCE 500 spectrometer from Bruker Instruments (Karlsruhe, Germany).

Results

A suspension of recombinant *E. coli* cells engineered for overexpression of the *xylB* gene was supplied with [U-$^{13}C_5$]1-deoxy-D-xylulose as described under *Experimental Procedures* (Fig. 2). ^{13}C NMR analysis of the cell-free extract obtained after cell disruption showed intense signals for 1-deoxy-D-xylulose 5-phosphate (25), indicating that exogenous 1-deoxy-D-xylulose is rapidly purged from the culture medium and accumulated into intracellular 1-deoxy-D-xylulose 5-phosphate in substantial concentrations (data not shown).

Capitalizing on this observation, we then constructed synthetic operons comprising the *xylB* gene and various terpenoid pathway genes endowed with artificial ribosomal binding sites under the control of a *lac* promoter/operator (Fig. 2) on high-copy plasmids (see *Experimental Procedures*). Bacterial suspensions with an OD_{600} of about 5 were incubated with [3,4,5-$^{13}C_3$]- or [U-$^{13}C_5$]-labeled 1-deoxy-D-xylulose for 2 h, harvested, disrupted by ultrasonic treatment in 10 mM NaF/D_2O in 50% (vol/vol) methanol-d_4, and centrifuged. To minimize the decomposition of potentially unstable metabolites, the supernatants were analyzed by one- and two-dimensional NMR spectroscopy without any pretreatment. This experimental strategy was expected to enhance the sensitivity and selectivity of the NMR analysis, and, indeed, ^{13}C NMR spectra of the crude extracts showed signals for products derived from the proffered ^{13}C-labeled 1-deoxy-D-xylulose, whereas the concentrations of unlabeled metabolites from the cell background turned out to be too low for ^{13}C NMR detection.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic	Ref. or source
E. coli		
XL1-Blue	*RecA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, [F′, *proAB*, *lac1*^{q}*ZΔM15*, *Tn10* (*tet*′)]	27, Stratagene
Plasmids		
pBluescript SKII^{-}	High copy cloning vector	Stratagene
pBSxylB	Expression of *xylB* from *E. coli*	This study
pBSxylBispC	Expression of *xylB* and *ispC* from *E. coli*	This study
pBSxylBispCDF	Expression of *xylB*, *ispC*, *ispD*, and *ispF* from *E. coli*	This study
pBScyclo	Expression of *xylB*, *ispC*, *ispD*, *ispE*, and *ispF* from *E. coli*	This study
pBSxispC-G	Expression of *xylB*, *ispC*, *ispD*, *ispE*, *ispF*, and *ispG* from *E. coli*	This study

Table 2. Oligonucleotides used in this study

Designation	5′-Sequence-3′
xylB*Eco*RI	CCGTCGGAATTCGAGGAGAAATTAACCATGTATATCGGGATAGATCTTGG
xylB*Hin*dIII	GCAGTGAAGCTTTTACGCCATTAATGGCAGAAGTTGC
dxr*Hin*dIII	CTAGCCAAGCTTGAGGAGAAATTAACCATGAAGCAACTCACCATTCTGG
dxr*Sal*I	GGAGATGTCGACTCAGCTTGCGAGACGC
ygbPB*Sal*I	CCGGGAGTCGACGAGGAGAAATTAACCATGGCAACCACTCATTTGGATG
ygbPB*Xho*I	TATCAACTCGAGTCATTTTGTTGCCTTAATGAG
ychB*Xho*I	GCGAACCTCGAGGAGGAGAAATTAACCATGCGGACACAGTGGCCC
ychB*Kpn*I	CCTGACGGTACCTTAAAGCATGGCTCTGTGC
gcpE*Sac*II	GCGGGAGACCGCGGGAGGAGAAATTAACCATGCATAACCAGGCTCCAATTCAACG
gcpE*Not*I	CGCTTCCCAGCGGCCGCTTATTTTTCAACCTGCTGAACG

When [U-$^{13}C_5$]1-deoxy-D-xylulose was incubated with a recombinant strain engineered for hyperexpression of *xylB*, *ispC, ispD*, *ispE*, and *ispF* (experiment A), the ^{13}C NMR spectrum of the cell extract was dominated by the known (19) signals of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (Fig. 3*A*, signals in green). In an analogous experiment (experiment B) with a recombinant *E. coli* strain expressing *xylB*, *ispC, ispD*, *ispE*, *ispF,* and, in addition, *gcpE* (29) (subsequently designated *ispG*), the ^{13}C NMR spectrum of the cell extracts displayed, in addition to the signature of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, a set of ^{13}C-coupled signals with comparable intensities belonging to a five-carbon compound (Fig. 3*B*, signals in red).

The ^{13}C NMR chemical shifts of the unknown metabolite suggested a double bond (signals at 122.7 and 139.5 ppm), a methyl group (signal at 14.7 ppm), and two carbon atoms (signals at 64.5 and 68.6 ppm) connected to a heteroatom. Because the compound had been biosynthesized from uniformly ^{13}C-labeled 1-deoxy-D-xylulose, the carbon connectivity could be established by ^{13}C,^{13}C coupling analysis. The three signals accounting for carbon atoms with sp^3 hybridization (14.7, 64.5, and 68.5 ppm) showed ^{13}C coupling to one adjacent ^{13}C atom with coupling constants of 40–50 Hz (Table 3). The signal at 122.7 ppm showed ^{13}C coupling to two adjacent ^{13}C neighbors (coupling constants, 74 and 50 Hz), whereas the signal at 141.5 ppm showed ^{13}C coupling to three neighboring ^{13}C atoms (coupling constants, 74, 43, and 43 Hz).

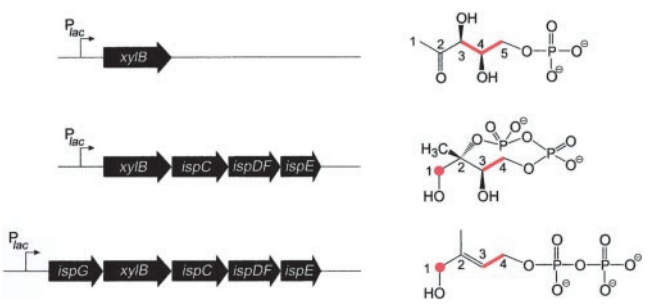

Fig. 2. Expression vectors comprising synthetic operons of *xylB* together with genes of the nonmevalonate pathway used for the *in vivo* production of intermediates in the nonmevalonate pathway. ^{13}C-labeled compounds detected in cell extracts obtained from *E. coli* cells overexpressing the indexed genes and supplied with [3,4,5-$^{13}C_3$]1-deoxy-D-xylulose are indicated (the ^{13}C label is in red).

^{1}H,^{13}C correlation experiments (heteronuclear multiple quantum correlation, heteronuclear multiple quantum correlation–total correlation spectroscopy, and heteronuclear multiple bond correlation) revealed the ^{1}H NMR chemical shifts, as well as the ^{13}C,^{1}H and ^{1}H,^{1}H spin systems summarized in Table 3. Together with the carbon connectivities, these data demonstrate the presence of a 1,4-dihydroxy-2-methyl-2-butenyl derivative.

In a similar experiment with proffered [3,4,5-$^{13}C_3$]1-deoxy-D-xylulose, only enriched signals accounting for C-1, C-3, and C-4 of the novel metabolite were observed (compare Fig. 2). Because the complexity of the ^{13}C,^{13}C coupling pattern was lower by comparison with the earlier experiment, it could be established beyond doubt that C-4 and C-3 resonating at 64.5 and 122.7 ppm show ^{13}C,^{31}P coupling with coupling constants of 5.5 and 8.0 Hz, respectively, thus suggesting the presence of a phosphate or diphosphate group at position 4.

The ^{1}H-decoupled ^{31}P NMR spectrum of the compound displayed a doublet at −9.2 (^{31}P,^{31}P coupling constant, 20.9 Hz) and a double-double doublet at −10.6 ppm (^{31}P,^{31}P coupling constant, 20.9 Hz; ^{31}P,^{13}C coupling constants, 5.8 and 7.4 Hz). Without ^{1}H decoupling, the ^{31}P NMR signal at −10.6 ppm was broadened, whereas the signal at −9.2 ppm was not affected by ^{1}H decoupling. The chemical shifts as well as the observed coupling patterns confirmed the presence of a diphosphate group at position 4. Conclusive proof for the structure of the compound was eventually obtained by comparison with the NMR data of a synthetic specimen of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, in which the geometry of the double bond was established by nuclear Overhauser effect (spectroscopy) experiments (data to be published elsewhere). Whereas this

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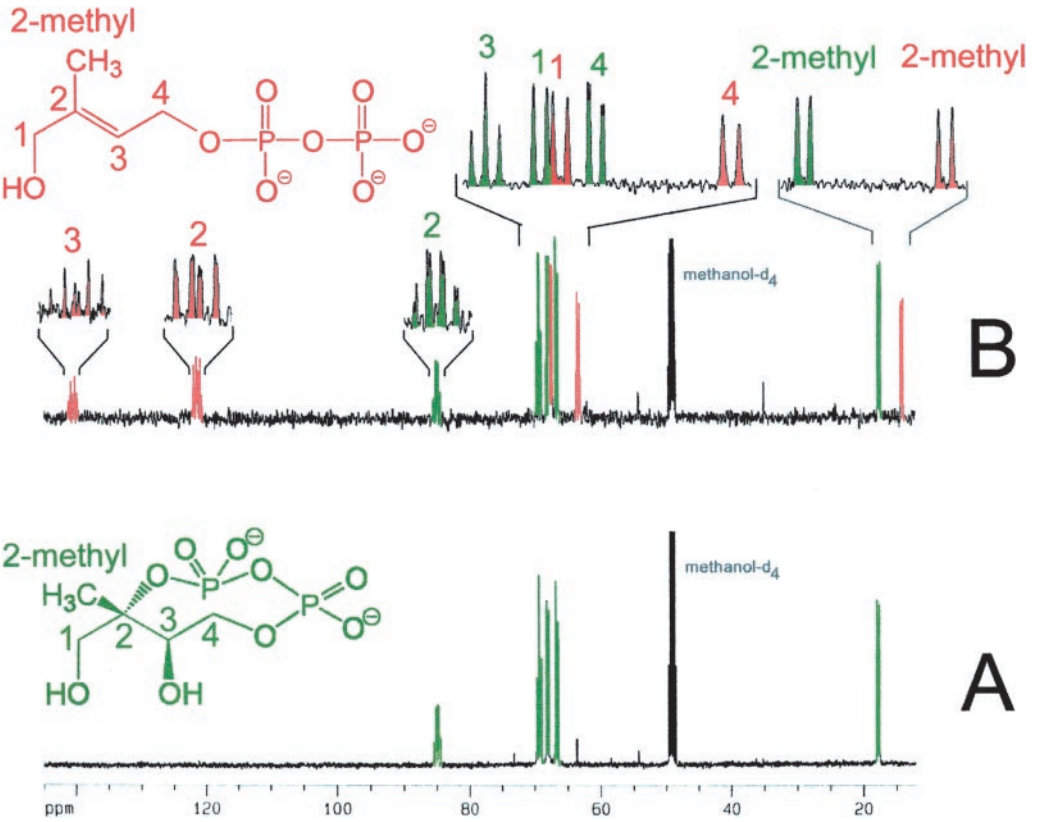

Fig. 3. ^{13}C NMR spectra of crude extracts obtained from the feeding of [U-$^{13}C_5$]1-deoxy-D-xylulose to recombinant cells of *E. coli* overexpressing (*A*) the *xylB*, *ispC*, *ispD*, *ispE,* and *ispF* genes and (*B*) the *xylB*, *ispC*, *ispD*, *ispE*, *ispF,* and *ispG* (*gcpE*) genes.

compound had not yet been isolated from natural sources, the structurally related 1-*O*-β-D-glucoside of 1,4-dihydroxy-2-methyl-2-(*E*)-butene has been detected in *Ornithogalum montanum* (30).

The metabolite was not formed in detectable amounts with the recombinant strain of experiment A lacking the overexpressed IspG protein, nor was it formed when the *ispG* gene of the recombinant strain used in experiment B was replaced by *lytB* (data not shown). Because 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate appears to be formed at the expense of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, we propose that it represents one of the missing links in the formation of isopentenyl diphosphate and dimethylallyl diphosphate.

Discussion

The available genetic and biochemical data show that the formation of the metabolite 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate requires the IspG protein. Enzymes involved in the transformation of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, in addition to IspG protein, if any, must have been specified in sufficient amounts by chromosomal genes in our experimental system, because the hyperexpression of *ispG* is sufficient for the formation of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate in relatively large amounts.

The cluster of the orthologous IspG (GcpE) group is characterized by three absolutely conserved cysteines (residues 269,

Table 3. NMR data of 1-hydroxy-2-(*E*)-methyl-2-butenyl 4-diphosphate

	Chemical shifts, ppm			Coupling constants, Hz			NMR correlation pattern	
Position	^1H*	^{13}C*	^{31}P†	J_{PC}	J_{PP}	J_{CC}	HMQC-TOCSY	HMBC
1	3.91	68.6‡§				43.0,§ 5.5,‡ 3.5‡	1	2-Methyl, 2, 3
2		139.5‡§				74.3,§ 43.3,§ 43.3§		
2-Methyl	1.51	14.7§				42.2,§ 4.0,§ 4.0§	2-Methyl	1, 3, 2
3	5.57	122.7§		8.0‡		73.9,§ 49.8,‡ 4.0‡	3, 4	
4	4.46	64.5‡§		5.5‡		49.3,‡ 5.5‡	4, 3	3, 2
P_β			−9.2		20.9			
P_α			−10.6	5.8,‡ 7.4‡	20.9			

HMQC, Heteronuclear multiple quantum correlation spectroscopy; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy. The solvent was a mixture of deuterated water and methanol (1:1, vol/vol).

*Referenced to external trimethylsilylpropane sulfonate.

†Referenced to external 85% orthophosphoric acid.

‡Observed with [1,3,4-$^{13}C_3$]1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate.

§Observed with [U-$^{13}C_5$]1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate.

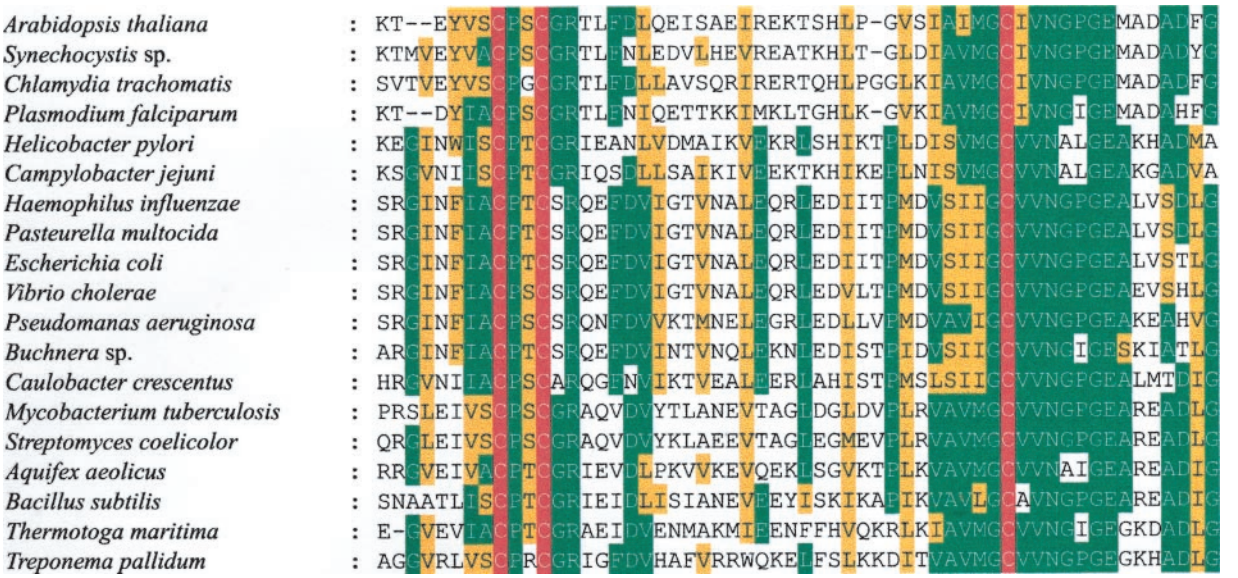

Fig. 4. Highly conserved amino acid region of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthases (IspG proteins) from various organisms. Identical and similar residues conserved in more than 60% of the sequences are shown in green and yellow, respectively. Conserved cysteine residues (corresponding to residues 269, 272, and 304 of the *E. coli* protein) are shown in red.

272, and 305 of the *E. coli* enzyme; Fig. 4). Although these residues might be part of a transition metal-binding site (e.g., for iron or zinc), no direct evidence for metal binding has been obtained. The overall reaction catalyzed by the IspG protein is a two-electron reduction process involving the cleavage of two carbon–oxygen bonds. From previous work on the *E. coli* pathway to terpenes it can be inferred that all of the carbon-bonded hydrogen atoms of the cyclodiphosphate precursor are preserved in the reaction product (16, 31, 32). This stringent requirement puts severe limitations on the choice of a plausible reaction mechanism for the unusual biochemical transformation; among the remaining possibilities, we would like to focus attention on the two hypothetical reaction sequences summarized in Figs. 5 and 6, both of which critically depend on the presence of at least two cysteinyl residues.

The mechanism illustrated in Fig. 5 is patterned after the mode of action of vitamin K epoxyquinone reductase (34). The aldehydo group generated in the first step with $NADP^+$ as a putative cofactor is expected to accelerate the subsequent nucleophilic displacement of the pyrophosphate group at C-2 by a thiolate ion and provides the necessary electron sink in the step that leads to the formation of a disulfide bond. After β-elimination of water from the resulting enolate, the original oxidation state of C-1 is then restored by back transfer from the cofactor of the hydride ion removed in the first step.

The alternative presented in Fig. 6 is a radical process akin to the one exploited by ribonucleotide reductase (35), in which the

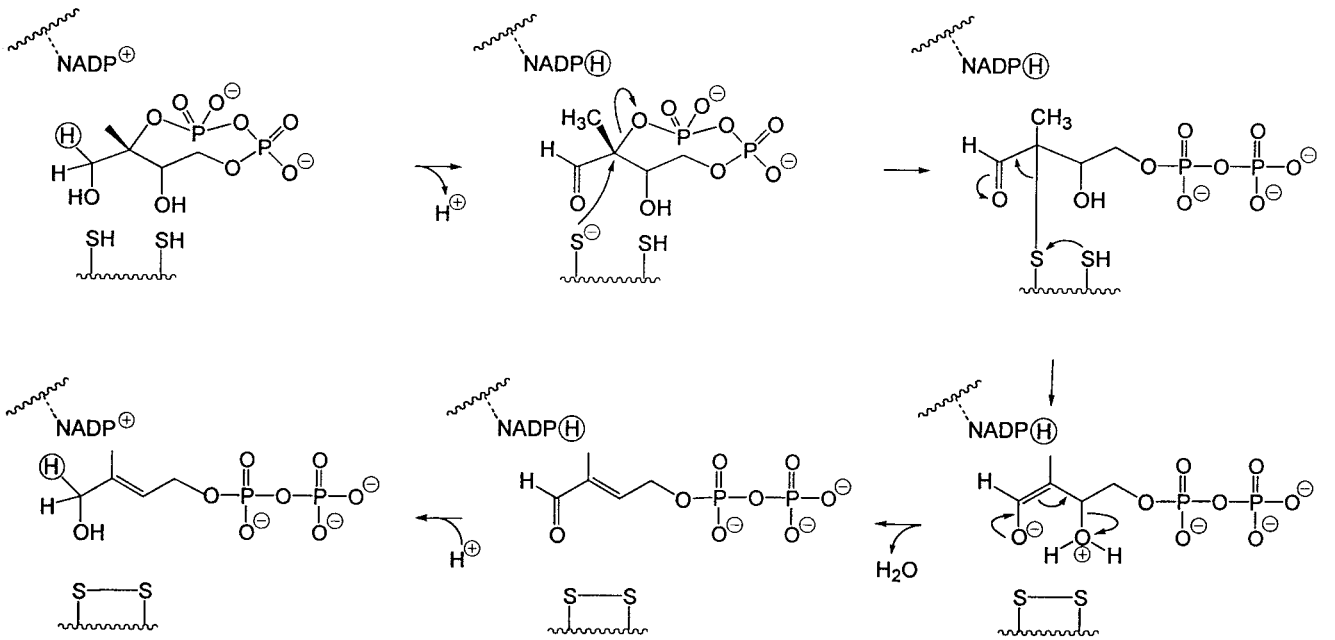

Fig. 5. Hypothetical mechanism of the IspG protein-mediated reaction patterned after the mode of action of vitamin K epoxyquinone reductase (30).

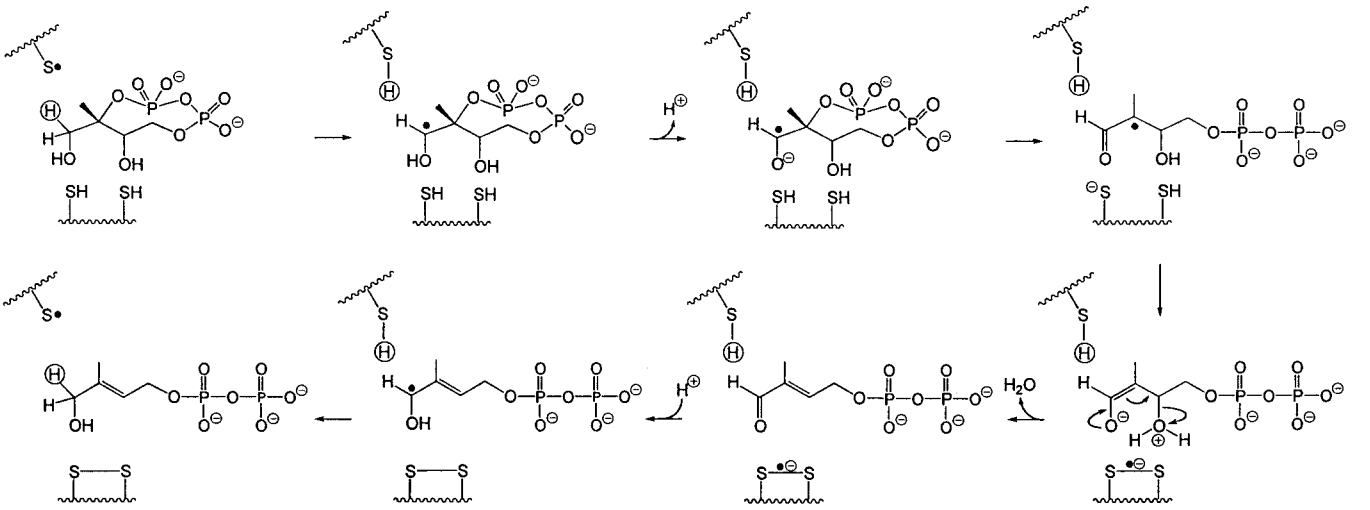

Fig. 6. Hypothetical mechanism of the IspG protein-mediated reaction patterned after the mode of action of ribonucleotide reductase (31).

role of the immediate initiator is assigned, by analogy, to a thiyl radical derived from the third conserved cysteine residue of the enzyme. Participation of such a radical without or with negligible exchange of the resulting thiol group with solvent protons is well precedented (34).

In both schemes reductive cleavage of the disulfide bond is necessary for multiple turnover. No clues are yet available concerning the nature of the reducing agent. An unusual sensitivity of the latter may explain why 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate is accumulated in certain bacteria under conditions of oxidative stress (35).

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