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Isoprenoid Biosynthesis via the MEP Pathway: *In Vivo* Mössbauer Spectroscopy Identifies a [4Fe-4S]$^{2+}$ Center with Unusual Coordination Sphere in the LytB Protein

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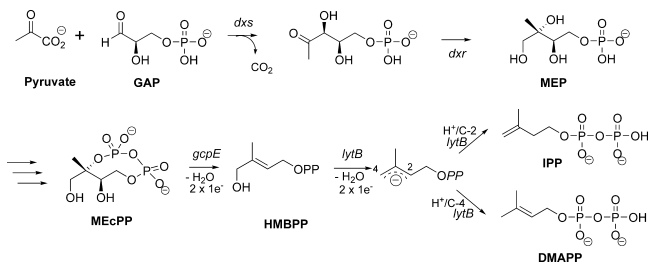
In many bacteria, including *Mycobacterium tuberculosis* responsible for tuberculosis, in the plant chloroplasts and in the malaria parasite *Plasmodium falciparum*, the biosynthesis of isoprenoids occurs according to the methylerythritol phosphate (MEP) pathway, an alternative to the well-known mevalonate pathway.[1] The MEP pathway (Scheme 1) does not exist in humans and is therefore a valuable target for the development of new specific antibacterial and antiparasitic drugs.[2]

The first steps leading from pyruvate and D-glyceraldehyde 3-phosphate (GAP) to methylerythritol 2,4-cyclodiphosphate (MEcPP) are rather well documented.[3,4] The last steps converting MEcPP into IPP and DMAPP represent, however, a bottleneck in the full elucidation of the pathway and are respectively catalyzed by the GcpE and LytB proteins. These two oxygen sensitive iron−sulfur enzymes are known for catalyzing unprecedented reactions (for a review see ref 5).

Scheme 1. Methylerythritol 4-Phosphate Pathway



LytB, also called IspH, the last enzyme of the MEP pathway converts (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) into a mixture of IPP and DMAPP using an iron−sulfur cluster as a prosthetic group. We showed by EPR spectroscopy that LytB from *E. coli* contains a 4Fe-4S cluster after reconstitution of the purified enzyme with $FeCl_3$, Na_2S, and DTT under an inert nitrogen atmosphere.[6] EPR data presented by Rohdich and co-workers indicate that LytB as-isolated contains a [3Fe-4S]$^+$ cluster.[7] The X-ray structures of the LytB enzyme from *Aquifex aeolicus*[8] and very recently from *E. coli*[9] also show [3Fe-4S]$^+$ clusters. Because of these contradictory reports, the exact nature of the iron−sulfur cluster of LytB is still a matter of debate. Here, we report Mössbauer studies performed on the anaerobically purified LytB and of *E. coli* cells overexpressing LytB, which show unequivocally that LytB contains *in vivo* a [4Fe-4S]$^{2+}$ cluster.

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Figure 1A shows the Mössbauer spectrum of the ^{57}Fe-LytB protein obtained at 77 K. Component 1 exhibits 50% of the total spectral area with isomer shift $\delta_1 = 0.42$ mm s^{-1} and quadrupole splitting $\Delta E_{Q1} = 1.21$ mm s^{-1}. These parameters are characteristic of tetrahedrally sulfur-coordinated $Fe^{2.5+}$ centers of mixed-valenced iron pairs ($S_p = {}^9/_2$) with a delocalized excess electron which is typical for [4Fe-4S]$^{2+}$ clusters in iron−sulfur proteins.[10] Component 2 has $\delta_2 = 0.37$ mm s^{-1} and $\Delta E_{Q2} = 0.89$ mm s^{-1} (25% rel. contribution) and is characteristic of a ferric high-spin Fe^{3+} site, whereas component 3 yields $\delta_3 = 0.89$ mm s^{-1} and $\Delta E_{Q3} = 1.97$ mm s^{-1}, which is characteristic of a ferrous high-spin Fe^{2+} site (25% rel. contribution).

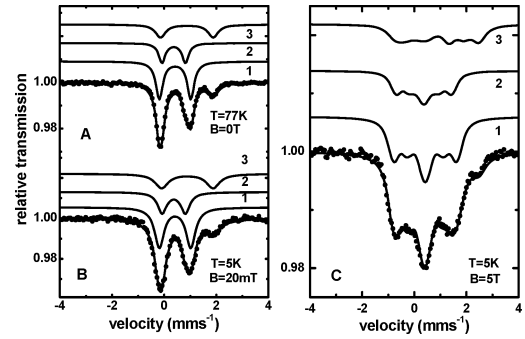

Figure 1. Mössbauer spectra of 400 μM ^{57}Fe-LytB at 77 K (A), at 5 K and a field of 20 mT (B) and 5 T (C) applied perpendicular to the γ-beam. The solid lines represent the result of a best fit analysis with the parameters given in the text and in Table 1S (Supporting Information).

The Mössbauer spectrum at $T = 5$ K (Figure 1B) basically resembles the pattern of the 77 K spectrum and also shows a ratio of 2:1:1 for components 1, 2, and 3. The Mössbauer data suggest that LytB contains an unusual [4Fe-4S]$^{2+}$ center with one pair of the iron sites having a delocalized excess electron ("$Fe^{2.5+}$-$Fe^{2.5+}$") and another pair of valence-trapped iron sites ("Fe^{3+}-Fe^{2+}"). This suggestion is unequivocally confirmed by the high-field Mössbauer spectrum displayed in Figure 1C. This spectrum shows magnetic splittings which are only due to the external magnetic field and therefore proves the diamagnetic ground state of the iron cluster in LytB. Based on the present data we cannot decide which of the three irons with almost identical ligands participates in the $Fe^{2.5+}$−$Fe^{2.5+}$ pair and which in the valence trapped Fe^{2+}−Fe^{3+} pair. The pairs might indeed change continuously in the nonfrozen active enzyme. Conventional [4Fe-4S]$^{2+}$ clusters, as in bacterial ferredoxins, contain four tetrahedrally sulfur-coordinated iron sites which exhibit $\delta = 0.43 \pm 0.02$ mm s^{-1}.[10] Contrary to this, component 3 in Figure 1 represents a high-spin ferrous iron site which

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clearly belongs to a diamagnetic [4Fe-4S] center but, according to its large isomer shift, can not have a tetrahedral sulfur-ligand sphere; most likely it is penta- or hexacoordinated. In fact, examples are known for such a unique ferrous iron site in $[4Fe\text{-}4S]^{2+}$ clusters. In the pyruvate formate-lyase activating enzyme such a Fe^{2+} site exhibits $\delta = 0.72$ mm s^{-1} when adenosylmethionine is coordinated either via its amino and carboxylate groups or via the two ribose hydroxyls.[11] For the 3S3N coordinated iron site of subsite-differentiated model complexes of $[4Fe\text{-}4S]^{2+}$ clusters, isomer shifts in the range 0.80−0.91 mm s^{-1} have been reported.[12] X-ray crystallography[13] and ENDOR studies[14] of substrate bound aconitase show that a unique iron site of its $[4Fe\text{-}4S]^{2+}$ center has a 6-fold coordination sphere with three sulfur and three oxygen atoms arising from a carboxylate and a hydroxyl group of the substrate and a water molecule. Indeed this iron site with a 3S3O coordination exhibits isomer shifts of 0.84−0.89 mm s^{-1} depending on the type of substrate.[15] We thus conclude from analogy to aconitase that LytB contains a $[4Fe\text{-}4S]^{2+}$ cluster with an unusual coordination sphere including 3 S and 3 N/O ligands, but we cannot totally exclude five-coordination. Model complexes with the dihydrobis(1-pyrazolyl) borate ligand exhibit also comparable Mössbauer parameters.[12]

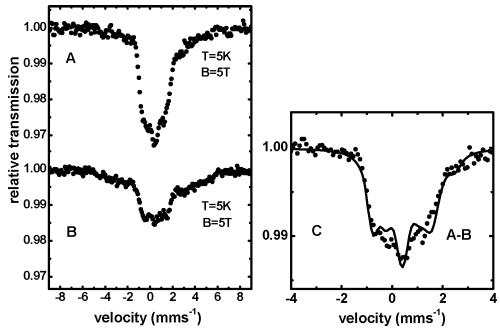

Figure 2. High-field Mössbauer spectra of LytB overexpressing *E. coli* cells (A) and of cells which do not overexpress LytB (B). The difference spectrum is shown in (C). The solid line represents a simulation with the parameters of pure LytB (Table 1S) as obtained from the analysis of the spectra displayed in Figure 1. The small deviation from the simulation might be caused by a different Fe(III) content, probably ferritin, within the two samples.

The specific activity of ^{57}Fe-LytB was 700 nmol min^{-1} mg^{-1}. That is similar to the activity of the LytB enzyme of *E. coli* for which a $[3Fe\text{-}4S]^{+}$ cluster was reported on the basis of EPR studies.[7] As both enzymes display the same activity, they might be identical and harbor the same Fe/S cluster. As in this latter work[7] neither the enzyme sample was reduced nor was the EPR signal quantified; a diamagnetic $[4Fe\text{-}4S]^{2+}$ cluster could not be detected by EPR even if it were present.

To check whether the iron center of LytB is affected by the purification procedure, high-field Mössbauer spectra of *E. coli* cells overexpressing LytB have been recorded (Figure 2A). After subtracting the spectrum of the WT strain cells grown under the same conditions as the cells overexpressing LytB (Figure 2B) a spectrum was obtained, which can be reproduced with the Mössbauer parameters of the purified ^{57}Fe-LytB (Figure 2C). This experiment confirms that LytB harbors a $[4Fe\text{-}4S]^{2+}$ center *in vivo* with one iron being in an unusual 3S-3N/O coordination sphere. It should be mentioned that, during the review process of this work, Mössbauer data have been published in this journal which resemble the low field spectra presented here. However, due to the lack of measurements in high magnetic fields a full assignment of the components has not been possible.[16]

The LytB family has only three conserved cysteines (C12, C96, C197) which have been shown to be essential for the binding of iron−sulfur center to the apoprotein.[7] The binding of the substrate to the iron sulfur center implies that at least one ligand of its noncysteine coordinated iron must be labile to be replaced by the substrate. In fact, the addition of HMBPP to ^{57}Fe-LytB decreases the isomer shift of the unique fourth iron site from $\delta_3 = 0.89$ mm s^{-1} to $\delta_{3'} = 0.53$ mm s^{-1} (Figure 4S). This unequivocally shows that HMBPP coordinates to the unique fourth iron site of the $[4Fe\text{-}4S]^{2+}$ cluster of LytB. From HMBPP docking studies based on the structure of the LytB protein of *Aquifex aeolicus* in its [3Fe-4S] form, it was observed that the hydroxyl group of the substrate is located at a distance of 1.9 Å to the fourth iron.[8] It was also proposed that E126 is the proton donor required for the protonation of the allylic anion intermediate.[8] Docking the fourth iron to the [3Fe-4S] cluster of the structure from *E. coli*[9] yields as possible ligands two water molecules and the conserved T167 (Figure 5S). Replacement of T167 by a valine or an asparagine leads to insoluble protein, whereas its mutation to serine leads to a fully active enzyme.[9] We suggest that T167 is a structural element that is important for the folding of the enzyme by coordinating the unique fourth iron via its hydroxyl group. This can also be achieved in the fully active T167S mutant.

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Note Added after ASAP Publication. The unit for specific activity of ^{57}Fe-LytB was corrected in the version published September 16, 2009.

Supporting Information Available: Experimental procedures; UV/visible spectrum of ^{57}Fe-LytB; Mössbauer parameters and additional Mössbauer spectra of *E. coli* cells and of ^{57}Fe-LytB plus HMBPP. Structural model of the prosthetic group of LytB. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bloch, K. *Steroids* **1992**, *57*, 378–383.
(2) Rohmer, M.; Grosdemange-Billiard, C.; Seemann, M.; Tritsch, D. *Curr. Opin. Invest. Drugs* **2004**, *5*, 154–162.
(3) Rohmer, M. *Nat. Prod. Rep.* **1999**, *16*, 565–574.
(4) Eisenreich, W.; Rohdich, F.; Bacher, A. *Trends Plant Sci.* **2001**, *6*, 78–84.
(5) Seemann, M.; Rohmer, M. *C. R. Chimie* **2007**, *10*, 748–755.
(6) Wolff, M.; Seemann, M.; Tse Sum Bui, B.; Frapart, Y.; Tritsch, D.; Garcia-Estrabot, A.; Rodriguez-Concepción, M.; Boronat, A.; Marquet, A.; Rohmer, M. *FEBS Lett.* **2003**, *1*, 115–120.
(7) 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.
(8) Rekittke, I.; Wiesner, J.; Röhrich, R.; Demmer, U.; Warkentin, E.; Xu, W.; Troschke, K.; Hintz, M.; No, J. H.; Duin, E. C.; Oldfield, E.; Jomaa, H.; Ermler, U. *J. Am. Chem. Soc.* **2008**, *130*, 17206–17207.
(9) Gräwert, T.; Rohdich, F.; Span, I.; Bacher, A.; Eisenreich, W.; Eppinger, J.; Groll, M. *Angew. Chem., Int. Ed.* **2009**, *48*, 5756–5759.
(10) (a) Beinert, H.; Holm, R. H.; Münck, E. *Science* **1997**, *277*, 653–659. (b) Schünemann, V.; Winkler, H. *Rep. Prog. Phys.* **2000**, *63*, 263–353.
(11) Krebs, C.; Broderick, W. E.; Henshaw, T. F.; Broderick, J. B.; Huynh, B. H. *J. Am. Chem. Soc.* **2002**, *124*, 912–913.
(12) Ciurli, S.; Carrie, M.; Weigel, J. A.; Carney, M. J.; Stack, T. D. P.; Papaefthymiou, G. C.; Holm, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 2654–2664.
(13) Beinert, H. J. *Biol. Inorg. Chem.* **2000**, *5*, 2–15.
(14) Werst, M. M.; Kennedy, M. C.; Houseman, A. L. P.; Beinert, H.; Hoffman, B. M. *Biochemistry* **1990**, *29*, 10533–10540.
(15) Kent, T. A.; Emptage, M. H.; Merkle, H.; Kennedy, M. C.; Beinert, H.; Münck, E. *J. Biol. Chem.* **1985**, *260*, 6871–1881.
(16) Xiao, Y.; Chu, L.; Sanakis, Y.; Liu, P. *J. Am. Chem. Soc.* **2009**, *131*, 9931–9933.

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