LytB protein catalyzes the terminal step of the 2-*C*-methyl-D-erythritol-4-phosphate pathway of isoprenoid biosynthesis

Boran Altincicek[a,1,*], Evert C. Duin[b,1], Armin Reichenberg[a], Reiner Hedderich[b], Ann-Kristin Kollas[c], Martin Hintz[a], Stefanie Wagner[a], Jochen Wiesner[a], Ewald Beck[c], Hassan Jomaa[a]

[a] *Jomaa Pharmaka GmbH, Frankfurter Strasse 50, 35392 Giessen, Germany*
[b] *Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Strasse, 35043 Marburg, Germany*
[c] *Biochemisches Institut, Justus-Liebig-Universität Giessen, Friedrichstrasse 24, 35392 Giessen, Germany*

Received 28 October 2002; revised 15 November 2002; accepted 15 November 2002

First published online 27 November 2002

Edited by Felix Wieland

Abstract Recombinant LytB protein from the thermophilic eubacterium *Aquifex aeolicus* produced in *Escherichia coli* was purified to apparent homogeneity. The purified LytB protein catalyzed the reduction of (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) in a defined in vitro system. The reaction products were identified as isopentenyl diphosphate and dimethylallyl diphosphate. A spectrophotometric assay was established to determine the steady-state kinetic parameters of LytB protein. The maximal specific activity of 6.6 ± 0.3 μmol min^{-1} mg^{-1} protein was determined at pH 7.5 and 60°C. The k_{cat} value of the LytB protein was 3.7 ± 0.2 s^{-1} and the K_m value for HMBPP was 590 ± 60 μM.

Key words: Isoprenoid biosynthesis; 2-*C*-Methyl-D-erythritol-4-phosphate pathway; LytB

1. Introduction

In a variety of organisms isoprenoids are synthesized by the mevalonate-independent 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Although the first five enzymatic steps of this pathway are well characterized only indirect evidence has been provided so far for two additional enzymes, GcpE and LytB, being involved in the terminal steps of the reaction cascade (Kollas et al., this issue). This article reports on the purification of LytB protein and analysis of its enzymatic activity in a defined in vitro system.

2. Materials and methods

2.1. Cloning of the gene encoding LytB protein

The *lytB* (also named AQ_1739 or *ispH*) gene of *Aquifex aeolicus* (GenBank accession number AE000754) encoding LytB protein was amplified by polymerase chain reaction (PCR) using *A. aeolicus* genomic DNA (generous gift of Dr. Klaus Reuter, Marburg, Germany) and the oligonucleotides Aa-LytB-cHis-for (5′-CCATGGTTGACATAATAATCGCA-3′) and Aa-LytB-cHis-rev (5′-AGATCTGGAGGAAACCAATTGCCC-3′), introducing an *Nco*I and *Bgl*II restriction site, respectively. The PCR was performed in a total volume of 20 μl using a Stratagene Robocycler with heated lid and the Expand high-fidelity PCR system (Roche). Conditions used were 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 60 s, followed by a final 7 min step at 72°C to allow complete extension of the product. The PCR product was ligated into the pCR-TOPO-T/A-vector (Invitrogen), verified by restriction endonuclease analysis and sequencing, and subsequently cloned into the pQE60 vector (Qiagen) using the restriction enzymes *Nco*I and *Bgl*II resulting in the plasmid pQE60-Aa-LytB. This construct allowed the production of the *A. aeolicus* LytB protein (Swiss-Prot accession number O67625) in *Escherichia coli* with a carboxy-terminal extension of eight amino acids (RSHHHHHH).

2.2. Expression and purification of recombinant LytB protein

XL1-Blue *E. coli* cells harboring a plasmid encoding tRNAs for enhanced translation of A/U-rich sequences (Stratagene) were transformed with pQE60-Aa-LytB. Transformants were grown with aeration at 30°C in 500 ml standard I medium (Merck) supplemented with ampicillin (150 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}), and $FeCl_3$ (50 μg ml^{-1}). Optimal protein production was observed when cultures were grown without induction of the *lac* promotor to an optical density of 5–6 at 600 nm. The cells were harvested by centrifugation and cell pellets were stored at −40°C until use.

All purification steps were carried out in a Coy glove box under an atmosphere of N_2/H_2 (95%/5%) and with dioxygen-free solutions prepared as described [1]. The cell pellet (3.5 g) was resuspended in 50 ml buffer A (30 mM Tris–HCl (pH 8.0), 100 mM NaCl) and disintegrated by ultrasonic treatment at 0°C. After centrifugation of the cell lysate at 15 000 × *g* for 20 min at 4°C the supernatant was incubated at 65°C for 30 min in a water bath. After centrifugation at 15 000 × *g* for 30 min at 4°C the supernatant containing the thermostable *A. aeolicus* LytB protein was filtrated over a 0.2 μm filter (Renner) and imidazole was added to a final concentration of 10 mM. The hexa-histidine-LytB fusion protein was purified by immobilized metal affinity chromatography on Talon superflow resin (BD Clontech) using a HR 10/10 column (Amersham Pharmacia) and a Pharmacia fast protein liquid chromatography (FPLC) device. Buffers used were: A, 30 mM Tris–HCl (pH 8.0), 100 mM NaCl; B, 30 mM Tris–HCl (pH 8.0), 100 mM NaCl, 100 mM imidazole. Chromatography was done with a flow rate of 2 ml min^{-1} as follows: equilibration, 30 min 90% A and 10% B; loading of protein solution; washing, 15 min 80% A and 20% B; elution, 15 min 100% B. The eluted LytB protein (approximately 48 mg) was rebuffered and desalted in 20 mM Tris–HCl (pH 8.0) using a prepacked PD10 Sephadex G-25 gel filtration column (Amersham Pharmacia). When necessary the protein was concentrated using ultrafree centrifugal filter devices (Millipore). All purification steps were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Protein concentrations were measured using the method of Bradford with bovine

*Corresponding author. Fax: (49)-641-9947529.
E-mail address: boran.altincicek@jomaa.de (B. Altincicek).

[1] These authors contributed equally to this work.

Abbreviations: DMAPP, dimethylallyl diphosphate; HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate; IPP, isopentenyl diphosphate; MEP, 2-*C*-methyl-D-erythritol-4-phosphate

0014-5793/02/$22.00
PII: S0014-5793(02)03726-2

serum albumin as standard. The concentration of purified LytB protein was determined in 20 mM sodium phosphate buffer (pH 6.5) containing 6 M guanidium hydrochloride by ultraviolet (UV) spectroscopy ($\varepsilon_{280} = 26.0$ mM^{-1} cm^{-1}). Recombinant LytB protein was stored at −40°C until use.

2.3. Assay of (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) reductase activity of LytB protein and analysis of the reaction products by high-performance liquid chromatography (HPLC) and HPLC-electrospray ionization-mass spectrometry (ESI-MS)

Assays were carried out in a Coy glove box under an atmosphere of N_2/H_2 (95%/5%) and with dioxygen-free solutions. The standard assay system consisted of 20 mM Tris–HCl (pH 7.5), 5 mM HMBPP, 10 mM sodium dithionite, and 3 μM LytB protein in a final volume of 250 μl. After incubation for 1 h at 60°C the analysis was performed on a 250×2 mm, 13 μm AS11-HC column (Dionex), and a 50×2 mm AG11-HC precolumn, using a Dionex DX-600 device equipped with an electrochemical detector (ED50). Solvents used were: A, 20 mM KOH; B, 100 mM KOH. Chromatography was done with a flow rate of 1 ml min^{-1} as follows: 3 min 90% A and 10% B; linear gradient to 100% B in 30 min; equilibration, 12 min 90% A and 10% B. HPLC-ESI-MS analysis was performed as described by Kollas et al. (this issue). Mass peaks were detected by single ion recording in negative mode [M-H]$^-$ for HMBPP (*m/z* 261), isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (*m/z* 245).

2.4. Initial velocity assays for LytB protein

All solutions were made dioxygen free before used. To monitor the activity of LytB protein methyl viologen was used as reductant. The oxidation of methyl viologen was followed at 732 nm ($\varepsilon_{732} = 3150$ M^{-1} cm^{-1}) because the absorbance at absorption maximum ($\varepsilon_{604} = 13\,600$ M^{-1} cm^{-1}) [2] was too high. Standard assays were performed at 60°C in anaerobic gas-tight quartz cuvettes and contained 20 mM Tris–HCl (pH 7.5), 2 mM methyl viologen, and varying concentrations of HMBPP (0–2.8 mM) in a total volume of 800 μl. After reduction of methyl viologen with sodium dithionite (typically 0.3–0.7 mM) until an absorbance of 1.57 was reached, corresponding to 0.5 mM reduced methyl viologen (semiquinone form), the reactions were initiated by the addition of enzyme. Typically, assays contained 3 μM LytB protein but concentrations between 0.5 and 6 μM were also tested. Activity is expressed as μmol of HMBPP reduced per min and mg of protein. It was assumed that two equivalents reduced methyl viologen are needed to reduce one equivalent HMBPP. Kinetic data were fitted to the standard Michaelis–Menten equations using SigmaPlot 2000 (SPSS Science).

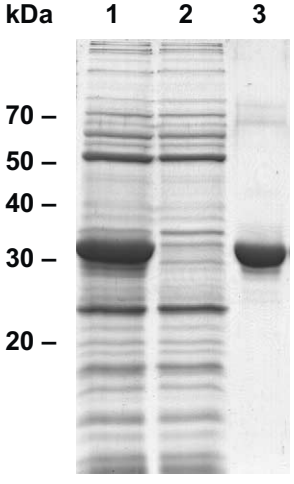

Fig. 1. SDS–PAGE analysis of the purification of LytB protein. After heat treatment, LytB protein was purified from the soluble fraction by immobilized metal affinity chromatography on Talon superflow resin. Lane 1, heat-stable protein fraction; lane 2, flow through; lane 3, purified LytB protein. Molecular mass standards are indicated in kDa. The migration velocity of the LytB protein is in accordance with the calculated molecular mass of 33.17 kDa.

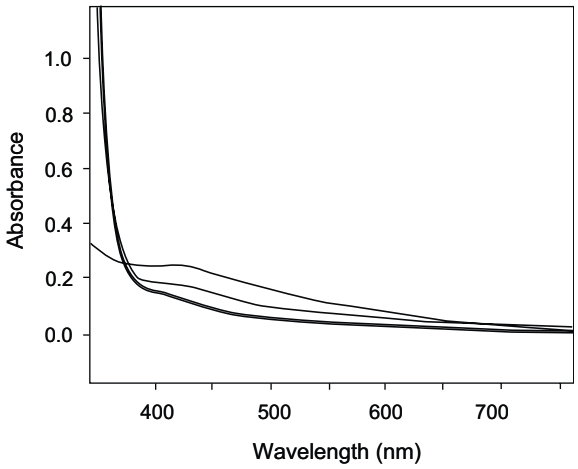

Fig. 2. UV-visible spectra of LytB protein. Purified LytB showed a broad absorption maximum at 420 nm that decreased after reduction with sodium dithionite, indicating the presence of an iron–sulfur cluster.

3. Results

For the analysis of LytB protein, the *lytB* gene from different organisms was expressed in *E. coli*. Optimal yields of recombinant protein were obtained with the *lytB* gene from *A. aeolicus*. The *A. aeolicus* LytB protein was purified in two steps under dioxygen-free conditions. After heat treatment at 65°C, the thermostable LytB protein was purified to over 95% homogeneity by immobilized metal affinity chromatography (Fig. 1). The protein sample showed a brownish color with a broad absorption maximum at 420 nm that decreased after reduction with sodium dithionite, indicating the presence of an iron–sulfur cluster (Fig. 2). Further analysis of the iron–

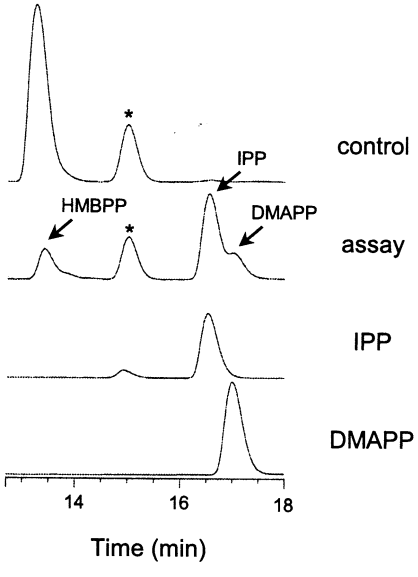

Fig. 3. Analysis of the reaction products of LytB protein by anion exchange HPLC. HMBPP was incubated in absence (control) and presence of LytB protein (assay) for 1 h at 60°C with sodium dithionite as reductant. IPP and DMAPP were generated in a ratio of approximately 5:1. Authentic IPP and DMAPP were used as standards. The peak marked with an asterisk resulted from sodium dithionite.

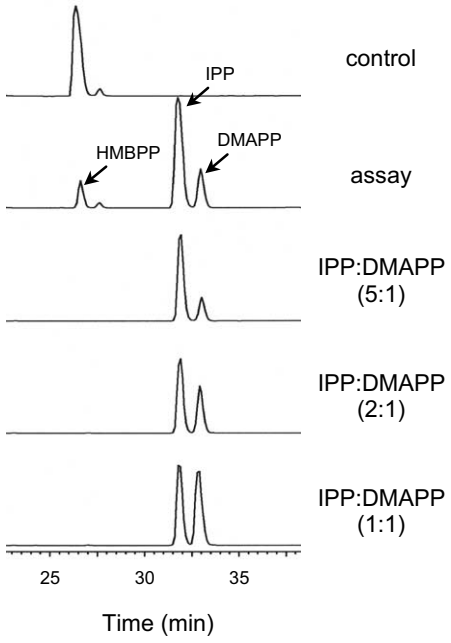

Fig. 4. Analysis of the reaction products of LytB protein by anion exchange HPLC-ESI-MS. Assay mixtures in absence (control) and presence of LytB protein (assay) were analyzed and compared to mixtures of authentic IPP and DMAPP in order to estimate the ratio of produced IPP to DMAPP.

sulfur cluster was performed by EPR and Raman spectroscopy and will be reported in due course.

Incubation of LytB protein and HMBPP in a defined assay mixture with dithionite as reductant resulted in consumption of HMBPP and formation of IPP and DMAPP, in a ratio of approximately 5:1, as estimated by anion exchange HPLC (Fig. 3). No reaction was observed in a control sample without enzyme. Additionally, mass peaks of HMBPP, IPP and DMAPP were recorded by anion exchange HPLC-ESI-MS (Fig. 4). In order to quantify the ratio of IPP to DMAPP produced by the enzymatic reaction more precisely, defined mixtures of authentic IPP and DMAPP in different ratios were analyzed in parallel. Again, the ratio of IPP to DMAPP

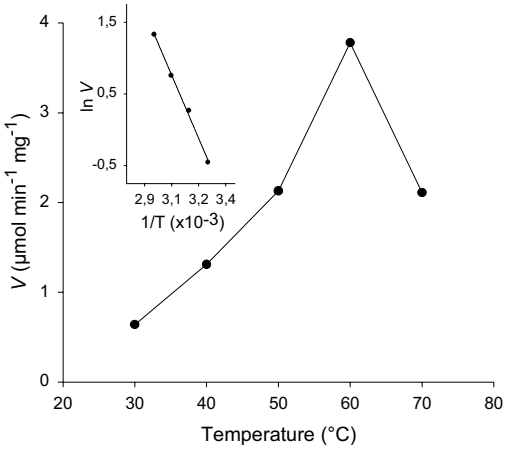

Fig. 5. Temperature dependence of LytB protein activity. The effect of temperature on LytB protein activity was investigated over a range of 30–70°C. The inset shows the Arrhenius plot used to estimate the activation energy. All data are average values of at least duplicate determinations.

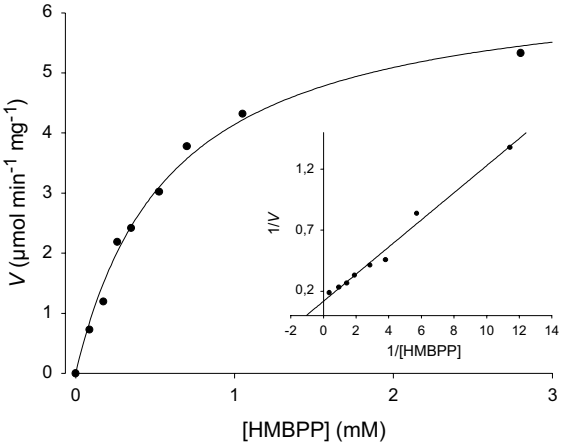

Fig. 6. Michaelis–Menten plot of LytB protein activity. HMBPP concentrations were varied from 0 to 2.8 mM and the respective initial velocities determined. The K_m value for HMBPP was 590 ± 60 µM. The inset shows the Lineweaver–Burk plot.

produced by the lytB protein-catalyzed reaction was in a range between 4:1 and 5:1 (Fig. 4).

For the determination of the kinetic parameters of LytB protein, a spectrophotometric assay was established. The pH optimum of LytB protein was in a range of pH 7.0–7.5. The effect of temperature on the enzyme activity was investigated over a range of 30–70°C. Maximal activity was observed at 60°C (Fig. 5). The activation energy was estimated to be 49 ± 2 kJ mol^{-1} based on an Arrhenius plot (Fig. 5, inset). The K_m value for HMBPP of 590 ± 60 µM was calculated based on a Michaelis–Menten plot (Fig. 6). The specific activity was 6.6 ± 0.3 µmol min^{-1} mg^{-1} protein corresponding to a k_{cat} value of 3.7 ± 0.2 s^{-1} for LytB protein. The enzyme retained more than 95% of the activity after freezing at −20°C in 20 mM Tris–HCl (pH 8.0) for 2 weeks. However, it was highly sensitive to dioxygen. After incubation at air for 10 min only 21% of the activity was retained.

4. Discussion

The value of the specific activity of LytB protein determined in the present study is over 3000 times higher than previously reported for *E. coli* LytB protein (approximately 2 nmol min^{-1} mg^{-1} protein), which was purified without exclusion of dioxygen and assayed in a crude *E. coli* cell extract based on conversion of radioactively labeled HMBPP [3]. Under our assay conditions, the formation of IPP and DMAPP in a ratio of approximately 5:1 was observed. A similar ratio was previously reported when the synthesis of IPP and DMAPP from ^{13}C-labeled 1-deoxy-D-xylulose was studied in *E. coli* cells overexpressing several genes of the MEP pathway [4]. The formation of IPP and DMAPP by a single enzyme is in striking contrast to the mevalonate pathway where DMAPP is successively synthesized from IPP by IPP isomerase. Branching of the MEP pathway for the formation of IPP and DMAPP has already been suggested earlier based on isotope labeling experiments [5] and the fact that *E. coli* deletion mutants for IPP isomerase are viable [6]. Elucidation of the catalytic mechanism of LytB protein for this unusual formation of two products from one substrate remains the subject of future studies. A hypothetical mechanism may include the elimination of the hydroxyl group leading to

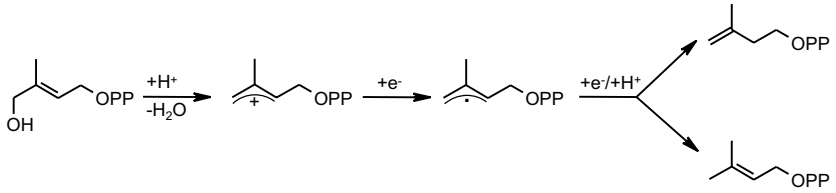

Fig. 7. Hypothetical reaction mechanism for the conversion of HMBPP into IPP and DMAPP by LytB protein.

a resonance stabilized allyl cation, which is reduced to the allyl anion and protonated either at carbon 1 or 3 (Fig. 7).

It was shown by Kollas et al. (this issue) that also the *gcpE* gene product is enzymatically active in converting 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate into HMBPP with dithionite as artificial electron donor without the need for any additional protein factors. Therefore, the complete MEP pathway must be considered as proven. Since it is absent in humans, the MEP pathway provides an attractive target for the development of novel antimicrobial and herbicidal agents. Previously, the natural antibiotic fosmidomycin was shown to be an inhibitor of 1-deoxy-D-xylulose-5-phosphate reductoisomerase [7–9], the second enzyme of the MEP pathway, and proved to be efficient in curing urogenital tract infections and malaria in a number of clinical studies [10,11]. Similar to 1-deoxy-D-xylulose-5-phosphate reductoisomerase, GcpE and LytB represent highly conserved enzymes without any homologies to mammalian enzymes. Consequently, broad spectrum activity without mechanism-based toxicity to humans can be expected from specific inhibitors. For the development of such inhibitors, the data presented are of considerable relevance. In particular, the convenient photometric assay may be useful for testing a larger numbers of candidate compounds. Also, solving the crystal structures of GcpE and LytB may now be achievable, taking their sensitivity to dioxygen into account.

Acknowledgements: This work was supported in part by grants of the Bundesministerium für Bildung und Forschung (Biochance 0312588) and the Deutsche Forschungsgemeinschaft (SPP1131). We thank Matthias Eberl for critical reading of the manuscript and Y. Kletschek, I. Steinbrecher, T. Kuhl, and C. Haug for excellent technical assistance.

References

[1] Beinert, H., Orme-Johnson, W.H. and Palmer, G. (1978) Methods Enzymol. 54, 111–132.
[2] Mayhew, S.G. (1978) Eur. J. Biochem. 85, 535–547.
[3] Adam, P., Hecht, S., Eisenreich, W.G., Kaiser, J., Grawert, T., Arigoni, D., Bacher, A. and Rohdich, F. (2002) Proc. Natl. Acad. Sci. USA 99, 12108–12113.
[4] Rohdich, F., Hecht, S., Gartner, K., Adam, P., Krieger, C., Amslinger, S., Arigoni, D., Bacher, A. and Eisenreich, W. (2002) Proc. Natl. Acad. Sci. USA 99, 1158–1163.
[5] Hoeffler, J.F., Hemmerlin, A., Grosdemange-Billiard, C., Bach, T.J. and Rohmer, M. (2002) Biochem. J. 366, 573–583.
[6] Hahn, F.M., Hurlburt, A.P. and Poulter, C.D. (1999) J. Bacteriol. 181, 4499–4504.
[7] Kuzuyama, T., Shizimu, T., Takahashi, S. and Seto, H. (1998) Tetrahedron Lett. 39, 7913–7916.
[8] Zeidler, J., Schwender, J., Müller, C., Wiesner, J., Weidemeyer, C., Beck, E., Jomaa, H. and Lichtenthaler, H.K. (1998) Z. Naturforsch. C 53, 980–986.
[9] Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Turbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H.K., Soldati, D. and Beck, E. (1999) Science 285, 1573–1576.
[10] Kuemmerle, H.P., Murakawa, T., Sakamoto, H., Sato, N., Konishi, T. and De Santis, F. (1985) Int. J. Clin. Pharmacol. Ther. Toxicol. 23, 521–528.
[11] Missinou, M.A., Borrmann, S., Schindler, A., Issifou, S., Adegnika, A., Matsiegui, P.B., Binder, R., Lell, B., Wiesner, J., Baranek, T., Jomaa, H. and Kremsner, P.G., Lancet, in press.