

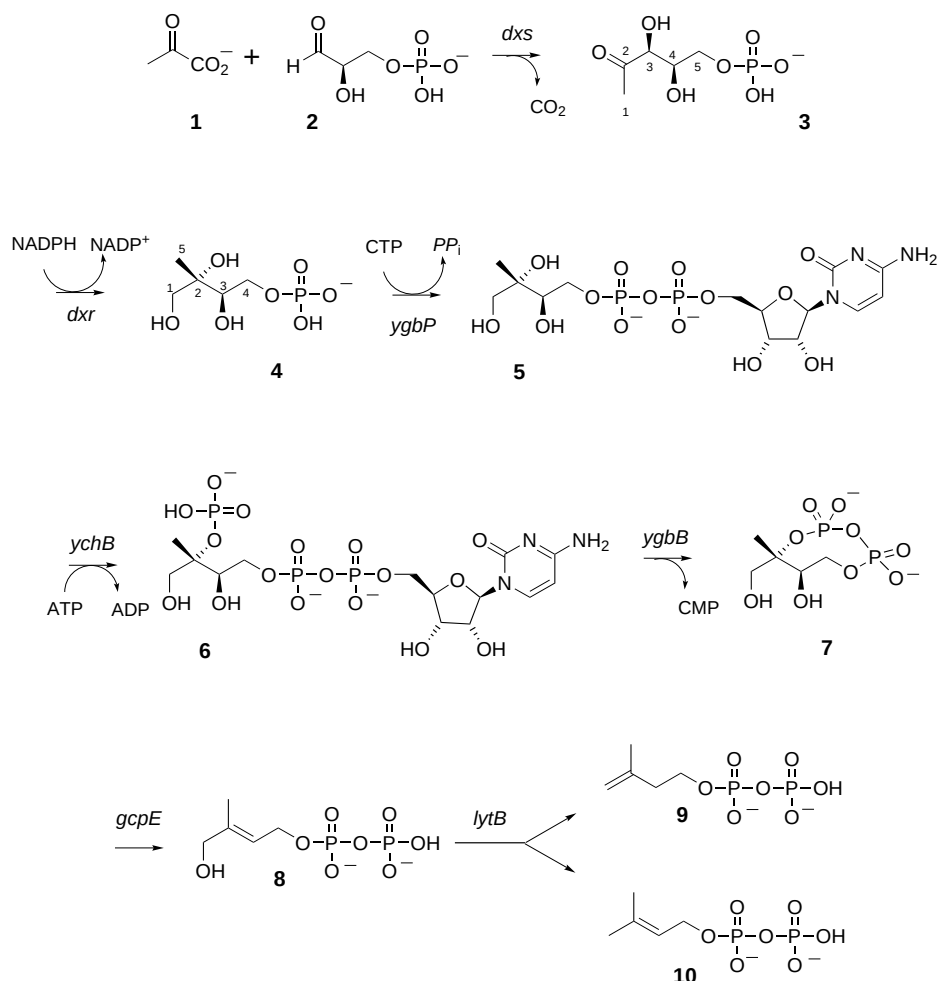


Isoprenoid Biosynthesis through the Methylerythritol Phosphate Pathway: The (*E*)-4-Hydroxy-3-methylbut-2-enyl Diphosphate Synthase (GcpE) is a [4Fe-4S] Protein**

Myriam Seemann, Bernadette Tse Sum Bui, Murielle Wolff, Denis Tritsch, Narciso Campos, Albert Boronat, Andrée Marquet, and Michel Rohmer*

Isopentenyl diphosphate (IPP, **9**), the fundamental unit in terpenoid biosynthesis, was widely accepted to be solely formed through the mevalonate pathway.^[1] An alternative metabolic route, the methylerythritol phosphate (MEP) pathway (Scheme 1), was later discovered for the formation of isoprenoids in many bacteria, green algae, and plant chloroplasts.^[2] Whereas the first steps leading from pyruvate (**1**) and glyceraldehyde 3-phosphate (**2**) to 2-methylerythritol (ME) 2,4-cyclodiphosphate (**7**) are well-known,^[2] the last steps leading to IPP (**9**) and dimethylallyl diphosphate (DMAPP, **10**) and involving the *gcpE*^[3] and *lytB*^[4] genes are still a matter of investigation. Cyclodiphosphate **7** is the substrate of the GcpE protein.^[5] A crude cell-free system of an *Escherichia coli* strain that overexpresses the *gcpE* gene converts **7** into (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP, **8**).^[6] *In vivo* studies were in accord with these results. Cyclodiphosphate **7** was accumulated from ¹³C-labeled 1-deoxy-D-xylulose (DX) by an *E. coli* strain that overexpresses xylulose kinase (which is capable of phosphorylating DX to yield deoxyxylulose phosphate **3**) and

the four genes of the MEP pathway (*dxr*, *ygbP*, *ychB*, and *ygbB*). Additional overexpression of the *gcpE* gene (or *ispG*) was followed by the formation of HMBPP (**8**).^[7] The reaction catalyzed by the GcpE protein formally involves elimination and reduction steps. The reducing cofactor was, however, not identified, as no assay with the purified enzyme was available. In a bioinformatic search on the *gcpE* gene,^[6b] we found strong homologies between the GcpE protein and enzymes



Scheme 1. Methylerythritol 4-phosphate pathway for isoprenoid biosynthesis in *E. coli*.

[*] Prof. Dr. M. Rohmer, Dr. M. Seemann, M. Wolff, Dr. D. Tritsch
Université Louis Pasteur, UMR 7123 CNRS, Institut Le Bel
4 rue Blaise Pascal, 67070 Strasbourg Cedex (France) Fax:
(+33)3-9024-1345
E-mail: mirohmer@chimie.u-strasbg.fr

Dr. B. T. S. Bui, Prof. Dr. A. Marquet
Laboratoire de Chimie Organique Biologique
Université Paris VI, UMR 7613 CNRS
4 place Jussieu, 75252 Paris Cedex 05 (France)

Dr. N. Campos, Prof. Dr. A. Boronat
Departament de Bioquímica i Biologia Molecular
Facultat de Química, Universitat de Barcelona
Martí i Franquès 1, 09028 Barcelona (Spain)

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that possess a [4Fe-4S] cluster. Herein we describe a purified His₆-tagged GcpE protein (His-GcpE), which is active after reconstitution of its Fe/S cluster.

A crude cell-free system obtained from *E. coli* strain XL1-blue [pBAD-HisGcpE], which overexpresses His-GcpE, was capable of catalyzing the formation of HMBPP (**8**) from [¹⁴C]**7** under the conditions described for the nontagged protein.^[6] The His-GcpE (30 mg from a 500-mL transformed cell culture) was purified on a Ni²⁺ nitrilotriacetic acid agarose column. The enzyme was found to be 95% pure by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and presented an apparent molecular mass of 43 kDa. The purified protein was inactive, even in the presence of the reducing systems detailed below. Such a lack of catalytic activity was probably a result of the predominant

production of the apoenzyme without the Fe/S cluster. The Fe/S cluster was therefore restored using FeCl_3 and Na_2S in the presence of dithiothreitol as reductant.^[8] As such prosthetic groups are often oxygen-sensitive, all operations were performed under an inert argon atmosphere. The UV/Vis spectrum of the khaki solution of the reconstituted enzyme displayed an absorption band at 410 nm, which is characteristic for proteins that possess a $[\text{4Fe-4S}]^{2+}$ cluster (Figure 1).^[9] Exposure of the reconstituted enzyme solution to air led to bleaching, corresponding to the fast degradation of the $[\text{4Fe-4S}]^{2+}$ cluster to yield again the spectrum of the apoenzyme. The determination of the iron content revealed 4.4 iron ions per reconstituted GcpE molecule, affording additional evidence for the presence of a $[\text{4Fe-4S}]$ cluster.

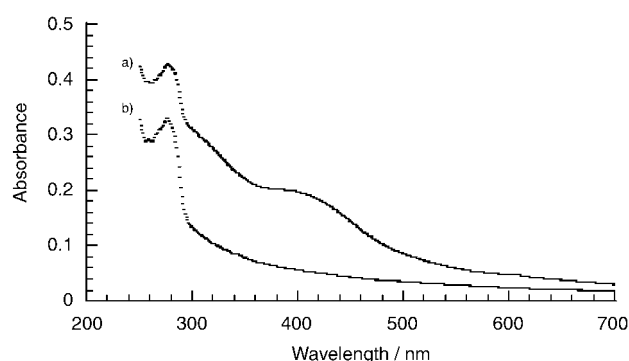
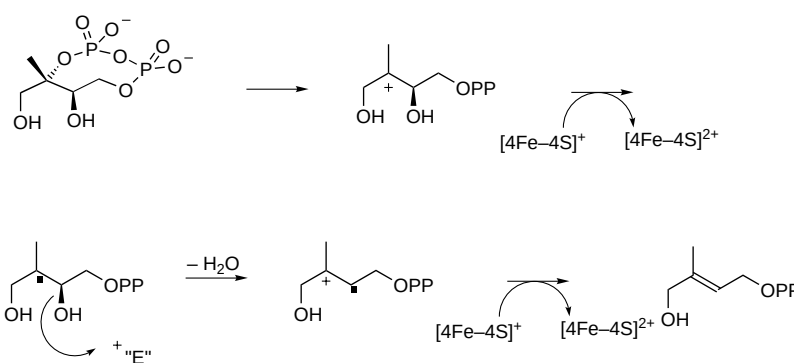


Figure 1. UV/Vis absorption spectra of His-GcpE: a) reconstituted His-GcpE, b) apoenzyme (protein concentration: 1.3 mg mL^{-1} , 50 mM Tris HCl, pH 8).

According to the assay conditions described for other enzymes with Fe/S clusters,^[8,10,11] the test with the purified His-GcpE was performed in the presence of flavodoxin, flavodoxin reductase, and NADPH as regeneration systems. In the presence of this reducing system, the purified enzyme quantitatively converted **7** into HMBPP (**8**) after 4 h. The formerly utilized crude cell-free system allowed only a 4–50 % conversion under the same conditions, depending on the method used to break the cells.^[6] The former reducing system was efficiently replaced by the 5-deazaflavin (DAF) semiquinone radical obtained by photoreduction of DAF in the presence of Tris-HCl and used in excess with respect to the substrate.^[8] Under these conditions, **7** was quantitatively converted into **8** after 15 min.

The presence of a $[\text{4Fe-4S}]$ cluster as a prosthetic group in GcpE suggested a radical reaction involving a reduction through two one-electron transfers (Scheme 2). This was efficiently performed in our assays in two different ways: either by an endogenous reducing system, already present in *E. coli* (starting from the reduced FMNH_2 of flavodoxin and leading to the oxidized FMN via the semiquinone radical $\text{FMNH}^\cdot$, and recycling of the oxidized flavodoxin cofactor by the flavodoxin reductase and NADPH), or by the partially reduced DAF semiquinone radical. Such a successive double



Scheme 2. Hypothetical biogenetic pathway for the GcpE-catalyzed reaction in the presence of flavodoxin, flavodoxin reductase, and NADPH (intermediates are not free species but enzyme-bound stabilized entities).

one-electron transfer has been proposed in a $[\text{2Fe-2S}]^+$ cluster in the biosynthesis of the 3,6-dideoxyhexose ascardylose.^[12] Cyclodiphosphate **7** is accumulated in some bacteria in the presence of benzylviologen, an inhibitor of one-electron transfer reactions.^[13] This feature may be explained by an interference of the inhibitor with the electron-transfer process catalyzed by GcpE. The two previously proposed mechanisms for the GcpE-catalyzed reaction now appear rather unlikely. The fact that GcpE is functional in the presence of the semiquinone form of DAF alone (in the absence of any other reducing cofactor such as NADPH) is in contradiction with a mechanism resembling that of the reaction catalyzed by the vitamin K epoxyquinone reductase.^[7] In contrast to other enzymes that have a $[\text{4Fe-4S}]^{2+}$ prosthetic group (e.g. biotin synthase, ribonucleotide reductase, pyruvate formate lyase, and lysine 2,3-aminomutase),^[11] an initiator of radical reactions such as *S*-adenosylmethionine is not required for the activity of GcpE, a feature that is not in favor of a mechanism resembling that of the reaction catalyzed by the ribonucleotide reductase.^[6b,7] Opening of the cyclodiphosphate ring and elimination of the C3 hydroxy group remain to be solved. The Lewis acid character of a Fe/S cluster, which may coordinate to a hydroxy group and facilitate its elimination, has been reported for a dehydratase,^[14] but it is not known whether the Fe/S cluster and/or other acidic groups are involved. Finally, the proposed mechanism (Scheme 2) is in accord with the fate of the hydrogen atoms in the MEP pathway. From incorporations performed with deuterium-labeled isotopomers of DX or ME into the prenyl chain of ubiquinone from *E. coli*, no deuterium loss from C1, C4, and C5 of MEP (**4**) was observed in its conversion into IPP (**9**) and DMAPP (**10**).^[15] It is also in accord with the retention of the C3 hydrogen atom of MEP (**4**) or cyclodiphosphate **7** in the isoprene units derived from DMAPP in *E. coli*^[16] or in those derived from DMAPP and from IPP in the tobacco BY-2 cell culture.^[17] The GcpE-catalyzed reaction does not lead to a premature loss of this hydrogen atom, which is still retained in HMBPP (**8**), the intermediate located at the branching towards IPP or DMAPP.^[18] It is the successive action of the *lytB*-encoded enzyme, the IPP isomerase, and the prenyl transferase that is responsible for its retention or elimination.^[17]

One of the last bottlenecks in the elucidation of the MEP pathway has been solved. A $[\text{4Fe-4S}]$ cluster is the prosthetic

group of the GcpE protein in *E. coli*. This enzyme efficiently converts cyclodiphosphate **7** into HMBPP (**8**) in the presence of flavodoxin, flavodoxin reductase, and NADPH. Whether the latter system is the effective regeneration system in *E. coli* still has to be determined. The oxygen sensitivity of the GcpE Fe/S cluster is one of the major reasons for the failure of crude cell-free systems to convert intermediates of this pathway into IPP (**9**), DMAPP (**10**), or further metabolites, and for the late discovery of the MEP pathway.

Experimental Section

Supporting information for this article (expression, purification, and characterization of the GcpE protein) is available on the WWW under <http://www.angewandte.org>.

Reconstitution of the FeS centers in the apoenzyme: The apo-GcpE solution (250 μ L, 197 μ M), a 20 mM Na₂S solution in argon-saturated buffer (50 mM Tris-HCl, pH 8), a 20 mM FeCl₃ and a 100 mM DTT solution in water were deoxygenated separately in Eppendorf tubes for 1 h under a stream of moist argon. All tubes were transferred to a glovebox (Jacomex BS531 NMT) equipped with an oxymeter (ARELCO ARC) and filled with argon containing less than 2 ppm O₂, where they were left to stand overnight at 15 °C before beginning the reconstitution procedure. DTT (to a final concentration of 5 mM) as well as FeCl₃ and Na₂S (in a fivefold molar excess with respect to the apoenzyme) were added successively to the enzyme solution. After 5 h, the reaction mixture was desalted on a PD 10 column (Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 8). For recording the UV/Vis absorption spectrum, a fraction of the reconstituted protein was directly transferred into a cuvette, which was closed with a septum before being removed from the glove box.

GcpE assay with flavodoxin and flavodoxin reductase: Flavodoxin and flavodoxin reductase from *E. coli* were obtained as previously described.^[8] A mixture (70 μ L final volume) containing [¹⁴C]7 (39 μ M, 0.04 μ Ci), DTT (5 mM), NADPH (1 mM), flavodoxin (1 μ M), flavodoxin reductase (0.5 μ M) in Tris-HCl (50 mM, pH 8) was degassed for 45 min at room temperature under a stream of moist argon before adding the previously reconstructed GcpE with a gas-tight syringe to a final concentration of 2 μ M. The incubation was performed in anaerobic conditions at 37 °C for 4 h.

GcpE assay with 5-deazaflavin: A mixture (70 μ L final volume) containing [¹⁴C]7 (39 μ M, 0.04 μ Ci), DTT (5 mM) in Tris-HCl (50 mM, pH 8) was degassed for 45 min at room temperature under a stream of wet argon before adding successively, through a gas-tight syringe, the GcpE solution (final concentration: 2 μ M) and a degassed 5-deazaflavin solution (final concentration: 50 μ M) in the above-mentioned buffer. After generation of the reducing semiquinone radical from 5-deazaflavin and Tris-HCl by irradiation with a white fluorescent tube (30 °C, 1 h), the assay was incubated at 37 °C for 15 min or up to 3 h.

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