

Functional characterization of GcpE, an essential enzyme of the non-mevalonate pathway of isoprenoid biosynthesis

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Abstract The *gcpE* gene product controls one of the terminal steps of isoprenoid biosynthesis via the mevalonate independent 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. This pathway is utilized by a variety of eubacteria, the plastids of algae and higher plants, and the plastid-like organelle of malaria parasites. Recombinant GcpE protein from the hyperthermophilic bacterium *Thermus thermophilus* was produced in *Escherichia coli* and purified under dioxygen-free conditions. The protein was enzymatically active in converting 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) into (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) in the presence of dithionite as reductant. The maximal specific activity was 0.6 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ at pH 7.5 and 55°C. The k_{cat} value was 0.4 s^{-1} and the K_{m} value for HMBPP 0.42 mM.

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Key words: 2-C-Methyl-D-erythritol-2,4-cyclodiphosphate; GcpE; (*E*)-4-Hydroxy-3-methyl-but-2-enyl diphosphate; Isoprenoid biosynthesis; LytB; 2-C-Methyl-D-erythritol-4-phosphate pathway

1. Introduction

Isoprenoids are essentially involved in the metabolism of all organisms as in electron transfer, photosynthesis, membrane stability, and cell signaling. In animals, fungi, archaeobacteria, and certain eubacteria, biosynthesis of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), the precursors of all isoprenoids, proceeds exclusively via the mevalonate pathway. In contrast, in many eubacteria and the plastids of algae and higher plants, IPP and DMAPP are synthesized via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [1–4].

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Abbreviations: DMAPP, dimethylallyl diphosphate; DOXP, 1-deoxy-D-xylulose-5-phosphate; HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate; IPP, isopentenyl diphosphate; MEcPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; PBMC, peripheral blood mononuclear cells

While the first five enzymatic steps of the MEP pathway have been well established, the terminal steps are still not fully understood. The pathway is initiated by the formation of 1-deoxy-D-xylulose-5-phosphate (DOXP) through condensation of pyruvate and D-glyceraldehyde-3-phosphate by DOXP synthase (Dxs, EC 4.1.3.37) (Fig. 1). DOXP is then converted into MEP by DOXP reductoisomerase (Dxr, EC 1.1.1.267). The enzymes encoded by the genes *ygbP* (*ispD*, EC 2.7.7.60), *ychB* (*ispE*, EC 2.7.1.148), and *ygbB* (*ispF*, EC 4.6.1.12) mediate the formation of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) via three additional reaction steps [1–4]. Recent data demonstrated an additional role for the *gcpE* and *lytB* gene products in the terminal steps of the MEP pathway. *Escherichia coli* mutants deficient in *gcpE* and *lytB* were only viable when they were genetically engineered to utilize exogenously provided mevalonate for isoprenoid biosynthesis [5–8].

$\Delta gcpE$ and $\Delta lytB$ deletion mutants accumulate MEcPP and (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP), respectively, suggesting these metabolites to represent the putative substrate and reaction product of GcpE [9–11]. Also, in *E. coli* cells overexpressing all genes of the MEP pathway from *dxs* down to *gcpE* on an artificial operon, enrichment of HMBPP can be detected in the bacterial lysate [12]. Similarly, the production of IPP and HMBPP was observed when the operon additionally contained the *lytB* gene [13]. In another study, production of a phosphorylated derivative of (*E*)-2-methylbut-2-ene-1,4-diol (most likely representing HMBPP) from radioactively labeled MEcPP was observed in crude extracts of *E. coli* cells overexpressing the three genes *yfgA*, *yfgB*, and *gcpE*, which appear in a single gene cluster [14]. Yet, there have been no reports on the functional activity of purified GcpE or LytB in a defined in vitro system. Here, we describe the production of recombinant GcpE from the hyperthermophilic bacterium *Thermus thermophilus*. This protein was enzymatically active in converting MEcPP into HMBPP under dioxygen-free conditions, using dithionite as reductant.

2. Materials and methods

2.1. Plasmid construction

The *gcpE* gene of *T. thermophilus* (GenBank accession number bankit497118 AY167032) was identified by scanning the genome of *T. thermophilus* with the amino acid sequence of the *E. coli* GcpE

protein using the TBLASTN algorithm. The *T. thermophilus* genome is at >99.5% completion by a genome sequencing project currently pursued at Göttingen Genomics Laboratory. In order to overcome the G/C-rich codon bias of *T. thermophilus*, the amino acid sequence of the GcpE protein was back-translated into the codon usage of *E. coli* and produced as a fully synthetic gene by custom synthesis at ThermoHybaid (Ulm, Germany). The synthetic gene was cloned into the pQE-60 expression vector (Qiagen, Hilden, Germany) using the *NcoI* and *BglII* restriction sites. The carboxy-terminal His-tag was subsequently removed by digestion of the plasmid with *BglII* and *HindIII*, fill-in of the overhanging ends using the Klenow fragment of DNA polymerase I, and religation with T4 DNA ligase. The vector-derived sequences resulted in the addition of the sequence Gly-Ser-Ala at the carboxy-terminus.

2.2. Purification of *T. thermophilus* GcpE

Four flasks with 400 ml terrific broth (Difco, Augsburg, Germany) supplemented with 200 $\mu\text{g ml}^{-1}$ ampicillin and 50 μM FeCl_3 were inoculated each with 10 ml of an overnight culture of *E. coli* TOP10F' (Invitrogen) harboring the above construct, and incubated under vigorous agitation for 20 h at 37°C. The cells were harvested by centrifugation and frozen on dry ice. All subsequent steps were performed under exclusion of dioxygen in a Coy glove box (95% N_2 , 5% H_2). 80 ml buffer A (20 mM Tris-HCl, pH 8.0) containing 0.1 mg ml^{-1} DNase I were added to the frozen pellet, and the cells were disintegrated by sonication. After centrifugation of the cell lysate at 16000 rpm for 20 min, the supernatant was subjected to heat denaturation at 65°C for 30 min. Precipitated material was removed by centrifugation at 25000 rpm for 25 min, and the supernatant was filtered over a 0.2 μm filter. The solution was loaded onto a 1.6 \times 40 cm diethylaminoethyl (DEAE) Sepharose column (fast flow; Pharmacia, Freiburg, Germany) equilibrated with buffer A. The column was eluted using a linear gradient from 0 to 1 M NaCl in buffer A. The main fraction was rebuffed in buffer A using prepacked PD10 Sephadex G-25 gel filtration columns (Pharmacia). The protein was further purified on a 1 \times 10 cm source 15Q anion exchange column (Pharmacia) with a linear gradient of 0–1 M NaCl in buffer A. The peak fraction was concentrated to 1.3 ml using a Centricon ultrafiltration unit with 30 kDa cut-off (Millipore, Eschborn, Germany). Final purification was carried out by gel filtration using an XK 16/60 Superdex 200 column (Pharmacia) equilibrated with buffer A containing 100 mM NaCl. The protein solution was concentrated to 18 mg ml^{-1} and stored at -80°C until further use.

2.3. GcpE activity assay by high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS)

MEcPP was isolated from benzyl viologen-treated cultures of *Corynebacterium ammoniagenes* as described previously [15,16]. HMBPP used as reference for HPLC analysis was obtained by chemical synthesis (Reichenberg et al., submitted for publication). For identification of the reaction product, 20 mM Tris-HCl (pH 7.5), 12 mM MEcPP, 8 mM sodium dithionite, and 20 μM GcpE were incubated in a total volume of 130 μl for 1 h at 55°C. The mixture was analyzed by HPLC-ESI-MS using a Micromass ZQ 4000 (Waters, Milford, MA, USA). Chromatography was performed on a 250 \times 2 mm, 13 μm AS11-HC column (Dionex Corporation, Sunnyvale, CA, USA), and a 50 \times 2 mm AG11-HC precolumn, with a binary gradient applied by a Waters separation module 2790 pump (Waters). Solvent A was 0.05% ammonia in water (v/v), 9% methanol, pH 10.6; solvent B was 1 M ammonium formate, 9% methanol, pH 9.3. The injection volume was 18 μl , the flow rate was 250 $\mu\text{l min}^{-1}$ directly into the mass spectrometer (MS) via the electrospray ionization (ESI) interface. The gradient applied for separation was: 95% A, 5% B; linear gradient to 100% B in 40 min, followed by 20 min equilibration. Mass peaks were detected at a transfer capillary temperature of 250°C and a capillary voltage of 3.5 kV by single ion recording in negative mode $[\text{M}-\text{H}]^-$, for MEcPP (m/z 277), HMBPP (m/z 261), and sulfate (m/z 97).

2.4. Photometric GcpE activity assay

The initial velocity was determined at 55°C in anaerobic gas-tight quartz cuvettes in a total volume of 750 μl by monitoring the oxidation of methyl viologen at 732 nm ($\epsilon_{732} = 3150 \text{ M}^{-1} \text{ cm}^{-1}$). The assay mixture consisted of 20 mM Tris-HCl (pH 7.5), 2 mM methyl viologen, 5.4 μM GcpE, and varying concentrations of MEcPP. After

reduction of methyl viologen with sodium dithionite until an absorbance of 2.0 at 732 nm was reached, corresponding to 0.63 mM reduced methyl viologen, the reaction was initiated by the addition of the enzyme.

2.5. $\gamma\delta$ T cell activation assay

2×10^5 peripheral blood mononuclear cells (PBMC) were seeded in 200 μl RPMI-1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 25 $\mu\text{g ml}^{-1}$ gentamycin, 100 U ml^{-1} human interleukin-2 (all from Life Technologies, Karlsruhe, Germany), and 10% human AB serum (Bayerisches Rotes Kreuz, Augsburg, Germany) [10]. Aliquots from the GcpE activity assay were tested at serial dilutions ranging from 1:10⁶ to 1:10¹⁰; HMBPP was used as positive control. Cells were analyzed after 6 days on a four-color Epics XL flow cytometer supported with Expo32 software (Beckman Coulter, Krefeld, Germany), using CD3-ECD (UCHT1) and V γ 9-PC5 (Immu360) monoclonal antibodies (Beckman Coulter).

3. Results and discussion

In order to obtain high-quality protein for biochemical characterization, the *gcpE* gene of a variety of bacteria was expressed in *E. coli* using different vector systems. In most

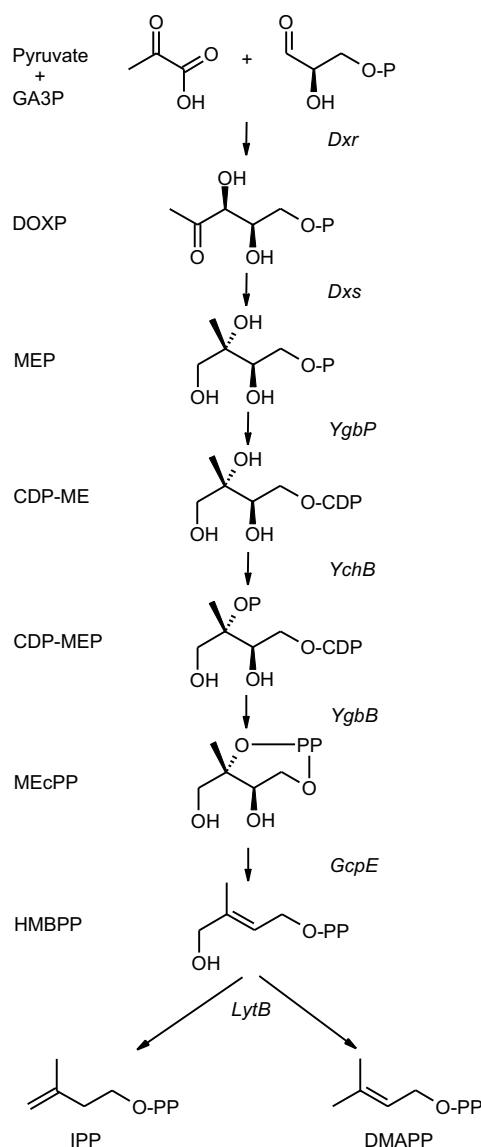


Fig. 1. Proposed reaction steps of the MEP pathway.

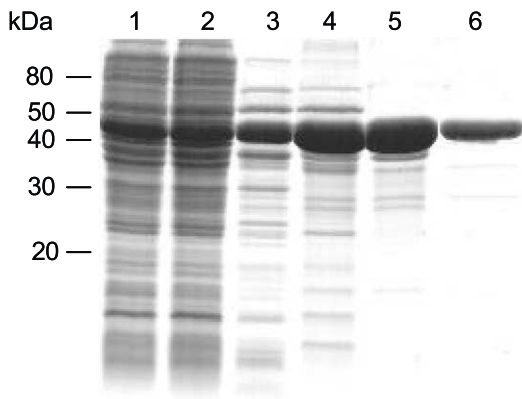


Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of different fractions obtained during the purification of recombinant GcpE. 1, Crude bacterial lysate; 2, soluble proteins; 3, heat stable proteins; 4, GcpE fraction after weak anion exchange chromatography on DEAE Sepharose; 5, GcpE fraction after strong anion exchange chromatography on 15 Q Sepharose; 6, GcpE fraction after Superdex 200 gel filtration.

cases the expression level or yield of soluble protein was insufficient. Also, instability of the recombinant GcpE protein during purification was observed in some instances (not shown). In contrast, GcpE from *T. thermophilus* was produced at high levels and turned out to be stable after the original coding sequence was adapted to the *E. coli* codon usage using a fully synthetic gene. The protein was purified under dioxygen-free conditions to approximately 90% homogeneity by heat denaturation and three chromatographic steps (Fig. 2). The protein as purified was brown in color and the ultraviolet (UV)-visible absorption spectrum revealed a pronounced shoulder at 413 nm. After reduction with titanium(III) citrate the absorption at 413 nm decreased, a property characteristic for proteins containing an Fe–S cluster (Fig. 3). Further preliminary data obtained by EPR and Raman spectroscopy (not shown) indicated the presence of an [4Fe–4S] cluster as suggested earlier based on the existence of three

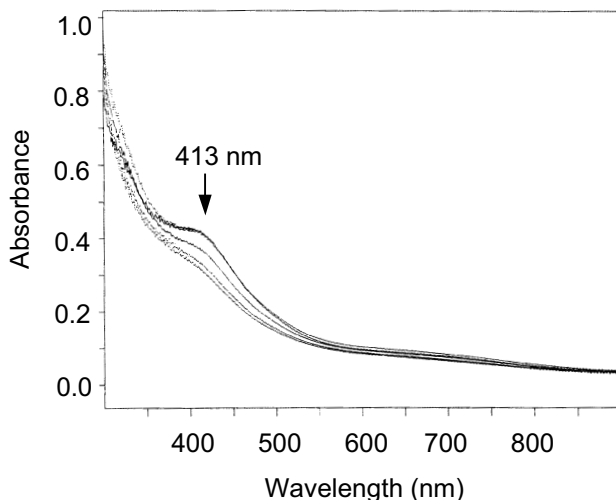


Fig. 3. UV-visible absorption spectra of the GcpE protein. The upper spectrum was obtained with the protein as purified, the lower spectra were recorded 5, 10, and 20 min after reduction with titanium(III) citrate, respectively.

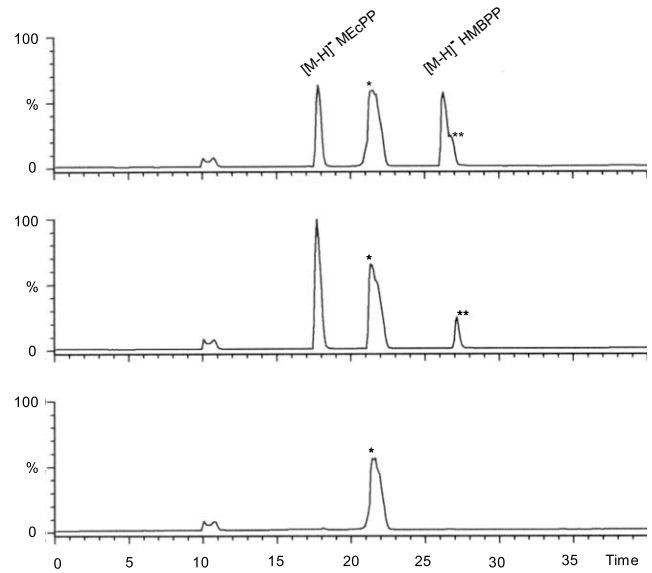


Fig. 4. HPLC-ESI-MS analysis of the GcpE reaction product. The reaction mixture containing 12 mM MEcPP, 8 mM dithionite and 20 μ M GcpE was incubated for 1 h at 55°C (upper panel). As control, GcpE (middle panel) or MEcPP (lower panel) were omitted. Mass chromatograms were recorded for MEcPP (m/z 277) and HMBPP (m/z 261). As an additional major component, sulfate (m/z 97) derived from dithionite was recorded (asterisk). An unknown compound of m/z 277 was detected as an impurity of the MEcPP sample (two asterisks).

conserved cysteine residues [17]. The detailed spectroscopic data will be reported in due course.

When the GcpE protein was incubated with MEcPP in the presence of dithionite as reductant, the formation of HMBPP was observed by HPLC-ESI-MS analysis (Fig. 4). In control experiments, the specific mass peak for HMBPP (m/z 261) was not observed when GcpE or MEcPP were omitted from the reaction mixture. The partial reduction of the MEcPP peak

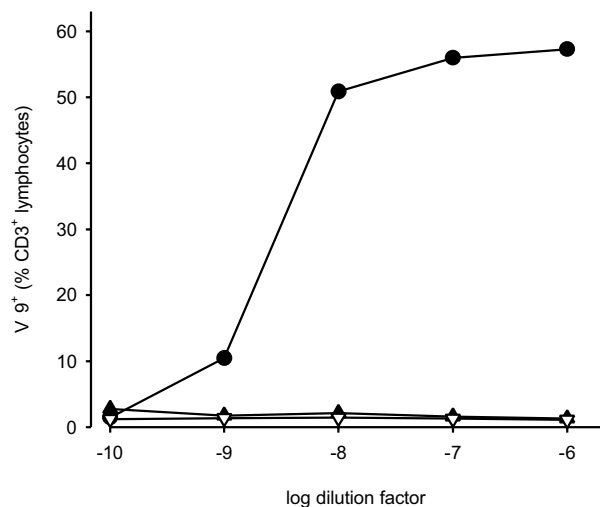


Fig. 5. Expansion of V γ 9/V δ 2 T cells as a result of GcpE-mediated conversion of MEcPP into HMBPP. PBMC were incubated over a period of 6 days with the reaction product of the GcpE activity assay at dilutions between 1:10¹⁰ and 1:10⁶, and analyzed by flow cytometry. Results were expressed as percentage of V γ 9⁺ cells among total CD3⁺ cells (circles). As control, GcpE (triangles) or MEcPP (inverted triangles) were omitted from the activity assay.

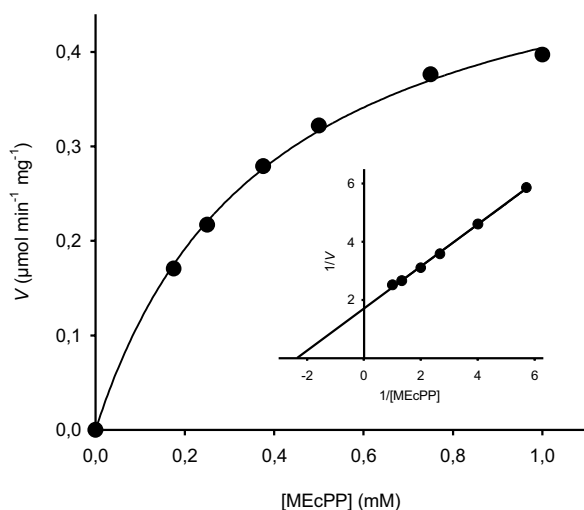


Fig. 6. Michaelis–Menten plot of the GcpE activity. The initial velocity was determined at 55°C with 5.4 μM GcpE and varying concentrations of MEcPP from 0 to 1 mM. The inset shows the deduced Lineweaver–Burk plot.

(*m/z* 277) compared with the control is in accordance with the excess of MEcPP used in this experiment. Also, HMBPP was detected by its biological activity based on specific stimulation of human $\gamma\delta$ T cells. Incubation of PBMC with the reaction product of the GcpE activity assay resulted in a considerable expansion of V γ 9/V δ 2 T cells among CD3⁺ T cells, while neither the substrate nor enzyme alone led to any significant response (Fig. 5). A similar result was obtained when analyzing upregulation of the activation markers CD25 or CD45RO on the surface of V γ 9/V δ 2 T cells (not shown).

In order to measure the initial velocity of the reaction, an assay based on the photometric measurement of the oxidation of reduced methyl viologen was established. Maximal activity was observed at 55°C and pH values between 7.5 and 8.0 (not shown). For the determination of the kinetic parameters, the GcpE activity was measured with different MEcPP concentrations ranging from 0 to 1 mM (Fig. 6). Based on a Michaelis–Menten and a Lineweaver–Burk plot, the K_m value for MEcPP was calculated to be 0.42 mM. The maximal specific activity was 0.6 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, corresponding to a k_{cat} value of 0.4 s^{-1} .

The presented data provide formal evidence that HMBPP is the direct reaction product of the enzymatic conversion of MEcPP by GcpE. The reaction is dependent on a [4Fe–4S] cluster as cofactor, which is sensitive to dioxygen, and can be reduced by dithionite as an artificial one-electron donor. This result is of considerable interest since it shows a novel function for an Fe–S cluster in the reductive elimination of a hydroxyl group. Different hypothetical mechanisms for the GcpE-mediated reaction were suggested earlier assuming the three conserved cysteine residues being part of the active site directly interacting with the substrate instead of constituting an Fe–S cluster [12]. As a possible alternative mechanism the

reaction may be initiated by ring opening of the cyclic diphosphate yielding a relatively stable tertiary carbocation, which is reduced by two successive one-electron transfer steps under the concerted elimination of the hydroxyl group (Fig. 7).

As suggested by recent data, the physiological relevance of HMBPP clearly exceeds its role as merely an intermediate in isoprenoid biosynthesis. Most strikingly, HMBPP has been identified as a highly potent immunomodulator leading to proliferation and expression of activation markers of human $\gamma\delta$ T cells [9,10]. Elevated levels of $\gamma\delta$ T cells are commonly observed in patients suffering from infections with pathogens known to possess the MEP pathway such as tuberculosis or malaria. It has also been shown that HMBPP acts as the precursor for the synthesis of the plant hormone zeatin by *Agrobacterium tumefaciens* [18]. Whether HMBPP is involved in additional regulatory and biosynthetic functions remains to be elucidated.

4. Note added in proof

In a paper published after submission of this manuscript Seemann et al. have reported the in vitro reconstitution of the Fe–S cluster of *E. coli* GcpE and its enzymatic activity using photoreduced 5-deazaflavin or a flavodoxin/flavodoxin reductase regeneration system [19].

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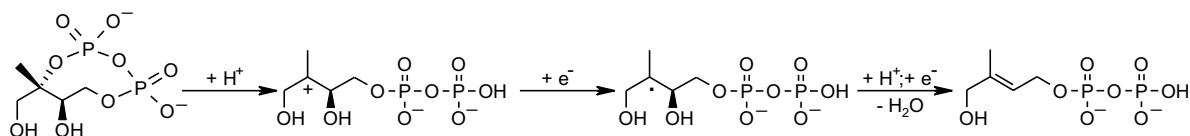


Fig. 7. Possible reaction mechanism for the conversion of MEcPP into HMBPP by GcpE.

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