Enzyme Inhibitors

DOI: 10.1002/anie.201104562

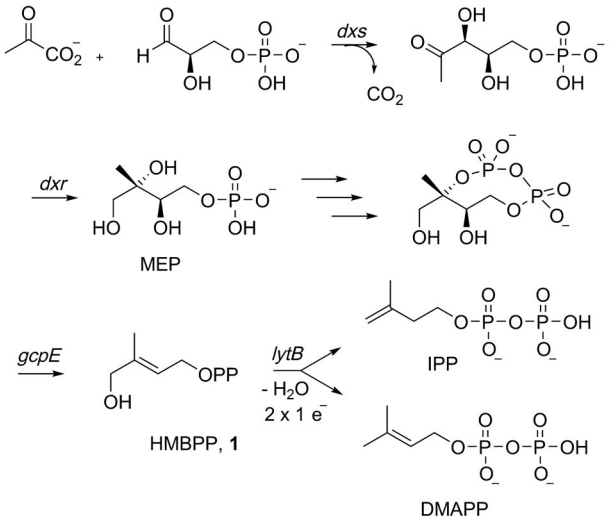
Biosynthesis of Isoprene Units: Mössbauer Spectroscopy of Substrate and Inhibitor Binding to the [4Fe-4S] Cluster of the LytB/IspH Enzyme**

*Annegret Ahrens-Botzong, Karnjapan Janthawornpong, Juliusz A. Wolny, Erasmienne Ngouamegne Tambou, Michel Rohmer, Sergiy Krasutsky, C. Dale Poulter, Volker Schünemann, and Myriam Seemann**

The biosynthesis of isoprenoids in many bacteria and in the malaria parasite *Plasmodium falciparum* occurs according to the methylerythritol phosphate (MEP) pathway,[1] an alternative to the mevalonate pathway.[2] The MEP pathway is a valuable target for the development of new antimicrobial agents, as it is essential for microorganisms and absent in humans.[3] In the last step of this biosynthetic route (Scheme 1), 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP, **1**) is converted into a mixture of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are both precursors of isoprenoids. This reaction is catalyzed by a peculiar [4Fe-4S] center of the LytB/IspH protein.[4]

LytB has a molecular weight of 72 kDa and is a homodimer. It contains a highly O_2-sensitive $[4Fe\text{-}4S]^{2+}$ cluster, which is diamagnetic and therefore shows no signal in the electron paramagnetic resonance (EPR) spectrum.[5] Field-dependent Mössbauer spectroscopy indicated that the four iron centers in the $[4Fe\text{-}4S]^{2+}$ cluster of LytB in its substrate-free form are not equivalent as in conventional ferredoxin-type $[4Fe\text{-}4S]^{2+}$ clusters.[6] Instead, one of the iron sites has an isomer shift ($\delta = 0.89\ mms^{-1}$) that is identical within experimental error to that of an unusual fourth iron site in the citrate-bound form of aconitase.[4] Accordingly, it was concluded that the coordination sphere of this special iron site comprises three inorganic sulfur atoms from the cluster and additional three or two nonsulfur ligands (O or N) in a binding motive similar to those of substrate-bound aconitase.[7,8] Two X-ray structures of substrate-free LytB from *Aquifex aeolicus*[9] and *Escherichia coli*[10] were reported for the $[3Fe\text{-}4S]^{+}$ form. On the basis of our spectroscopic results, the structure of LytB from *E. coli* has been refined from a structure with a [3Fe-4S] cluster[10] to a structure with a [4Fe-4S] cluster in the presence of HMBPP.[11] However, the crystal structure of the substrate-free LytB in its $[4Fe\text{-}4S]^{2+}$ state as well as structures with potential inhibitors have not been reported to date. Instead, EPR and electron nuclear double-resonance (ENDOR) spectroscopy studies on the dithionite-reduced enzyme showed that alkynes bind at or very close to the unique fourth iron center in the one-electron-reduced $[4Fe\text{-}4S]^{+}$ cluster, and thus alkynes could be quite potent inhibitors of this enzyme.[12] Very recently pyridine diphosphates have been reported to inhibit LytB. Pulsed-EPR techniques using hyperfine sublevel correlation (HYSCORE) spectroscopy showed that the pyridine diphosphates directly coordinate to the fourth iron site of the reduced $[4Fe\text{-}4S]^{+}$ cluster of LytB.[13]

Note that the studies mentioned above have been performed on dithionite-reduced enzyme, a procedure that



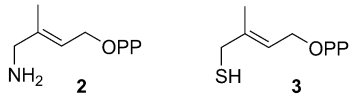
Scheme 1. Methylerythritol phosphate (MEP) pathway; *dxs*, *dxr*, and *gcpE* are the genes coding for the enzymes that catalyze the corresponding reactions.

[*] Dr. K. Janthawornpong, Dr. E. N. Tambou, Prof. M. Rohmer, Dr. M. Seemann
Université de Strasbourg/CNRS, UMR 7177, Institut Le Bel
4 rue Blaise Pascal, CS 90032, 67081 Strasbourg Cedex (France)
E-mail: mseemann@unistra.fr
A. Ahrens-Botzong, Dr. J. A. Wolny, Prof. V. Schünemann
Fachbereich Physik, University of Kaiserslautern
Erwin-Schrödinger-Strasse 46, 67653 Kaiserslautern (Germany)
Dr. S. Krasutsky, Prof. C. D. Poulter
Department of Chemistry, University of Utah
315 South 1400 East RM 2020, Salt Lake City, Utah 84112 (USA)

[**] We are grateful to Prof. A. Boronat (University of Barcelona, Spain) and his group for providing us with the *E. coli* strain overexpressing LytB. We thank M. Parisse for technical assistance. This work was supported by the "Agence Nationale de la Recherche" (ANR-05-JCJC-0177-01) to M.S., by the Thai government to K.J., by NIH grant GM25521 to C.D.P., and by the German Federal Ministry of Education and Research (05K10UKA) and NANOKAT to V.S.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201104562.

might alter the properties of the unique fourth iron site of the LytB iron–sulfur cluster. Herein we present a field-dependent Mössbauer spectroscopy study on the EPR-silent $[4Fe\text{-}4S]^{2+}$ cluster of the substrate-bound form of LytB as well as its interaction with two new HMBPP analogues **2** and **3** (Scheme 2). The latter were both anticipated to be recognized by LytB and to tightly bind to the [4Fe-4S] cluster with their amino or thiol functional groups, respectively.



Scheme 2. Structures of the amino (**2**) and thiol (**3**) analogues of HMBPP.

The inhibition studies of *E. coli* LytB by **2** and **3**, performed under the same conditions as those described for the alkynes[12] and pyridine diphosphates,[13] led to IC_{50} values of 0.15 μM for the amino analogue **2** and 0.21 μM for the thiol analogue **3** (See Figures 2S and 4S in the Supporting Information). These values are even better than that reported for 3-butynyl diphosphate, the best inhibitor published to date ($IC_{50} = 0.45$ μM for *Aquifex aeolicus* LytB)[12] and underline the promising inhibition potential of **2** and **3**.

Figure 1a shows Mössbauer spectra of HMBPP-bound LytB taken at $T = 77$ K in zero field as well as at $T = 5$ K in a high external field of $B = 5$ T. The spectra have been analyzed by means of three components (see Table 1S in the Supporting Information). Component 1 has an isomer shift of $\delta_1 = 0.42\ mm\,s^{-1}$ and a quadrupole splitting of $\Delta E_{Q1} = 1.33\ mm\,s^{-1}$. These parameters are characteristic of tetrahedrally sulfur-coordinated $Fe^{2.5+}$ centers of mixed-valence iron pairs with a delocalized excess electron typical for $[4Fe\text{-}4S]^{2+}$ clusters in iron–sulfur proteins. Component 2 shows $\delta_2 = 0.38\ mm\,s^{-1}$ and $\Delta E_{Q2} = 0.92\ mm\,s^{-1}$. The low value of the isomer shift is indicative of an iron site with high-spin Fe^{3+} character. Component 3 on the contrary exhibits $\delta_3 = 0.64\ mm\,s^{-1}$ and $\Delta E_{Q3} = 1.22\ mm\,s^{-1}$. The latter parameters are characteristic of a high-spin Fe^{2+} component. The relative contribution of the three components to the total area of the spectrum is exactly 2:1:1. The Mössbauer spectrum of substrate-bound LytB taken at 5 K and an external magnetic field of 5 T displays a magnetic hyperfine splitting which is due only to the external field. This finding confirms the diamagnetic ground state of the $[4Fe\text{-}4S]^{2+}$ cluster in HMBPP-bound LytB.

For comparison the Mössbauer spectra of substrate-free LytB obtained under the same experimental conditions are shown in Figure 1b.[4] The isomer shift of component 3, which has been shown to originate from the unusual fourth iron site of the $[4Fe\text{-}4S]^{2+}$ cluster in substrate-free LytB,[4] changes significantly from 0.89 to $0.64\ mm\,s^{-1}$ after addition of the substrate HMBPP. This means that HMBPP (**1**) is bound to the unique fourth iron site of the iron–sulfur cluster with its OH group, as also indicated by the crystal structure.[11] The other two N or O ligands of substrate-free LytB have dissociated upon substrate binding.

Figure 1c shows the Mössbauer spectra of substrate-free LytB after addition of the amino analogue **2**. Again, the spectra have been analyzed with three components with a spectral ratio of 2:1:1. Component 1 exhibits $\delta_1 = 0.45\ mm\,s^{-1}$ and $\Delta E_{Q1} = 1.18\ mm\,s^{-1}$. This component is related to a tetrahedrally sulfur-coordinated $Fe^{2.5+}$ pair, and its Mössbauer parameters remain almost unchanged upon binding of **2** in comparison to the substrate-free and substrate-bound forms (Table 1S in the Supporting Information). Component 2 shows $\delta_2 = 0.28\ mm\,s^{-1}$ and $\Delta E_{Q2} = 1.14\ mm\,s^{-1}$ and is related to a tetrahedrally sulfur-coordinated Fe^{3+} site. Component 3 has $\delta_3 = 0.61\ mm\,s^{-1}$ and $\Delta E_{Q3} = 1.08\ mm\,s^{-1}$. The isomer shift of the unique fourth high-spin Fe^{2+} site is much smaller now than in substrate-free LytB but is comparable to the case of the HMBPP-LytB complex. Obviously also the amino substrate analogue coordinates with its amino function

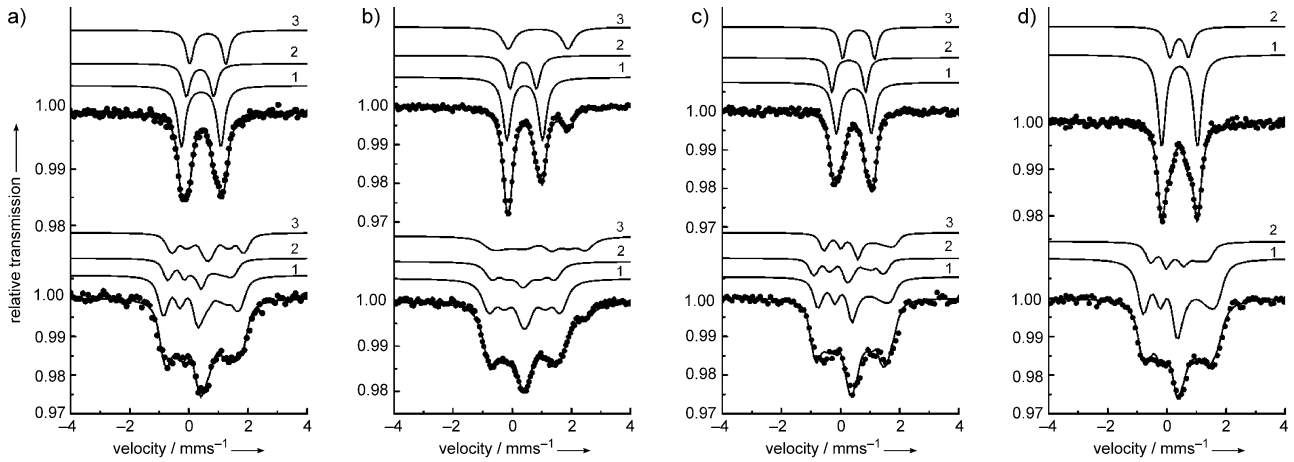


Figure 1. Mössbauer spectra taken at 77 K, $B = 0$ T (upper traces) and at 5 K with an external magnetic field of 5 T applied perpendicular to the γ-beam (lower traces) of a) HMBPP (**1**)-bound LytB, b) substrate-free LytB, c) the amino analogue **2** LytB complex, and d) the thiol analogue **3** LytB complex. The solid lines represent the result of a best-fit analysis assuming a diamagnetic ground state of the $[4Fe\text{-}4S]^{2+}$ cluster with the parameters given in Table 1S in the Supporting Information. Traces 1, 2 (a–d), and 3 (a–c) represent component contributions (see text for details).

directly to the unique fourth Fe^{2+} site of the $[4Fe\text{-}4S]^{2+}$ cluster of LytB.

Figure 1 d shows the Mössbauer spectra of the thiol analogue **3** added to substrate-free LytB. Now the spectra can be analyzed by means of only two components. Component 1 has $\delta_1 = 0.43\ mm\,s^{-1}$, and $\Delta E_{Q2} = 1.21\ mm\,s^{-1}$ and contributes with 75% to the total spectral area. Component 2 (25% relative contribution) shows $\delta_2 = 0.42\ mm\,s^{-1}$ and $\Delta E_{Q2} = 0.63\ mm\,s^{-1}$. The spectral simulation of the high-field Mössbauer spectrum displayed in Figure 1 d confirms the diamagnetic ground state of the $[4Fe\text{-}4S]^{2+}$ cluster for the thiol- analogue-bound form of LytB as well.

The diamagnetic ground state as well as the fact that all four iron sites exhibit $\delta \approx 0.45\ mm\,s^{-1}$ indicates that after binding of **3** all four iron centers are in a $Fe^{2.5+}$ state, as in a conventional $[4Fe\text{-}4S]^{2+}$-cluster-containing protein such as oxidized ferredoxin.[6] However, the fourth special iron site shows only half the value of the quadrupole splitting of the other three $Fe^{2.5+}$ sites. This finding can be explained by this site's slightly different ligand geometry. Nevertheless, it is clear from the experimental data displayed in Figure 1 d that the addition of the thiol analogue **3** restores electron delocalization of the previously localized mixed-valence iron pair in the $[4Fe\text{-}4S]^{2+}$ cluster of LytB.

To relate our Mössbauer-spectroscopic study to structural models of the amino and thiol analogues bound to LytB, we have performed quantum chemical density functional theory (DFT) calculations based on the published X-ray structure[11] of HMBPP-bound LytB. The resulting structures of the substrate/inhibitor–cluster complexes obtained after total-energy minimization with the Gaussian ONIOM method[14] are shown in Figure 2. On the basis of these structures and the experimentally determined diamagnetic ground state of the $[4Fe\text{-}4S]^{2+}$ cluster, the Mössbauer parameters (Table 2S in the Supporting Information) have been obtained by preliminary DFT calculations with the software package ORCA[15] using the closed-shell approach. The comparison of the experimentally determined Mössbauer parameters (Table 1S in the Supporting Information) and the calculated parameters (Table 2S in the Supporting Information) shows a reasonable agreement. This fact gives confidence that the structures of the amino inhibitor **2** and the thiol inhibitor **3** displayed in Figure 2 do indeed represent the structures of the substrate/inhibitor bound to LytB, at least in solution in vitro, but most probably also in vivo.

In conclusion, we report the first field-dependent Mössbauer spectroscopic study of the $[4Fe\text{-}4S]^{2+}$ cluster of LytB binding HMBPP and two new inhibitors. Indeed, the unique fourth iron site of the iron–sulfur cluster coordinates to the hydroxy group of HMBPP and to the amino and thiol moieties in **2** and **3**, respectively. These results unequivocally confirm that the first step in the LytB-catalyzed reaction involves the binding of the hydroxy group of the substrate to the apical iron site of the oxidized [4Fe-4S] cluster.[4,16–18] This feature has already led to the design of two promising LytB inhibitors, for which the complete characterization is under investigation.

Experimental Section

For the enzyme preparation and the determination of the IC_{50} values, see the Supporting Information.

A solution of ^{57}Fe-LytB (393 μM) and HMBPP (4 mM) was prepared in a glove box, transferred to the Mössbauer sample holder, and frozen in liquid nitrogen until the measurement. The samples of ^{57}Fe-LytB (393 μM) alone or in presence of inhibitor **2** or **3** (4 mM) were prepared in the same way.

The samples were measured using a conventional spectrometer in the constant-acceleration mode. Isomer shifts are given relative to α-Fe at room temperature. Field-dependent Mössbauer spectra were recorded with a spectrometer from WissEL GmbH coupled to a closed-cycle cryostat from CRYO Industries of America Inc. equipped with a superconducting magnet. The measurements at 77 K were performed in a conventional bath cryostat (Oxford Instruments). Isomer shifts are given relative to α-Fe at room temperature. The analysis of the spectra has been performed with the Software package Vinda[19] assuming Lorentzian line shape for the measurements at low fields and using the spin-Hamiltonian formalism[6] for the simulation of the spectra taken at 5 K and 5 T.

The structure of the whole protein with the corresponding substrates was calculated by a combined quantum-mechanics (QM) and molecular-mechanics (MM) approach using the ONIOM[14]

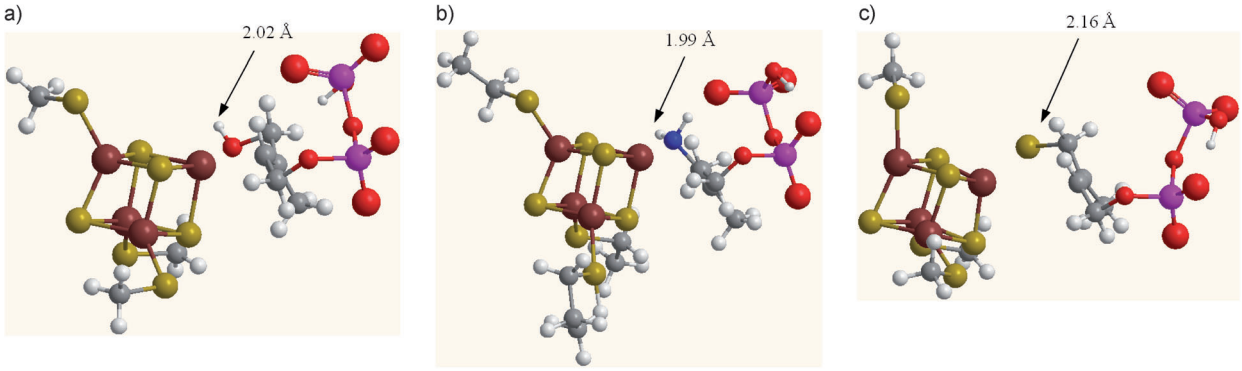


Figure 2. a) Structure of the HMBPP (**1**)-bound cluster in LytB taken from the pdb data file 3KE8.pdb.[11] b) Structure of the amino analogue **2**-bound $[4Fe\text{-}4S]^{2+}$ cluster in LytB as obtained by energy minimization of the whole LytB protein using the ONIOMmethod. c) Structure of the thiol analogue **3**-bound cluster in LytB obtained with the same procedure as used in (b). Fe···OH, Fe···NH_2, and Fe···S distances are given. H white, C gray, Fe brown, S green, N blue, P purple, O red. Corresponding structure files in pdb format are given in the Supporting Information.

option of Gaussian09.[20] Mössbauer parameters were calculated for the high-layer (DFT) fragment of the optimized geometries using the DFT software package ORCA.[15] For details, see the Supporting Information.

Received: July 1, 2011
Published online: October 25, 2011

Keywords: inhibitors · iron · metalloenzymes · Mössbauer spectroscopy · sulfur

[1] a) M. Rohmer, *Nat. Prod. Rep.* **1999**, *16*, 565 – 574; b) W. Eisenreich, F. Rohdich, A. Bacher, *Trends Plant Sci.* **2001**, *6*, 78 – 84.
[2] K. Bloch, *Steroids* **1992**, *57*, 378 – 383.
[3] M. Rohmer, C. Grosdemange-Billiard, M. Seemann, D. Tritsch, *Curr. Opin. Invest. Drugs* **2004**, *5*, 154 – 162.
[4] M. Seemann et al., *J. Am. Chem. Soc.* **2009**, *131*, 13184 – 13185, see the Supporting Information.
[5] M. Wolff, M. Seemann, B. Tse Sum Bui, Y. Frapart, D. Tritsch, A. Garcia-Estrabot, M. Rodriguez-Concepción, A. Boronat, A. Marquet, M. Rohmer, *FEBS Lett.* **2003**, *541*, 115 – 120.
[6] A. X. Trautwein, E. Bill, E. L. Boominar, H. Winkler, *Struct. Bonding (Berlin)* **1991**, *78*, 1 – 95.
[7] S. Ciurli, M. Carrie, J. A. Weigel, M. J. Carney, T. D. P. Stack, G. C. Papaefthymiou, R. H. Holm, *J. Am. Chem. Soc.* **1990**, *112*, 2654 – 2664.
[8] H. Beinert, *J. Biol. Inorg. Chem.* **2000**, *5*, 2 – 15.
[9] I. Rekittke et al., *J. Am. Chem. Soc.* **2008**, *130*, 17206 – 17207, see the Supporting Information.
[10] T. Gräwert, F. Rohdich, I. Span, A. Bacher, W. Eisenreich, J. Eppinger, M. Groll, *Angew. Chem.* **2009**, *121*, 5867 – 5870; *Angew. Chem. Int. Ed.* **2009**, *48*, 5756 – 5759.
[11] T. Gräwert, I. Span, W. Eisenreich, F. Rohdich, J. Eppinger, A. Bacher, M. Groll, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1077 – 1081.
[12] K. Wang, W. Wang, J. H. No, Y. Zhang, Y. Zhang, E. Oldfield, *J. Am. Chem. Soc.* **2010**, *132*, 6719 – 6727.
[13] W. Wang, J. Li, K. Wang, T. I. Smirnova, E. Oldfield, *J. Am. Chem. Soc.* **2011**, *133*, 6525 – 6528.
[14] S. Dapprich, I. Komáromi, K. S. Byun, K. Morokuma, M. J. Frisch, *J. Mol. Struct. (Theochem)* **1999**, *461 – 462*, 1 – 21.
[15] Orca- an ab initio density functional and semiempirical program package, Vers. 2.6. Rev. 35, 28.2.2008.
[16] T. Gräwert, I. Span, A. Bacher, M. Groll, *Angew. Chem.* **2010**, *122*, 8984 – 8991; *Angew. Chem. Int. Ed.* **2010**, *49*, 8802 – 8809.
[17] W. Wang, K. Wang, Y. L. Liu, J. H. No, J. Li, M. J. Nilges, E. Oldfield, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 4522 – 4527.
[18] Y. Xiao, Z. K. Zhao, P. Liu, *J. Am. Chem. Soc.* **2008**, *130*, 2164 – 2165.
[19] http://whome.phys.au.dk/~hpg/vinda.htm.
[20] Gaussian 09 (Revision A.02): M. J. Frisch et al., **2009** see the Supporting Information.

