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Isoprenoid biosynthesis via the methylerythritol phosphate pathway: the (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB/IspH) from *Escherichia coli* is a [4Fe–4S] protein

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Abstract **The last enzyme (LytB) of the methylerythritol phosphate pathway for isoprenoid biosynthesis catalyzes the reduction of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate into isopentenyl diphosphate and dimethylallyl diphosphate. This enzyme possesses a dioxygen-sensitive [4Fe–4S] cluster. This prosthetic group was characterized in the *Escherichia coli* enzyme by UV/visible and electron paramagnetic resonance spectroscopy after reconstitution of the purified protein. Enzymatic activity required the presence of a reducing system such as flavodoxin/flavodoxin reductase/reduced nicotinamide adenine dinucleotide phosphate or the photoreduced deazaflavin radical.**

Key words: Dimethylallyl diphosphate; Isopentenyl diphosphate; Isoprenoid; LytB; 2-*C*-Methyl-D-erythritol 4-phosphate pathway; (*E*)-4-Hydroxy-3-methylbut-2-enyl diphosphate

1. Introduction

Isoprenoid biosynthesis follows two biosynthetic pathways depending on the organisms. The long known mevalonate pathway is found in animals, fungi, the cytoplasm of phototrophic organisms, some eubacteria and archaea, whereas the recently discovered methylerythritol phosphate pathway is present in most eubacteria, in unicellular green algae, in the chloroplasts of phototrophic organisms [1,2] as well as in some unicellular eukaryotes related to photosynthetic phyla such as the *Plasmodium* spp. [3] and *Prototheca wickerhamii* [4]. A combination of biochemical and genetic methods resulted in the nearly complete elucidation of the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Fig. 1) [1,2,5–8]. The last two steps catalyzed by the GcpE (IspG) and LytB (IspH) proteins, however, presented puzzling problems for the complete elucidation of the pathway.

We have recently shown that GcpE is a metallo-enzyme possessing a $[4Fe–4S]^{2+}$ center after reconstitution of the purified protein [9]. The enzyme catalyzes the reduction of 2-*C*-methyl-D-erythritol (ME) cyclodiphosphate (**3**) into (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) (**4**) via two successive one-electron transfers. The reduction is very likely mediated by the reduced $[4Fe–4S]^{1+}$ form of the cluster. The oxidized $[4Fe–4S]^{2+}$ form can be reduced in vitro by photoreduced deazaflavin [9], dithionite [10] or reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of flavodoxin/flavodoxin reductase [9], which is probably the in vivo reducing system in *Escherichia coli*.

The last step of the MEP pathway is catalyzed by LytB (or IspH), which converts HMBPP (**4**) into isopentenyl diphosphate (IPP) (**5**) or dimethylallyl diphosphate (DMAPP) (**6**) [8]. Very recently, in vitro activity tests under anaerobic conditions have been described for LytB from two bacteria, *Aquifex aeolicus* [11] and *E. coli* [12]. The reaction was followed using either a colorimetric test with methylviologen and dithionite as reducing system [11], or by ^{13}C nuclear magnetic resonance or high-performance liquid chromatography using photoreduced deazaflavin or flavodoxin/flavodoxin reductase/NADPH [12]. It has been proposed that LytB is a Fe/S protein catalyzing the reduction of HMBPP (**4**) into IPP (**5**) or DMAPP (**6**) via two successive one-electron transfers [11,12]. A convincing characterization of the protein has not yet been provided, since the described UV/visible spectrum did not allow any quantification. In this paper, we characterize the LytB protein from *E. coli*, showing by electron paramagnetic resonance (EPR) the presence of a $[4Fe–4S]^{1+}$ cluster after reconstitution of the isolated protein and reduction with dithionite, and we describe an alternative in vitro radioactive test using the natural flavodoxin/flavodoxin reductase/NADPH system from *E. coli*, which already proved successful with the GcpE enzyme [9].

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Abbreviations: DAF, 5-deazaflavin (10-methyl-5-deazaisoalloxazine); DMAPP, dimethylallyl diphosphate; EPR, electron paramagnetic resonance; HMBPP, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; ME, 2-*C*-methyl-D-erythritol; MEP, 2-*C*-methyl-D-erythritol 4-phosphate

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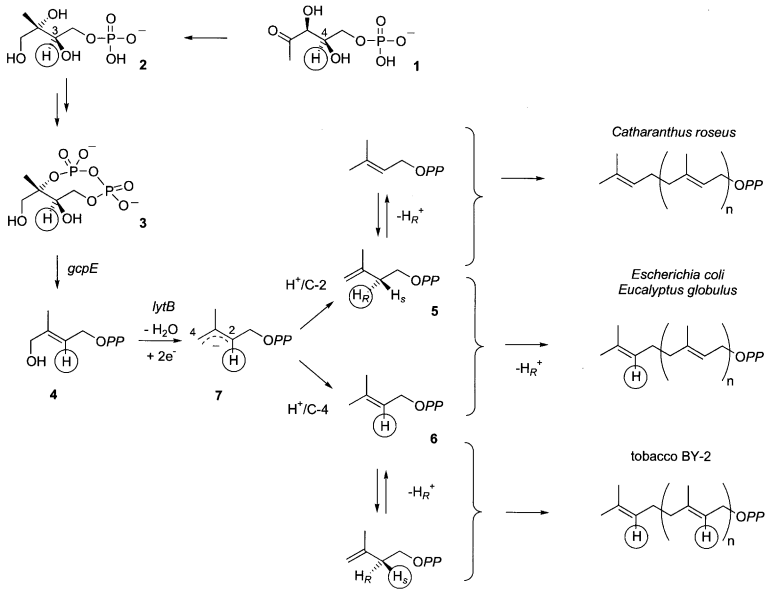

Fig. 1. Methylerythritol phosphate pathway for isoprenoid biosynthesis and hypothetical mechanism for the LytB-catalyzed reaction in the presence of flavodoxin, flavodoxin reductase and NADPH.

2. Materials and methods

2.1. Expression and purification of LytB protein from E. coli

The *lytB* (*ispH*) gene was amplified by polymerase chain reaction (PCR) using *E. coli* XL1-Blue DNA as template and the oligonucleotides 5′-GTTCGAGCTCATGCAGATCCTGTTGGCCAACC-3′ and 5′-CATAACTTAGGCTGCAGTGACTTAATCG-3′ as primers. In this reaction *Sac*I and *Pst*I restriction sites were respectively generated at the 5′ and 3′ end of the sequence. The product was digested with *Sac*I and *Pst*I and cloned between the corresponding sites of the pQE-30 vector (Qiagen). After sequencing, plasmid pQE30-LytB encodes the entire LytB protein from *E. coli* extended at the N-terminus with Met-Arg-Gly-Ser-His_6-Gly-Ser-Ala-Cys-Glu-Leu. Plasmid pQE30-LytB was used to transform *E. coli* M15[pREP4]. The resulting recombinant strain *E. coli* M15[pREP4, pQE30-LytB] was grown at 30°C on Luria–Bertani (LB) medium (9×500 ml) containing ampicillin (100 μg ml^{-1}) and kanamycin (25 μg ml^{-1}) until a 0.54 OD_{600} was reached. Induction was performed with 100 μM isopropyl thiogalactose (IPTG) for 4.5 h at 30°C instead of at 37°C with 2 mM IPTG, to obtain higher yields of soluble protein [8]. Cells were harvested by centrifugation (5000×*g*, 10 min), resuspended in 50 mM Tris–HCl pH 8 (55 ml) and sonicated (5×30 s with 2 min cooling in melting ice). After centrifugation (11 000×*g*, 15 min), the supernatant was collected and loaded onto a Ni^{2+}-nitrilotriacetic acid agarose resin column (Qiagen, 1.2 cm×7.5 cm column), equilibrated with 50 mM Tris–HCl pH 8. The resin was first washed with a 20 mM imidazole solution in 50 mM Tris–HCl pH 8 (40 ml). The His_6-LytB protein was eluted using a linear imidazole gradient (20–200 mM, 90 ml, 1 ml min^{-1} in 50 mM Tris–HCl pH 8, yielding LytB (18 mg) after pooling the fractions containing the protein (>90% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Fig. 2). Upon overnight dialysis, concentration on Centricon 10 (Amicon) and addition of glycerol (20%), the brown protein solution was frozen in liquid nitrogen and stored at −80°C. Protein concentration was measured by the method of Bradford using bovine serum albumin (BSA) as a standard [13]. Iron was assayed by the method of Fish [14] and inorganic sulfide was quantified spectrophotometrically as described by Beinert [15].

To determine the apparent molecular size of native and reconstituted LytB, a sample (0.4 mg, 100 μl) was gel-chromatographed on a Superdex 200 HR 10/30 (Pharmacia) that was pre-equilibrated at room temperature with 50 mM Tris–HCl buffer pH 8. Proteins for column calibration were ovalbumin (45 kDa), BSA (66 kDa), His_6-tagged biotin synthase (78 kDa), β-galactosidase (116 kDa) and yeast alcohol dehydrogenase (150 kDa).

2.2. Expression and purification of the flavodoxin reductase from E. coli

The *fpr* gene encoding flavodoxin reductase was amplified by PCR using *E. coli* XL1-Blue DNA as template and the oligonucleotides 5′-TGGAGGGGATCCATGGCTGATTGGGTAACAGGC-3′ and 5′-GCATTTCTGCAGTTACCAGTAATGCTCCGCTGTC-3′ as primers. In this reaction *Bam*HI and *Pst*I restriction sites were respectively generated at the 5′ and 3′ end of the sequence. The product was digested with *Bam*HI and *Pst*I and cloned between the corresponding sites of the pQE-30 vector. After sequencing, plasmid pQE30-HisFPR encodes the entire flavodoxin reductase from *E. coli* extended at the N-terminus with Met-Arg-Gly-Ser-His_6. *E. coli* M15[pREP4] carrying plasmid pQE30-HisFPR was grown at 37°C on LB medium (500 ml) containing ampicillin (100 μg ml^{-1}) and kanamycin (25 μg ml^{-1}) until a 0.5 OD_{600} was reached. Induction was performed with 100 μM IPTG for 4 h. Cells were harvested, opened, and purified flavodoxin reductase (10 mg) was obtained as already described for LytB. Protein concentration was calculated by using the absorbance of bound flavin adenine dinucleotide (FAD) (ε_{456nm} = 7100 M^{-1} cm^{-1}) [16].

2.3. Reconstitution of the FeIS center in the as-purified LytB protein

The solutions of the purified LytB protein (two 200 μl samples,

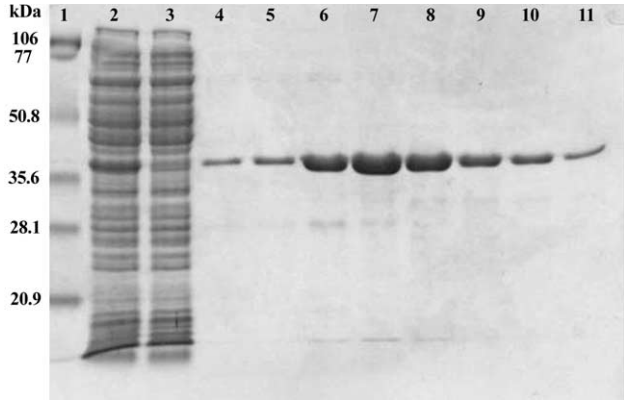

Fig. 2. SDS–PAGE analysis of the purification of LytB protein. Lane 1, standards (Low Range, Bio-Rad); lane 2, crude cell extract of recombinant *E. coli* expressing LytB/IspH protein; lane 3, flow through; lanes 4–11, fractions containing pure LytB protein. These fractions were pooled together to afford pure LytB (>90%).

660 μM in 50 mM Tris–HCl pH 8), 20 mM Na_2S in argon-saturated buffer (50 mM Tris–HCl pH 8), 20 mM $FeCl_3$ and 100 mM dithiothreitol (DTT) (both dissolved in water) were deoxygenated in separate tubes under a stream of wet argon for 1 h. The tubes were transferred into a glove box (Jacomex BS531 NMT) equipped with an oxymeter (Arelco Arc), filled with a N_2 atmosphere containing less than 2 ppm O_2, where they were left to stand overnight at 15°C before beginning the reconstitution procedure. DTT was added to the enzyme to a final concentration of 5 mM. $FeCl_3$ and Na_2S in a five-fold molar excess with respect to the enzyme were added to the solution. After 3 h, the mixture was desalted on a PD 10 column (Pharmacia) equilibrated with 50 mM Tris–HCl pH 8. The reconstituted LytB protein solution was concentrated to 208 μM in the glove box using a Microcon YM-30 (Millipore). For recording the UV/visible absorption spectrum, a fraction of the reconstituted protein was directly transferred into a cuvette, which was closed with an air-tight rubber septum before being removed from the glove box.

2.4. As-purified or reconstituted LytB assay with flavodoxin, flavodoxin reductase and NADPH

All assays were performed in anaerobic conditions. [3-^{14}C]HMBPP was synthesized as already described [9]. A mixture (70 μl, final volume) containing [3-^{14}C]HMBPP (100 μM, 5 nCi), DTT (5 mM), NADPH (1.1 mM), flavodoxin (3 μM), flavodoxin reductase (1 μM) and LytB solution (2.1 μM) in Tris–HCl (50 mM, pH 8) was incubated at 37°C. An aliquot (4 μl) of one assay was directly plotted on thin-layer chromatography silica gel plates eluted either with isopropanol/water/EtOAc (6:3:1) or isopropanol/30% NH_4OH/H_2O (6:3:1). The reaction product coeluted with IPP and DMAPP in the two previous solvent systems ($R_f = 0.29$ and $R_f = 0.34$, respectively).

In order to estimate the relative amounts of IPP and DMAPP, the incubation was performed in duplicate. The first assay was hydrolyzed with alkaline phosphatase (50 mM Tris–HCl pH 8, 5 mM $MgCl_2$, 4 h, 25°C). The resulting ^{14}C-labeled isopentenol and dimethylallyl alcohol respectively derived from IPP (**5**) and DMAPP (**6**) were extracted with hexane (7×600 μl). The combined organic layers were passed through a silica column (Kieselgel Si 60, 40–63 μm, 0.7 cm×1.5 cm), eluted with hexane/ethyl acetate (6:4, 4 ml), and radioactivity was measured by liquid scintillation counting. For DMAPP determination [17], a MeOH/10 M aqueous HCl solution (3:1, 200 μl) was added to the second assay, and the reaction mixture was incubated for 15 min at 37°C. The alcohols derived from labile allylic diphosphate DMAPP (**5**) under acidic conditions were extracted with hexane (3×700 μl). The radioactivity of the combined organic layers was measured using a liquid scintillation counter.

2.5. As-purified or reconstituted LytB assay with photoreduced 5-deazaflavin

A mixture (70 μl, final volume) containing [3-^{14}C]HMBPP (100 μM, 5 nCi), DTT (5 mM), in Tris–HCl pH 8 (50 mM) was degassed for 45 min at room temperature under a stream of wet argon. A degassed 5-deazaflavin (DAF) solution (170 μM final concentration) and a degassed LytB solution (1.3 μM final concentration) were added with a gas-tight syringe in the sample. The assay was incubated in anaerobic conditions under irradiation with a white fluorescent tube at 37°C for 15 min and analyzed as mentioned above.

2.6. EPR spectroscopy of as-purified and reconstituted LytB

EPR experiments have been performed on a Bruker Elexsys 500 spectrometer with an SHQ001 cavity fitted with an ESR900 cooling system. The spectra have been recorded at 10 K (Fig. 4) or at 20 K. For a power saturation study at 20 K, a two-dimensional experiment was performed by measuring intensity against magnetic field at different microwave powers from 200 to 2×10^{-4} mW. EPR parameters were as follows: modulation amplitude 1 mT, field modulation frequency 100 kHz, time constant 0.01024 s, microwave frequency 9.44342 GHz. Spin quantification was performed by double integration of the signal and comparison to the signal of a 15 μM Cu-EDTA standard solution. Both EPR spectra were recorded under non-saturated conditions.

Reduction of reconstituted LytB protein was performed in the anaerobic glove box. Sodium dithionite prepared in argon-saturated 50 mM Tris–HCl pH 8 (245 mM) was added in a 50-fold excess (3 μl) to the reconstituted LytB protein solution (70 μl, 208 μM), yielding a final 200 μM protein concentration. The effective dithionite concentration was previously determined with potassium ferricyanide. After incubation at room temperature and transfer into the EPR tubes (50 min), performed in the glove box, the EPR tubes were frozen in liquid nitrogen outside the glove box and stored in liquid nitrogen.

3. Results

The *lytB* (*ispH*) gene of *E. coli* was cloned into the pQE-30 vector, and the His_6-tagged LytB protein was produced in a similar way as already described [8] and purified by chromatography on a Ni^{2+}-nitrilotriacetic agarose column. The calculated molecular mass deduced from the amino acid sequence is 36591 Da. The apparent molecular mass of LytB, as determined by analytical gel filtration chromatography, is ca. 72 kDa, indicating that LytB is a dimer.

The as-purified protein solution, which served for the reconstitution of the prosthetic group, was pale brown in color and displayed a UV/visible spectrum with absorption characteristics reminiscent of a Fe–S cluster (Fig. 3b). Chemical analysis of this protein gave 0.8 iron and 0.6 sulfur atoms, respectively, per monomer. These two features suggested the presence of traces of an iron–sulfur center as prosthetic group. Reconstitution of the cluster was therefore performed under an inert nitrogen atmosphere using $FeCl_3$ and Na_2S in the presence of DTT [9], which previously revealed to be successful for the reconstitution of GcpE. After reconstitution in a dioxygen-free environment, the dark khaki solution of the reconstituted enzyme showed an absorption band at 410 nm ($\varepsilon = 18\,750$ M^{-1} cm^{-1}) characteristic of a $[4Fe–4S]^{2+}$ cluster [18]. Chemical determination also showed a large increase in iron and sulfur content, five and six per protein monomer, respectively. These values, higher than the expected ones of four, may be due to non-specific binding to the protein. Overnight exposure of the reconstituted enzyme solution to air led to bleaching, corresponding to the degradation of the $[4Fe–4S]^{2+}$ cluster. LytB, however, degrades much more slowly than GcpE. The UV/visible spectrum of the reconstituted enzyme did not significantly change after 30 min exposure to air at room temperature. Upon 4 h exposure, absorbance at 410 nm was still present. In the same conditions, the absorbance of reconstituted GcpE nearly disappeared after 2 h (results not shown).

Confirmation of the presence of a [4Fe–4S] cluster was obtained by EPR spectroscopy (Fig. 4). Reconstituted LytB is EPR-silent. This is in accordance with the presence of a [4Fe–

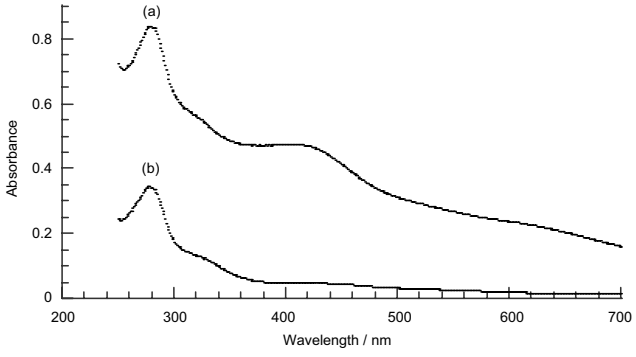

Fig. 3. UV/visible absorption spectra of His-LytB (0.5 mg ml^{-1}, 50 mM Tris–HCl pH 8). a: Reconstituted His-LytB. b: As-isolated protein (spectrum recorded after storage).

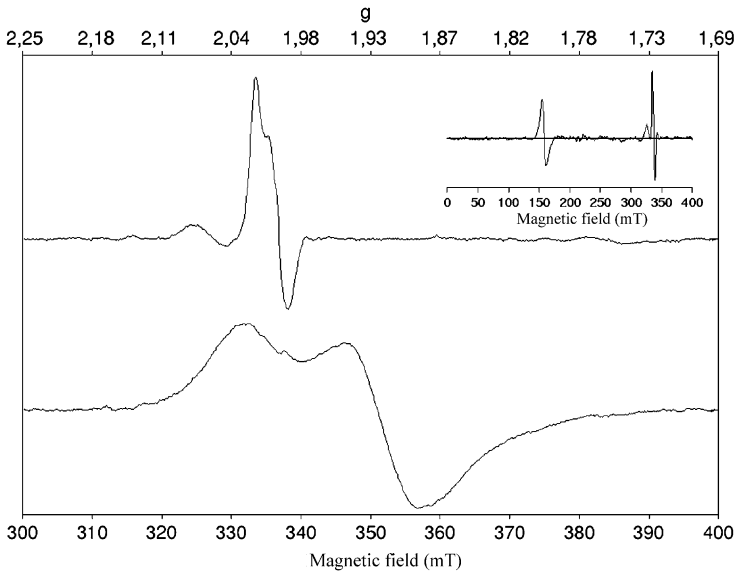

Fig. 4. EPR spectra of LytB in Tris–HCl pH 8 (50 mM) in as-isolated oxidized form (660 μM, upper spectrum) and reconstituted reduced form (200 μM, lower spectrum). EPR conditions: 10 K, modulation amplitude 1 mT, field modulation frequency 100 kHz, time constant 0.01024 s, microwave frequency 9.44342 GHz.

4S]$^{2+}$ center. Reduction of the reconstituted enzyme with sodium dithionite (50 equivalents) induced the appearance of a signal with g values at $g = 1.92(1)$ and 2.03(7), typical of an $S = 1/2$ [4Fe–4S]$^{1+}$ cluster [19–22], to be compared to g values obtained by calculation (1.889, 1.927, 2.038). The microwave power half-saturation ($P_{1/2}$) is currently used to characterize the Fe–S nuclearity. [2Fe–2S]$^{1+}$ clusters are characterized by a very low $P_{1/2}$, as found for the ferredoxins from spinach ($P_{1/2} = 3.8$ mW) or from maize ($P_{1/2} = 10.5$ mW) [23]. [4Fe–4S]$^{1+}$ clusters are, in contrast, characterized by a much higher $P_{1/2}$, as found, for instance, for the *Chromatium* high-potential iron–sulfur protein ($P_{1/2} = 95$ mW) and the ferredoxin from *Bacillus stearothermophilus* ($P_{1/2} = 330$ mW) [23]. Power saturation experiments on the dithionite-reduced reconstituted LytB at 20 K yielded a $P_{1/2}$ (153 mW), indicating the presence of a [4Fe–4S]$^{1+}$. The LytB signal of the [4Fe–4S]$^{1+}$ species integrating for 144 μM, when compared to the signal of Cu-EDTA standard (15 μM), indicated that the reduction yield with sodium dithionite was 72%. In contrast, the as-purified enzyme gave an EPR signal with a g value at 2.02(1) (Fig. 4) (g values obtained by simulation: 1.956, 2.012 and 2.013), which is very similar to that of the [3Fe–4S]$^{1+}$ center of the activase of anaerobic ribonucleotide reductase, lysine 2,3-aminomutase and certain ferredoxins [22,24,25]. Power saturation experiments yielded a $P_{1/2}$ of 135 mW at 20 K. In addition, a strong $g = 4.29$ signal was assigned to ferric ions (Fig. 4, inset). Quantification of the signal yielded a 64 μM spin concentration for the signal of the [3Fe–4S]$^{1+}$ cluster in this non-reconstituted LytB sample, corresponding to ca. 10% of the enzyme concentration. The combined results suggest that LytB initially contains a [4Fe–4S]$^{2+}$ cluster, which degrades into a [3Fe–4S]$^{1+}$ form in the presence of dioxygen during purification, a phenomenon frequently observed for [4Fe–4S]$^{2+}$ proteins [19,22,24,25].

LytB reduces HMBPP into a mixture of IPP and DMAPP. The detection of the two reaction products was therefore adapted from an enzyme test designed to check the activity of the IPP isomerase. Each incubation of [3-^{14}C]HMBPP was run in duplicate. Alkaline phosphatase treatment of the reaction mixture from the first assay released isopentenol and dimethylallyl alcohol from IPP and DMAPP respectively, which were extracted with hexane. The radioactivity of this extract defined the conversion yield of the test. The 2-methylbut-2-ene-1,4-diol resulting from HMBPP hydrolysis remained in the water phase. The second assay was submitted to an acidic hydrolysis of the allylic diphosphate, DMAPP, which was converted into a mixture of allylic monoalcohols extracted with hexane. The IPP/DMAPP ratio was deduced from the comparison of the radioactivity of the two hexane extracts (Table 1). LytB produced IPP and DMAPP in a ratio comprised between 4:1 and 6:1, similar to those described in former assays [8,11,12]. Conversion yields were up to 60%

Table 1
Enzyme tests with reconstituted LytB: [3-^{14}C]HMBPP (5 nCi, 100 μM), 50 mM Tris–HCl pH 8, 37°C

	dpm		[IPP]/[DMAPP]	Conversion
	IPP+DMAPP	DMAPP		(%)
A				
–	4830	710	6:1	57
Mg^{2+} (0.5 mM)	5070	1240	3:1	61
Co^{2+} (0.5 mM)	2790	8202.	2.4:1	39
B				
–	4360	660	6:1	40

Reducing system: **A** flavodoxin (3 μM), flavodoxin reductase (1 μM), NADPH (1.1 mM), 4 h; **B** DAF (170 μM) photoreduced, 15 min.

Table 2
Comparison of the activities of as-isolated LytB and reconstituted LytB: [3-^{14}C]HMBPP (5 nCi, 100 μM), 50 mM Tris–HCl pH 8, 37°C

	[LytB] (μM)	IPP+DMAPP (dpm)	Conversion (%)	Specific activity (nmol mg^{-1} min^{-1})	Incubation time (min)
A					
As isolated[a)]	2.6	1030	10	1.7	60
Reconstituted[a)]	1.2	1145	11	18	15
Reconstituted[b)]	1.2	3111	31	44	15
B					
As isolated	2.6	1670	17	11	15
Reconstituted	2.6	4360	44	30	15

Reducing system: **A** a) flavodoxin (3 μM), flavodoxin reductase (1 μM), NADPH (1.1 mM), b) flavodoxin (17 μM), flavodoxin reductase (10 μM), NADPH (2 mM); **B** DAF (170 μM) photoreduced.

in the presence of flavodoxin/flavodoxin reductase/NADPH and 40% in the presence of DAF (Table 1).

According to our assay conditions described for GcpE, another enzyme of the MEP pathway [9], LytB, was tested in the presence of flavodoxin/His$_6$-tagged flavodoxin reductase/NADPH or in the presence of the DAF semiquinone radical as reducing system as recently described [12]. The protein was found to be active using both reducing systems (Tables 1 and 2) and showed no activity in their absence. The enzyme was required for the conversion of HMBPP. The substrate remained unchanged when treated with heat-inactivated LytB (15 min boiling). The activity found for the reconstituted enzyme with an intact [4Fe–4S] cluster was much higher than that of the as-isolated enzyme, which served for the reconstitution and still conserved some activity (Table 2). This is in accordance with the presence of remaining Fe–S cluster, which may be activated in the presence of the reducing agents.

The presence of divalent cations, preferably Co^{2+}, enhanced the LytB activity in enzyme tests performed with a crude cell-free system from *E. coli* [26]. Co^{2+} ions were also added in assays performed with the purified enzyme [12]. In our tests, purified reconstituted LytB did not require the addition of divalent cations such as Co^{2+} or Mg^{2+}. Activities of the same order of magnitude were found in their presence or in their absence, with even a yield decrease in the presence of Co^{2+} (Table 1).

4. Discussion

The nature of the Fe–S cluster in as-isolated and reconstituted LytB has been characterized by UV/visible and EPR spectroscopy as well as by chemical means. EPR analysis of the as-isolated enzyme shows a small signal characteristic of a $[3Fe–4S]^{1+}$ center together with a larger signal corresponding to adventitious ferric ions. Chemical analysis revealed the presence of 0.8 Fe and 0.6 S^{2-}, which corresponds to a substoichiometric amount of cluster, 0.15 $[3Fe–4S]^{1+}$, as based on the sulfide content, thus explaining the low signal observed in EPR.

Upon reconstitution with an excess of Fe^{3+} and S^{2-} under strict anaerobic conditions, a full complement of one $[4Fe–4S]^{2+}$ cluster per monomer is formed, as indicated by the ε_{410nm} value of the UV/visible spectrum. This is also in accordance with the iron and sulfide determination (five Fe and six S^{2-} per monomer), the excess most probably corresponding to non-specific binding. The presence of this $[4Fe–4S]^{2+}$ cluster is further confirmed by EPR spectroscopy since upon dithionite reduction, the characteristic signal of a $[4Fe–4S]^{1+}$ is observed. Our data hence suggest the presence of an oxygen-sensitive [4Fe–4S] cluster in LytB that converts to a $[3Fe–4S]^{1+}$ during aerobic isolation. The transformation of a $[4Fe–4S]^{2+}$ cluster into a $[3Fe–4S]^{1+}$ form in the presence of oxygen and the possibility of reversing the process in the presence of Fe^{2+} under strict anaerobic conditions is a widespread feature among such iron–sulfur proteins [19,22,24,25].

These data support the conclusion that the prosthetic group of LytB is a $[4Fe–4S]^{2+/1+}$ center, which is now found in all the iron–sulfur proteins performing mono-electronic reductions [22,24,25]. As expected, the reconstituted enzyme, containing a larger amount of cluster, has a higher activity.

This strongly suggests the reduction occurs via two successive one-electron transfers from the reduced $[4Fe–4S]^{1+}$ form [11], yielding the allylic anionic intermediate (**7**), which is protonated either at C-2 yielding IPP (**5**), or at C-4, yielding DMAPP (**6**) (Fig. 1). Reduction of the oxidized $[4Fe–4S]^{2+}$ cluster could be performed with dithionite [11,12]. This reducing system was efficiently replaced by those previously tested on GcpE [9], such as the DAF semiquinone radical or the endogenous flavodoxin/flavodoxin reductase/NADPH reducing system from *E. coli* [12]. Even if the latter system worked efficiently and is most likely the native regeneration system, this aspect of the LytB catalysis has to be further investigated.

The enantioselectivity of the protonation of intermediate (**7**) can be deduced from former results on the isoprenoid biosynthetic pathway in *E. coli*. According to the labeling pattern observed in the prenyl chains of ubiquinone and menaquinone after feeding of [4-^{2}H]deoxyxylulose or [3-^{2}H]methylerythritol, the hydrogen atom at C-4 of DXP (**1**) or at C-3 of MEP (**2**) (Fig. 1) is retained in DMAPP (**6**), yielding [2-^{2}H]DMAPP, and lost in IPP (**5**) [27,28]. This observation is consistent with the retention of this hydrogen atom throughout the MEP pathway [29], i.e. up to HMBPP (**4**) and the anionic allylic intermediate (**7**) (Fig. 1). Protonation of intermediate (**7**) has to occur from the *si* face. Indeed, in the chain elongation, the prenyl transferase eliminates the pro-*R* hydrogen of IPP [30], which is derived from C-4 of DXP, and preserves the pro-*S* hydrogen, which is introduced by LytB. This hypothesis is consistent with the observations on the fate of the deuterium atom of [4-^{2}H]DX in the biosynthesis of plastid isoprenoids in plants (Fig. 1). In *Eucalyptus globulus* twigs, labeling of the monoterpene cineol was similar to the labeling of the *E. coli* isoprenoids with deuterium retention in DMAPP and deuterium loss in IPP [31]. Complete deuterium loss was observed in phytol and carotenoids from a *Catharanthus roseus* cell culture [32]. This is compatible with the formation of non-labeled DMAPP from [2*R*-^{2}H]IPP by the IPP isomerase that

removes the pro-*R* hydrogen of IPP [33] and of non-labeled polyprenyl diphosphates from the two former intermediates by the prenyl transferase. Deuterium retention was found in all isoprene units from plastoquinone and phytoene of a tobacco BY-2 cell culture [29]. This resulted from the formation of [2*S*-^{2}H]IPP from [2-^{2}H]DMAPP by protonation of the *re* face of DMAPP by the IPP isomerase and of polyprenyl diphosphates with deuterium labeling on all isoprene units by elimination of the pro-2*R*-hydrogen by the prenyl transferase.

A previous experiment pointed out an unsolved problem concerning the reduction steps of the MEP pathway [34]. The bacterium *Zymomonas mobilis* was grown in the presence of [1-^{2}H]glucose as sole carbon and energy source. Owing to the peculiar metabolism of this bacterium, which synthesizes its NAD(P)H, and accordingly its whole reducing agent pool, from glucose catabolism, the two possible C-4 deuterium-labeled isotopomers of NAD(P)^{2}H are synthesized from [1-^{2}H]-glucose. This means that the reduction steps in a metabolic pathway may leave a deuterium signature on the carbon skeleton of the reduced metabolites. After feeding *Z. mobilis* with [1-^{2}H]glucose, two deuteration sites were found in the isoprene units of the bacterial triterpenoids of the hopane series. A first one was located on carbon atoms derived from C-4 of IPP and DMAPP and corresponded to the expected NADPH-dependent reduction involved in the conversion of DXP into MEP catalyzed by the DXP reductoisomerase. A second deuteration site was found in the isoprene units derived from IPP. It corresponded to carbon atoms derived from C-2 and was interpreted as the signature of an additional reduction step, which was later tentatively identified as the LytB catalyzed reduction of the isoprenyl diphosphate allylic cation by NADP^{2}H [29]. This hypothesis is not consistent with the present knowledge of the LytB-catalyzed reaction involving a Fe–S cluster enzyme and the anionic intermediate (**7**). Interpretation of this odd labeling pattern is not obvious, and two tentative explanations may be proposed. Either the formation of IPP and DMAPP proceeds in *Z. mobilis* in a different way from that in *E. coli*, or, if LytB works in a similar way in *Z. mobilis* and in *E. coli*, the deuterium from NADP^{2}H has to be finally delivered as $^2H^+$. This might be possible if LytB is coupled to a flavoprotein. Reduction of the oxidized flavin via NADP^{2}H yields the N-5 deuterated dihydroflavin, which should not exchange its deuterium. Reduction of the oxidized [4Fe–4S]$^{2+}$ cluster system then proceeds via two one-electron transfers from deuterated dihydroflavin and the loss of a $^2H^+$ ion from N-5, which might be available at the reduction site for the protonation of the allylic carbanion (**7**) derived from HMBPP.

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