

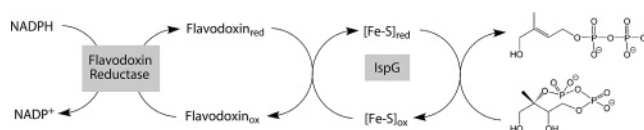
Biosynthesis of Isoprenoids. Purification and Properties of IspG Protein from *Escherichia coli*

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The IspG protein is known to catalyze the transformation of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate in the nonmevalonate pathway of isoprenoid biosynthesis. We have found that the apparent IspG activity in the cell extracts of recombinant *Escherichia coli* cells as observed by a radiochemical assay can be enhanced severalfold by coexpression of the *isc* operon which is involved in the assembly of iron–sulfur clusters. The recombinant protein was isolated by affinity chromatography under anaerobic conditions. With a mixture of flavodoxin, flavodoxin reductase, and NADPH as the reducing agent, stringent assay methods based on photometry or on ^{13}C NMR detection of multiply ^{13}C -labeled substrate/product ratios afforded catalytic activities greater than $60 \text{ nmol mg}^{-1} \text{ min}^{-1}$ for the protein “as isolated” (i.e., without reconstitution of any kind). Lower apparent activities were found using photoreduced deazaflavin as an artifactual electron donor, whereas dithionite was unable to serve as an artificial electron donor. The apparent Michaelis constant for 2-C-methyl-D-erythritol 2,4-cyclodiphosphate was $700 \mu\text{M}$. The enzyme was inactivated by EDTA and could be reactivated by Mn^{2+} . The pH optimum was at 9.0. The protein contained 2.4 iron ions and 4.4 sulfide ions per subunit. The replacement of any of the three conserved cysteine residues afforded mutant proteins which were devoid of catalytic activity and contained less than 6% of Fe^{2+} and less than 23% of S^{2-} as compared to the wild-type protein. Sequence comparison indicates that putative IspG proteins of plants, the apicomplexan protozoan *Plasmodium falciparum*, and bacteria from the Bacteroidetes/Chlorobi group contain an insert of about 170–320 amino acid residues as compared with eubacterial enzymes.

Introduction

Recent studies have established the existence of a second pathway, besides the well-known mevalonate pathway, for the biosynthesis of the universal isoprenoid precursors isopentenyl diphosphate (IPP, Compound 7, Figure 1) and dimethylallyl diphosphate (DMAPP, Compound 8, Figure 1).^{1,2} This nonmevalonate pathway is now known to supply the building blocks for the vast majority of terpenes in higher plants and has therefore already been evaluated as a target for new herbicides.^{3–7} It is also known to be the unique source for the biosyn-

thesis of terpenes in the majority of pathogenic eubacteria, with the notable exception of Gram-positive cocci which use the mevalonate pathway.^{8,9} Hence, the nonmevalonate pathway enzymes are potential targets for

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

(2) Schwarz, M. K. Ph.D. Thesis, ETH Zürich, Zürich, 1994.

(3) Eisenreich, W.; Menhard, B.; Hylands, P. J.; Zenk, M. H.; Bacher, A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6431–6436.

(4) Lichtenthaler, H. K.; Schwender, J.; Disch, A.; Rohmer, M. *FEBS Lett.* **1997**, *400*, 271–274.

(5) Arigoni, D.; Sagner, S.; Latzel, C.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10600–10605.

(6) Eichinger, D.; Bacher, A.; Zenk, M. H.; Eisenreich, W. *Phytochemistry* **1999**, *51*, 223–236.

(7) Eisenreich, W.; Sagner, S.; Zenk, M. H.; Bacher, A. *Tetrahedron Lett.* **1997**, *38*, 3889–3892.

(8) Laupitz, R.; Hecht, S.; Amslinger, S.; Zepeck, F.; Kaiser, J.; Richter, G.; Schramek, N.; Steinbacher, S.; Huber, R.; Arigoni, D.; Bacher, A.; Eisenreich, W.; Rohdich, F. *Eur. J. Biochem.* **2004**, *271*, 2658–2669.

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

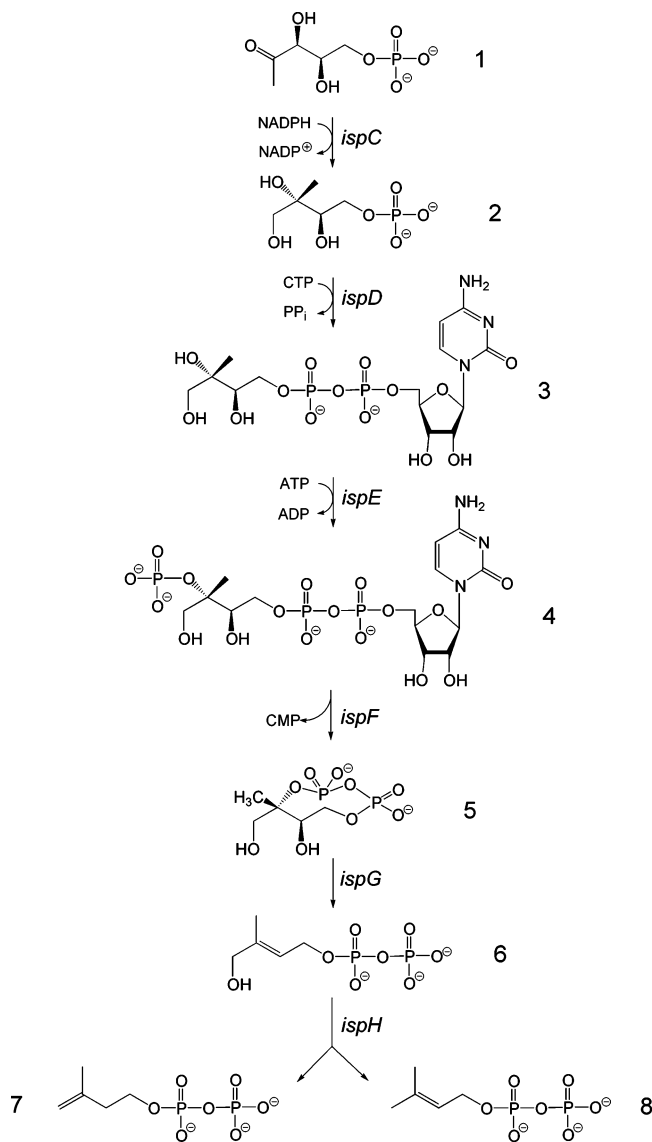


FIGURE 1. Nonmevalonate isoprenoid pathway.

inhibitors which could serve as antibacterial agents to cope with the rapid spreading of microbial resistance against all currently used antibiotics.^{10–13}

The first committed step of the nonmevalonate pathway involves the conversion of 1-deoxy-D-xylulose 5-phosphate (**1**) into 2-C-methyl-D-erythritol 4-diphosphate (**2**) by a sequence of skeletal isomerization and reduction, which are both catalyzed by the IspC protein (Figure 1).^{5,14–17} The branched chain polyol derivative **2** is

subsequently converted into the cyclic diphosphate **5** by a series of three reactions catalyzed by the IspD, IspE, and IspF proteins.^{18–20}

The cyclic diphosphate **5** is converted into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**6**) by a mechanistically complex reaction catalyzed by an iron–sulfur protein specified by the *ispG* gene.^{21–25} Reduction equivalents required for the enzyme-catalyzed process can be supplied via a cascade of redox proteins (flavodoxin and flavodoxin reductase) or by artificial electron transponders such as photoreduced deazaflavin or dithionite. The enzyme is as of yet incompletely characterized. The product **6** of the IspG protein can be converted into a mixture of IPP (**7**) and DMAPP (**8**) by an iron–sulfur protein specified by the *ispH* gene.^{26–30}

This paper reports the preparation of the recombinant IspG protein from *Escherichia coli* in high yields and in catalytically high active form and describes its properties.

Results

We have previously³⁰ shown that the recombinant expression of the *ispH* gene specifying the last enzyme in the nonmevalonate pathway in catalytically active form can be considerably improved by the coexpression of genes from the *isc* operon, whose translation products are involved in the assembly of iron–sulfur clusters.^{31,32} The overexpression of *ispH* and *isc* genes was confirmed

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

(19) Lüttgen, H.; Rohdich, F.; Herz, S.; Wungsintaweekul, J.; Hecht, S.; Schuhr, C. A.; Fellermeier, M.; Sagner, S.; Zenk, M. H.; Bacher, A.; Eisenreich, W. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1062–1067.

(20) Herz, S.; Wungsintaweekul, J.; Schuhr, C. A.; Hecht, S.; Lüttgen, H.; Sagner, S.; Fellermeier, M.; Eisenreich, W.; Zenk, M. H.; Bacher, A.; Rohdich, F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2486–2490.

(21) Hecht, S.; Eisenreich, W.; Adam, P.; Amslinger, S.; Kis, K.; Bacher, A.; Arigoni, D.; Rohdich, F. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14837–14842.

(22) Kollas, A. K.; Duin, E. C.; Eberl, M.; Altincicek, B.; Hintz, M.; Reichenberg, A.; Henschker, D.; Henne, A.; Steinbrecher, I.; Ostrovsky, D. N.; Hedderich, R.; Beck, E.; Jomaa, H.; Wiesner, J. *FEBS Lett.* **2002**, *532*, 432–436.

(23) Seemann, M.; Bui, B. T.; Wolff, M.; Tritsch, D.; Campos, N.; Boronat, A.; Marquet, A.; Rohmer, M. *Angew. Chem., Int. Ed. Engl.* **2002**, *41*, 4337–4339.

(24) Rohdich, F.; Zepeck, F.; Adam, P.; Hecht, S.; Kaiser, J.; Laupitz, R.; Gräwert, T.; Amslinger, S.; Eisenreich, W.; Bacher, A.; Arigoni, D. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1586–1591.

(25) Seemann, M.; Wegner, P.; Schunemann, V.; Bui, B. T.; Wolff, M.; Marquet, A.; Trautwein, A. X.; Rohmer, M. *J. Biol. Inorg. Chem.* **2005**, *10*, 131–137.

(26) Altincicek, B.; Duin, E. C.; Reichenberg, A.; Hedderich, R.; Kollas, A. K.; Hintz, M.; Wagner, S.; Wiesner, J.; Beck, E.; Jomaa, H. *FEBS Lett.* **2002**, *532*, 437–440.

(27) Rohdich, F.; Hecht, S.; Gartner, K.; Adam, P.; Krieger, C.; Amslinger, S.; Arigoni, D.; Bacher, A.; Eisenreich, W. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1158–1163.

(28) Adam, P.; Hecht, S.; Eisenreich, W.; Kaiser, J.; Gräwert, T.; Arigoni, D.; Bacher, A.; Rohdich, F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12108–12113.

(29) Wolff, M.; Seemann, M.; Bui, B. T.; Frapart, Y.; Tritsch, D.; Garcia Estrabot, A.; Rodríguez-Concepción, M.; Boronat, A.; Marquet, A.; Rohmer, M. *FEBS Lett.* **2003**, *541*, 115–120.

(30) Gräwert, T.; Kaiser, J.; Zepeck, F.; Laupitz, R.; Hecht, S.; Amslinger, S.; Schramek, N.; Schleicher, E.; Weber, S.; Haslbeck, M.; Buchner, J.; Rieder, C.; Arigoni, D.; Bacher, A.; Eisenreich, W.; Rohdich, F. *J. Am. Chem. Soc.* **2004**, *126*, 12847–12855.

(31) Zheng, L.; Cash, V. L.; Flint, D. H.; Dean, D. R. *J. Biol. Chem.* **1998**, *273*, 13264–13272.

(32) Tokumoto, U.; Nomura, S.; Minami, Y.; Mihara, H.; Kato, S.; Kurihara, T.; Esaki, N.; Kanazawa, H.; Matsubara, H.; Takahashi, Y. *J. Biochem. (Tokyo)* **2002**, *131*, 713–719.

(10) Eisenreich, W.; Bacher, A.; Arigoni, D.; Rohdich, F. *Cell. Mol. Life Sci.* **2004**, *61*, 1401–1426.

(11) Rohmer, M.; Grosdemange-Billiard, C.; Seemann, M.; Tritsch, D. *Curr. Opin. Investig. Drugs* **2004**, *5*, 154–162.

(12) Rohdich, F.; Hecht, S.; Bacher, A.; Eisenreich, W. *Pure Appl. Chem.* **2003**, *75*, 393–405.

(13) Rohdich, F.; Bacher, A.; Eisenreich, W. *Bioorg. Chem.* **2004**, *32*, 292–308.

(14) Radykewicz, T.; Rohdich, F.; Wungsintaweekul, J.; Herz, S.; Kis, K.; Eisenreich, W.; Bacher, A.; Zenk, M. H.; Arigoni, D. *FEBS Lett.* **2000**, *465*, 157–160.

(15) Proteau, P. J.; Woo, Y. H.; Williamson, R. T.; Phaosi, C. *Org. Lett.* **1999**, *1*, 921–923.

(16) Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9879–9884.

(17) Fox, D. T.; Poulter, C. D. *J. Org. Chem.* **2005**, *70*, 1978–85.

TABLE 1. Specific IspG Protein Activities in Cell Extracts of Recombinant and Wild-Type *E. Coli* Strains

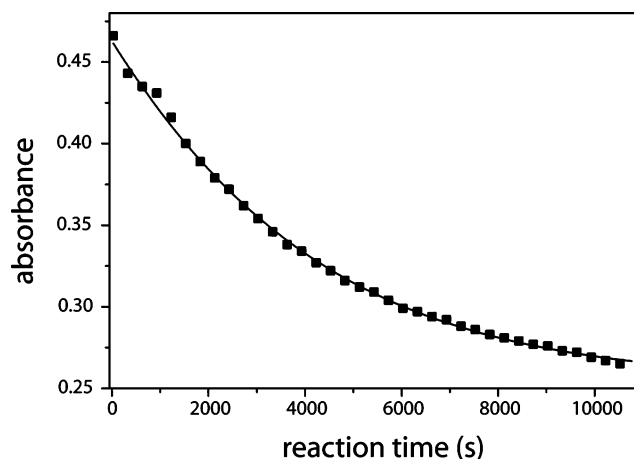
strain	FldA and Fpr	specific activity (nmol min ⁻¹ mg ⁻¹)
wild type	—	0.25
	+	0.5
XL1-pMALispG	—	2.1
	+	4.5
XL1-pMALispG-pACYCisc	—	8.2
	+	31

by two-dimensional acryl amide gel electrophoresis.³⁰ In the present study, we applied the same approach to the recombinant expression of the *ispG* gene of *E. coli*. Specifically, a recombinant *E. coli* *malE/ispG* fusion gene was overexpressed together with the *E. coli* *isc* operon in the *E. coli* strain XL1-Blue (for details, see Supporting Information).

Specific activities of cell extracts from wild-type and recombinant strains determined in radiochemical assays under different conditions are summarized in Table 1. Cell extracts of the recombinant *E. coli* strain expressing the *ispG* gene without an enhanced *isc* operon expression were shown to convert **5** into **6**, with an apparent rate of 2.1 nmol mg⁻¹ min⁻¹ when assayed without the electron transponder enzymes. The specific activity was increased to 4.5 nmol mg⁻¹ min⁻¹ when assays were performed in the presence of flavodoxin and flavodoxin reductase. Coexpression of the *isc* operon increased these values further to 8.2 and 31 nmol mg⁻¹ min⁻¹, respectively. This represents a 7-fold increase by comparison with cell extracts of recombinant strains expressing *ispG* alone and a 60-fold increase as compared with cell extracts of the wild-type strain, respectively.

The recombinant MalE/IspG fusion protein (expression level of about 40% with respect to the total soluble cell protein) was purified by affinity chromatography on an amylose resin FF column that was developed with a gradient of 0–10 mM maltose under anaerobic conditions, whereby the enzyme was enriched by a factor of 2.4 (Supporting Information). The 84 kDa protein migrated on the column as an intensely brown-colored band and appeared nearly homogeneous after elution, as judged by SDS polyacrylamide gel electrophoresis (Supporting Information). The absorption spectrum showed a maximum at about 410 nm which disappeared after exposure to oxygen (Supporting Information). Partial N-terminal Edman degradation afforded the sequence MKIEEGKLVI (i.e., the N-terminus of the MalE domain), as expected.

The catalytic properties of the recombinant protein were studied using [2-¹⁴C]-**5** as the substrate and a mixture of flavodoxin (from *E. coli*), flavodoxin reductase (from *E. coli*), and NADPH as the reducing agent. Product formation was assessed by high-pressure liquid chromatography (HPLC) monitored by real time scintillation counting. The recombinant IspG protein (as isolated) was active in the absence of added metal ions. When certain divalent metals, preferably Mn²⁺, were added to the assay mixture, the enzymatic activity was enhanced up to 2-fold (Supporting Information). Zn²⁺ decreased the enzyme activity. The enzyme was completely inactivated by the addition of EDTA; the activity of an EDTA-inactivated enzyme could be partially restored (to a level of about

**FIGURE 2.** Kinetics of IspG protein activity. For details, see Experimental Section. Absorbance at 340 nm is represented by filled squares (■) (experimental data). The line represents the absorbance at 340 nm obtained from numerical simulation using the program Biokin.³³

25%) by the addition of Mn²⁺. A significant excess of flavodoxin and flavodoxin reductase, as compared to the recombinant IspG protein, was required for maximum activity (Supporting Information).

More specifically, maximum activity was observed with 9-fold and 3-fold molar excesses of flavodoxin and flavodoxin reductase, respectively. The optimum pH for the enzyme-catalyzed reaction was 9.0 (Supporting Information). Ferredoxin and ferredoxin reductase from spinach could be used instead of the *E. coli* proteins with quantitatively similar results (data not shown).

Under optimal conditions (i.e., 1 mM Mn²⁺, a 9-fold molar excess of *E. coli* flavodoxin, a 3-fold molar excess of *E. coli* flavodoxin reductase, 4 mM NADPH, and pH 9.0), the radiochemical assay afforded a Michaelis constant (*K_M*) value of 560 μM for **5** and afforded a value of 74 nmol mg⁻¹ min⁻¹ for the specific activity (Supporting Information).

The enzyme-catalyzed reaction could be monitored photometrically (Figure 2). In an assay mixture containing 1 mM Mn²⁺, 100 μM [1,3,4-¹³C₃]-**5**, 1 mM NADPH, 40 μM flavodoxin, 12 μM flavodoxin reductase, and 0.13 μM IspG protein, the consumption of NADPH afforded apparent values of 700 μM for *K_M* and 99 nmol mg⁻¹ min⁻¹ for the specific activity.

Using photoreduced deazaflavin as a reducing agent instead of the electron transponder proteins, we detected apparent specific activities of 45 nmol min⁻¹ mg⁻¹. Dithionite could not serve as an electron donor for the enzyme-catalyzed reaction; the specific activity was below the limit of detection (<1 pmol mg⁻¹ min⁻¹) in reaction mixtures containing 1 mM [2-¹⁴C]-**5** and 10 mM dithionite at pH 8.0.

The enzyme activity was also assessed by quantitative ¹³C NMR analysis. Experiments using [1,3,4-¹³C₃]-**5** or [2,2'-¹³C₂]-**5** as substrates afforded the NMR spectra shown in Figure 3. Because of ¹³C–¹³C and ¹³C–³¹P coupling (cf. Supporting Information), the signals of the multiply ¹³C-labeled substrates appear as highly characteristic multiplets.²¹ Similarly, the ¹³C-enriched carbon atoms of the respective products [1,3,4-¹³C₃]- and [2,2'-¹³C₂]-**6** afforded characteristic multiplets. The conditions

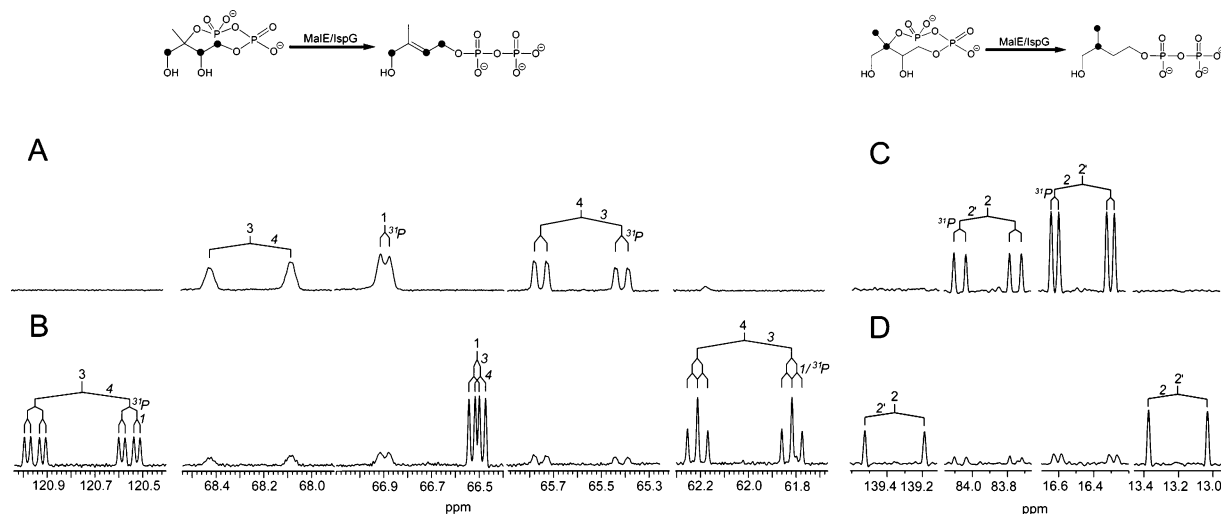


FIGURE 3. NMR signals detected in assay mixtures containing [1,3,4- $^{13}\text{C}_3$]-**5** (A and B) or [2,2'- $^{13}\text{C}_2$]-**5** (C and D).

TABLE 2. Catalytic Properties of IspG Mutant Proteins

mutation	iron content (mol mol $^{-1}$)	sulfide content (mol mol $^{-1}$)	specific activity	
			nmol min $^{-1}$ mg $^{-1}$	%
none	2.4	4.4	74	100
C270S	0.13	0.6	<0.001	<0.0014
C273S	0.14	0.7	<0.001	<0.0014
C306S	0.07	1	<0.001	<0.0014

of the experiments shown in Figure 3 were optimized for high product formation. Under initial rate conditions, an apparent catalytic rate of 100 nmol mg $^{-1}$ min $^{-1}$ was determined, in good agreement with the activities detected in the other assays. In a similar experiment monitored by ^{13}C NMR spectroscopy with photoreduced deazaflavin as the electron transponder, a catalytic rate of 64 nmol mg $^{-1}$ min $^{-1}$ was observed.

The assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 1 mM Mn $^{2+}$, 4 mM NADPH, 1 mM [1,3,4- $^{13}\text{C}_3$]-**5** or [2,2'- $^{13}\text{C}_2$]-**5**, 40 μM flavodoxin, 12 μM flavodoxin reductase, and 0.20 mg of Mal/IspG fusion protein (with a molar concentration of 4 μM) in a volume of 600 μL . The mixtures were incubated at 37 $^{\circ}\text{C}$ for 60 min and analyzed by NMR spectroscopy (cf. Experimental Section). A and C are the NMR signals after mixing of the assay components and B and D are the signals after incubation. The reaction schemes display the labeled substrate and product of the respective assay. The filled circles indicate ^{13}C -labeled atoms.

The recombinant IspG protein contained 2.4 iron ions per subunit, as shown by the method of Brumby and Massey.³⁴ The protein was also shown to comprise 4.4 labile sulfide ions per subunit. It should be noted that these values represent an average over all iron–sulfur clusters present in the analyzed protein and that it is not possible to assess the active form of the iron–sulfur cluster on the basis of these data. However, the data are useful in light of the respective values for the mutant proteins (Table 2).

A sequence alignment of 245 putative IspG proteins from eubacteria, plants, and the protozoan *Plasmodium falciparum* revealed 34 absolutely conserved residues (indicated by triangles in Figure 4).

The *E. coli* and *M. tuberculosis* (*P. falciparum*) proteins share 39% (36%) sequence identity and 54% (52%) sequence similarity. Notably, three cysteine residues were found to be absolutely conserved. More specifically, they formed a CPXCXRX_{27–33}GC motif and may well be involved in the binding of the iron–sulfur cluster. Site-directed mutagenesis was used to replace each of the cysteine residues by serine. The mutant genes were expressed together with *isc* genes, as described above for the wild-type enzyme. The recombinant mutant proteins were purified by affinity chromatography. In each case, the catalytic activity was <0.0014% (<1 pmol mg $^{-1}$ min $^{-1}$) as compared to the wild-type protein. The iron and sulfide contents were less than 6 and 23 %, respectively, as compared to the wild-type protein (Table 2).

By comparison with typical eubacterial IspG proteins, the IspG proteins of plants, *Plasmodium falciparum*, and a few bacterial species (predominantly from the Bacteroidetes/Chlorobi group) feature large inserts (ranging from amino acid position 290 to 612 of the *P. falciparum* sequence) with a length of approximately 170–320 amino acids. Sequence comparison using the insert sequence of *P. falciparum* as a template showed that the sequence of this insert is conserved among these species but retrieved no other sequences of significant similarity, except to certain IspG amino acid sequences under study. A dendrogram constructed from these insert sequences shows the expected phylogenetic clustering (Supporting Information).

A phylogenetic tree (Supporting Information) was developed using only the sequence parts which appear to be required for a minimum size catalytic domain without additional loops present in certain organisms. Without this restriction, the similarity analysis would have been dominated by the sequences which are only present in certain organisms. Specifically, sequences from 102 prototype representatives from the kingdoms of eubacteria and plants and the sequence of *P. falciparum* were used in the construction of the dendrogram. This analysis shows that a majority of species group according

(33) Kuzmic, P. *Anal. Biochem.* **1996**, *237*, 260–273.

(34) Brumby, P. E.; Massey, V. *Methods Enzymol.* **1967**, *10*, 463–474.

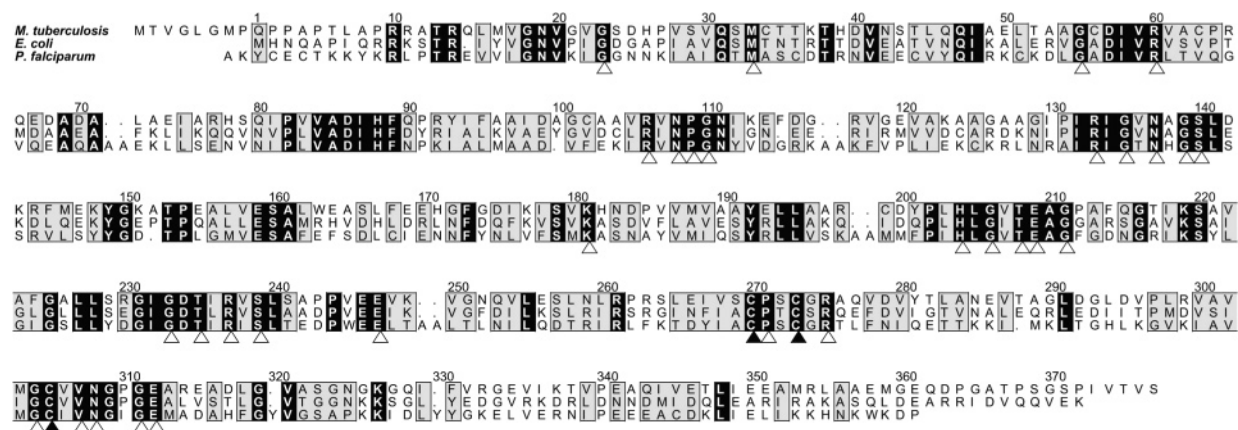


FIGURE 4. Alignment of the IspG proteins from *E. coli*, *M. tuberculosis*, and *P. falciparum*. Identical residues are shown in inverse contrast and similar residues are colored in gray. Symbols: <, N-extension; <>, extra-loop in the sequence of *P. falciparum* was omitted; ▲, conserved cysteine residue; △, residue absolutely conserved in all IspG amino acid sequences.

to their phylogenetic classification. Thus, Bacteroidetes/Chlorobi, Cyanobacteria, β - and γ -Proteobacteria, and Actinobacteria form clusters which are separated from other lineages (bootstrap values of 100%). Some α -Proteobacteria group together with β -Proteobacteria, whereas others group together with γ -Proteobacteria. Interestingly, *Chlamydia* sp. and *P. falciparum* group together with the plant species (bootstrap value of 80%). The remaining orders did not reveal any statistically supported relationship.

Discussion

In a previous study, we showed that the expression of the IspH protein in catalytically active form can be improved considerably by coexpression of the *isc* operon which specifies proteins involved in the assembly of iron–sulfur clusters.³⁰ The data reported in the present paper show that the expression of the IspG protein in catalytically active form is also considerably improved by hyperexpression of the *isc* operon (cf. Table 1). The essential role of an iron–sulfur cofactor in the IspG protein is additionally confirmed by the apparent lack of activity of mutant proteins, in which one of the three absolutely conserved cysteine residues was replaced by a serine residue. These data are in good agreement with similar studies on other iron–sulfur proteins such as IspH.³⁰ In accordance to our findings, Mössbauer studies indicated that a $[4\text{Fe}-4\text{S}]^{2+}$ cluster is bound to the IspG protein by three cysteine sulfurs.²⁵

Recombinant IspG proteins purified by affinity chromatography under anaerobic conditions catalyzed the conversion of **5** into **6** at apparent rates of at least 45 nmol mg⁻¹ min⁻¹ based on five different assay methods (using photoreduced deazaflavin or a mixture of flavodoxin, flavodoxin reductase, and NADPH as the reducing agent in conjunction with detection of product formation by online liquid scintillation counting or ¹³C NMR spectroscopy and the photometrical detection of NADPH consumption, respectively; cf. Table 3).

Notably, these data describe the catalytic activity of an as-isolated protein that had not been subject to any activating procedures. The K_M values and the specific activities calculated from radiometric as well as from photometric assays were also closely similar.

TABLE 3. K_M and Specific Activity Values of Recombinant IspG Proteins Determined by Various Assay Methods^a

method	K_M (μM)	specific activity (nmol min ⁻¹ mg ⁻¹)
radioassay		
FldA, Fpr, and NADPH	560	74
deazaflavin	n.d.	45
photometric assay	700	99
NMR assay		
FldA, Fpr, and NADPH	n.d.	100
deazaflavin	n.d.	64

^a n.d. = not determined.

Each of the various assay methods that have been shown to agree within narrow limits provides specific advantages. (i) The NMR method has by far the highest degree of selectivity. The multiple ¹³C labeling is conducive to spreading of the concentration data in the frequency domain. Depending on the type of labeling, the concentration of the substrate and product is represented by 16–22 NMR lines which are all individually proportional to the respective substrate or product concentration. Moreover, the ¹³C labeling enhances the sensitivity as well as the selectivity because the other assay components are hardly detectable due to their low ¹³C abundance of only 1.1%. However, it must also be noted that the NMR assays require relatively large sample volumes as well as relatively large amounts of ¹³C-labeled substrate. Moreover, they require extended measurement time on a medium-field NMR spectrometer. (ii) The radiochemical assays have the highest sensitivity and can tolerate small sample volumes. However, the radiochromatographic detection method requires the synthesis of radioactive substrates with high specific activity, and the real time liquid scintillation monitoring requires specialized equipment. (iii) The photometric assay method can be adapted to the handling of large numbers of samples to screen compound libraries for inhibitor activity.

The recombinant IspG protein of *E. coli* and *A. thaliana* isolated under aerobic conditions by Rohmer and co-workers had no detectable activity;^{23,25} treatment of the inactive proteins with a mixture of ferric chloride and sodium sulfide afforded detectable catalytic activity with photoreduced deazaflavin^{23,25} or, in the case of the *E. coli*

enzyme,²³ with a mixture of flavodoxin, flavodoxin reductase, and NADPH as the reducing agent.

Compared with that of the artificially reconstituted *E. coli* protein from the Rohmer group,²³ the recombinant IspG protein from *E. coli* prepared and studied as isolated by the present work was shown to exhibit significantly increased catalytic activity; e.g., when assayed with a mixture of flavodoxin, flavodoxin reductase, and NADPH, the catalytic rate of our protein is about 2 orders of magnitude higher than that of the artificially reconstituted protein from the Rohmer group. The artificially reconstituted *A. thaliana* protein is only active when assayed with deazaflavin.²⁵

Jomaa and co-workers²² studied the IspG protein from the hyperthermophilic eubacterium *Thermus thermophilus*. Their enzyme assays, performed with sodium dithionite as the reducing agent, afforded a comparatively high specific activity of 600 nmol mg⁻¹ min⁻¹ at a temperature of 55 °C, which is well below the optimum growth temperature of the microorganism (extrapolation would predict an even higher activity at the optimum growth temperature). In striking contrast, no catalytic activity was observed in our experiments with the *E. coli* enzyme using dithionite as a reducing agent under a variety of experimental conditions. In light of the detection limit, it can be concluded that the catalytic activity of the *E. coli* enzyme with dithionite as a reducing agent is below 1 pmol mg⁻¹ min⁻¹. Hence, it must be assumed that the enzymes from the mesophilic *E. coli* and the hyperthermophilic *T. thermophilus* differ quite substantially with respect to cofactor utilization. Notably, Jomaa and co-workers exclusively used dithionite as the reducing agent and did not report any experiments with more physiological electron donors such as flavodoxin.

Experimental Section

Engineering an *E. coli* Strain for Expression of *ispG* and *isc* Genes. The plasmid pACYCisc expressing the *isc* operon of *E. coli*³⁰ was transformed into the *E. coli* strain XL1-Blue cells³⁵ harboring the plasmid pMALispG,²⁴ which directs the expression of a recombinant *E. coli ispG* gene (fused to the 3' end of the *malE* gene) under the control of a *tac* promoter and a *lac* operator. The strain XL1-pMALispG-pACYCisc was grown in Terrific Broth (TB) medium containing chloramphenicol (25 mg L⁻¹) and ampicillin (180 mg L⁻¹) to enforce the maintenance of both plasmids.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by polymerase chain reaction (PCR) using the overlap extension technique.³⁶ PCR was performed with Dynazyme II DNA polymerase (Supporting Information). The general scheme of mutagenic PCR involved three rounds of amplification cycles using two mismatch and two flanking primers (gcpEBamHifw and gcpESalIrev). During the first round, 20 amplification cycles were carried out using one of the flanking primers and the corresponding mismatch primer with plasmid pMALispG as a template. During the second PCR round, 20 amplification cycles were carried out using the second flanking primer and the corresponding mismatch primer with plasmid pMALispG as a template. Amplificates were purified by agarose gel electrophoresis and isolated using the EZNA gel extraction kit. During the third round, the

products of round one and two were used as templates and 20 amplification cycles were carried out using the two flanking primers. The resulting amplificate was subjected to agarose gel electrophoresis, digested with BamHI and SalI, purified using the EZNA cycle pure purification kit, and ligated into the plasmid pMAL-C2, which had been digested with the same restriction enzymes. The ligation mixture was transformed into *E. coli* XL1-Blue cells. All gene constructs were verified by DNA sequencing.

Culturing Recombinant Bacteria. Recombinant *E. coli* strains were grown in TB medium containing ampicillin (180 mg L⁻¹) and chloramphenicol (25 mg L⁻¹) as appropriate. Cultures were incubated at 37 °C with shaking. At an optical density of 0.7 (600 nm), isopropylthiogalactoside was added to a final concentration of 2 mM and the cultures were incubated for 5 h. The cells were harvested by centrifugation, washed with 0.9% (w/v) sodium chloride, and stored at -20 °C under anaerobic conditions.

Preparation of the Recombinant MalE/IspG Fusion Protein. All steps were carried out under anaerobic conditions in a glovebox. A frozen cell mass (10 g) of *E. coli* XL1-pMALispG-pACYCisc was thawed in 40 mL of 200 mM Tris hydrochloride, pH 8.0, containing 0.5 M sodium chloride (buffer A). The suspension was passed through a french press and was then centrifuged. The supernatant was applied to a column of amylose resin FF (column volume = 60 mL) which had been equilibrated with buffer A at a flow rate of 1 mL min⁻¹. The column was washed with 200 mL of buffer A and was then developed with a gradient of 0–10 mM maltose in 180 mL of buffer A. The retention volume of the MalE/IspG fusion protein was 22 mL. Fractions were combined, dialyzed overnight against 100 mM Tris hydrochloride, kept at pH 8.0, and stored at -80 °C.

Radiochemical Assay of 1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate Synthase Activity. All steps were carried out under anaerobic conditions in a glovebox.

Method A. Assay mixtures containing 100 mM Tris hydrochloride, pH 8.0, 1 mM Mn²⁺, 4 mM NADPH, 1 mM [2-¹⁴C]-5 (0.83 μCi μmol⁻¹), 40 μM flavodoxin, 12 μM flavodoxin reductase, and 4 μM MalE/IspG fusion protein (0.07 mg) in a total volume of 200 μL were incubated at 37 °C. The reactions were terminated by the addition of 5 μL of 3 M trichloroacetic acid followed by immediate neutralization with 5 μL of 3 M sodium hydroxide. The mixtures were centrifuged, and the supernatants were subjected to ultrafiltration (100 kDa membrane). Aliquots (50 μL) were analyzed by reversed-phase ion-pair HPLC using a Luna C5 column (5 μm, 4 × 250 mm). The column was developed with 10 mM tetra-*n*-butylammonium phosphate, pH 6.0, containing 3% (v/v) methanol (10 mL) and then with a series of linear gradients containing 3–21% (v/v) methanol (2 mL), 21–35% (v/v) methanol (13 mL), 35–49% (v/v) methanol (10 mL), and 49–56% (v/v) methanol (5 mL) in the same buffer solution (flow rate of 1 mL min⁻¹). The effluent was monitored by real time liquid scintillation analysis. The retention volumes of **5** and **6** were 18 and 23 mL, respectively.

Method B. Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 1 mM Mn²⁺, 10 mM dithiothreitol, 1 mM [2-¹⁴C]-5 (0.83 μCi μmol⁻¹), and recombinant 4 μM MalE/IspG fusion protein (0.07 mg) in a volume of 200 μL. A solution (20 μL) containing 1.2 mM deazaflavin in dimethyl sulfoxide was added, and the mixture was placed on ice and was irradiated with a 100 W mercury vapor lamp placed at a distance of 15 cm. The samples were processed and analyzed by reversed-phase ion-pair HPLC as described above.

Photometric Assay of 1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate Synthase Activity. All steps were carried out under anaerobic conditions in a glovebox. Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 1 mM Mn²⁺, 100 μM [1,3,4-¹³C₃]-5, 1 mM NADPH, 40 μM flavodoxin, 12 μM flavodoxin reductase, and 0.13 μM MalE/IspG fusion protein (0.0053 mg) in a volume of 500 μL. The mixtures were incubated at 37 °C. The reaction was monitored photo-

(35) Bullock, W. O.; Fernandez, J. M.; Short, J. M. *BioTechniques* 1987, 5, 376–379.

(36) Horton, R. M.; Pease, L. R. *Directed Mutagenesis: A practical approach*; McPherson, M. J., Ed.; Oxford University Press: New York, 1991; pp 217–246.

metrically at 340 nm in a 0.2 cm cuvette. The reference assay contained 1 mM NADPH in 100 mM Tris hydrochloride with a pH of 8.0.

¹³C NMR Assay of 1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate Synthase Activity. All steps were carried out under anaerobic conditions in a glovebox.

Method A. Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 1 mM Mn²⁺, 4 mM NADPH, 1 mM [2,2'-¹³C₂]- or [1,3,4-¹³C₃]-**5**, 40 μM flavodoxin, 12 μM flavodoxin reductase, and 0.20 mg of Mal/IspG fusion protein (molar concentration of 4 μM) in a volume of 600 μL. The mixture was incubated at 37 °C. D₂O was added to a final concentration of 5% (v/v), and sodium [1,2-¹³C₂]acetate and EDTA were added to final concentrations of 2 mM and 5 mM, respectively. The solution was analyzed by ¹³C NMR spectroscopy.

Method B. Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 1 mM Mn²⁺, 10 mM dithiothreitol, 1 mM [2,2'-¹³C₃]- or [1,3,4-¹³C₃]-**5**, and 0.20 mg of MalE/IspG fusion protein in a volume of 600 μL. A solution (60 μl) containing 1.2 mM deazaflavin in dimethyl sulfoxide was added, and the mixture was placed on ice and irradiated with a 100 W mercury vapor lamp placed at a distance of 15 cm. D₂O was added to a final concentration of 5% (v/v), and sodium [1,2-¹³C₂]acetate and EDTA were added to final concentrations of 2 mM and 5 mM, respectively. The solution was analyzed by ¹³C NMR spectroscopy.

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Supporting Information Available: Materials, bacterial strains and plasmids, and oligonucleotides used in this study, purification of the recombinant MalE/IspG fusion protein from *Escherichia coli*, determination of iron, determination of sulfur, modulation of IspG enzymatic activity by divalent metal cations, UV-vis spectra of the recombinant IspG protein, activation of the IspG protein by (A) flavodoxin reductase and (B) flavodoxin, NMR spectroscopy, NMR data of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate and 1-hydroxy-2-(*E*)-methyl-2-butenyl 4-diphosphate, pH dependence of IspG protein activity, Lineweaver-Burk plot of catalytic rates of IspG protein vs substrate concentration, bioinformatics and consensus cladogram of IspG proteins from various microorganisms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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