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Biosynthesis of isoprene units. Mössbauer spectroscopy proofs on substrate- and inhibitor- binding to the [4Fe-4S] cluster of the LytB/IspH enzyme

Annegret Ahrens-Botzong,

Fachbereich Physik, University of Kaiserslautern, Erwin-Schrödinger-Strasse 46, D-67653 Kaiserslautern (Germany)

Karnjapan Janthawornpong,

Université de Strasbourg, UMR 7177 CNRS, Institut Le Bel, 4 rue Blaise Pascal, CS 90032, 67081 Strasbourg Cedex (France)

Juliusz A. Wolny,

Fachbereich Physik, University of Kaiserslautern, Erwin-Schrödinger-Strasse 46, D-67653 Kaiserslautern (Germany)

Erasmienne Ngouamegne Tambou,

Université de Strasbourg, UMR 7177 CNRS, Institut Le Bel, 4 rue Blaise Pascal, CS 90032, 67081 Strasbourg Cedex (France)

Michel Rohmer,

Université de Strasbourg, UMR 7177 CNRS, Institut Le Bel, 4 rue Blaise Pascal, CS 90032, 67081 Strasbourg Cedex (France)

Sergiy Krasutsky,

Department of Chemistry, University of Utah, 315 South 1400 East RM 2020, Salt Lake City, Utah 84112 (USA)

C. Dale Poulter,

Department of Chemistry, University of Utah, 315 South 1400 East RM 2020, Salt Lake City, Utah 84112 (USA)

Volker Schünemann, and

Fachbereich Physik, University of Kaiserslautern, Erwin-Schrödinger-Strasse 46, D-67653 Kaiserslautern (Germany)

Myriam Seemann

Université de Strasbourg, UMR 7177 CNRS, Institut Le Bel, 4 rue Blaise Pascal, CS 90032, 67081 Strasbourg Cedex (France) Fax: (+33) 3 68 85 13 45 mseemann@unistra.fr

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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, HMBPP **1** is converted into a mixture of IPP and DMAPP, which are both precursors of isoprenoids (Scheme 1). 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 O₂-sensitive [4Fe-4S]²⁺-cluster, which is diamagnetic and therefore EPR silent.^[5] Field-dependent Mössbauer spectroscopy indicated that the four iron centers in the [4Fe-4S]²⁺-cluster of LytB in its substrate-free form are not equivalent as it is the case in conventional ferredoxin-type [4Fe-4S]²⁺-clusters.^[6] Instead, one of the iron sites has an isomer shift ($\delta = 0.89 \text{ mms}^{-1}$) which 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 sulfurs from the cluster and additional 3 or 2 non-sulfur ligands (O and/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-4S]⁺ form. Based on 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 presence of HMBPP.^[11] The crystal structure of the substrate-free LytB in its [4Fe-4S]²⁺ state as well as structures with potential inhibitors have, however, not been reported yet. Instead electron paramagnetic resonance (EPR) and electron nuclear double-resonance (ENDOR) studies on the dithionite reduced enzyme showed that alkynes bound at or very close to the unique fourth iron in the one electron reduced [4Fe-4S]⁺-cluster and thus 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-4S]⁺-cluster of LytB.^[13]

One has to point out that the studies mentioned above have been performed on dithionite-reduced enzyme, a procedure that might alter the properties of the unique fourth iron site of the LytB iron sulfur cluster. Here we present a field dependent Mössbauer spectroscopy study on the EPR silent [4Fe-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 tightly bind to the [4Fe-4S]-cluster *via* their amino or thiol functional groups respectively. The inhibition studies of *E. coli* LytB by **2** and **3**, using the same conditions as those described for the alkynes^[12] and pyridine diphosphates^[13] led for the amino analogue **2** and the thiol analogue **3** to IC₅₀ values of 0.15 μM and 0.21 μM respectively (See supporting information, Figure 2S and Figure 4S). These values are even better as that reported for 3-butynyl diphosphate, the best inhibitor published to date (IC₅₀ = 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, Supporting Information). Component 1 has an isomer shift of $\delta_1 = 0.42 \text{ mms}^{-1}$ and a quadrupole splitting of $\Delta E_{Q1} = 1.33 \text{ mms}^{-1}$. 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-4S]²⁺-clusters in iron-sulfur proteins. Component 2 shows $\delta_2 = 0.38 \text{ mms}^{-1}$ and $\Delta E_{Q2} = 0.92 \text{ mms}^{-1}$. The low value of the isomer shift is indicative for an iron site with high-spin Fe³⁺ character. Component 3 on the contrary exhibits $\delta_3 = 0.64 \text{ mms}^{-1}$ and $\Delta E_{Q3} = 1.22 \text{ mms}^{-1}$. The latter parameters are characteristic of a ferrous high-spin Fe²⁺ component. The relative contribution of the four 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 only due to the external field. This confirms the diamagnetic ground state of the $[4\text{Fe-4S}]^{2+}$ -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]

From the inspection of Figure 1b it is clear that the isomer shift of component 3 which has been shown to originate from the unusual fourth iron site of the $[4\text{Fe-4S}]^{2+}$ -cluster in substrate-free LytB^[4] changes significantly from 0.89 mms^{-1} to 0.64 mms^{-1} after addition of the substrate HMBPP. This means that HMBPP **1** is bound to the unique fourth iron site of iron sulfur cluster via its OH-group as it is 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-substrate analogue **2**. Again the spectra have been analyzed with three components with a spectral ratio of 2:1:1. Component 1 exhibits $\delta_1 = 0.45 \text{ mms}^{-1}$ and $\Delta E_{Q1} = 1.18 \text{ mms}^{-1}$. This component is related to a tetrahedrally sulfur coordinated $\text{Fe}^{2.5+}$ pair and its Mössbauer parameters remain almost unchanged upon binding of **2** in comparison to the substrate-free and substrate bound form (Table 1S, Supporting Information).

Component 2 shows $\delta_2 = 0.28 \text{ mms}^{-1}$ and $\Delta E_{Q2} = 1.14 \text{ mms}^{-1}$ and is due to a tetrahedrally sulfur coordinated ferric Fe^{3+} site. Component 3 has $\delta_3 = 0.61 \text{ mms}^{-1}$ and $\Delta E_{Q3} = 1.08 \text{ mms}^{-1}$. The isomer shift of the unique fourth Fe^{2+} high-spin site is much smaller now than in substrate-free LytB, but comparable to the case of the HMBPP-LytB complex. Obviously also the amino-substrate analogue coordinates with its amino function directly to the unique 4th Fe^{2+} site of the $[4\text{Fe-4S}]^{2+}$ -cluster of LytB.

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

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

In order to relate our Mössbauer spectroscopic study to structural models of the amino- and thiol-substrate analogues bound to LytB we have performed quantum chemical density functional theory (DFT) calculations based on the published X-ray^[11] structure 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. Based on these structures and the experimentally determined diamagnetic ground state of the $[4\text{Fe-4S}]^{2+}$ -cluster the Mössbauer parameters displayed in the Supporting Information (Table 2S) have been obtained via preliminary DFT calculations with the software package ORCA^[15] using the closed shell approach. The comparison of the experimentally determined Mössbauer parameters (Table 1S) and the calculated parameters (Table 2S)

shows a reasonable agreement. This gives confidence fact, that the structures of the amino-inhibitor **2** and that of the thiol-inhibitor **3** displayed in Figure 2 do indeed represent the structures of the substrate/inhibitor bound to LytB at least *in vitro* solution, but most probable also *in vivo*.

In conclusion, we report the first field-dependent Mössbauer spectroscopic study of the $[4\text{Fe-4S}]^{2+}$ cluster of LytB binding HMBPP and two new inhibitors. Indeed, the unique fourth iron site of the iron sulfur cluster coordinates to the hydroxyl 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 hydroxyl group of the substrate to the apical iron site of the oxidized $[4\text{Fe-4S}]$ cluster. ^[4,16-18] This feature already led to the conception 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 Supporting Information.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.
Methylerythritol phosphate (MEP) pathway

Scheme 2.

Structures of the amino **2** and thiol **3** analogue of HMBPP

Figure 1. Mössbauer-spectra of HMBPP **1** bound LytB taken at 77 K and at 5 K with an external magnetic field of 5 T applied perpendicular to the γ -beam (a) and Mössbauer-spectra of substrate-free LytB (b), of the amino-substrate analogue **2** LytB complex (c) and of the thiol-substrate analogue **3** LytB complex (d) (experimental conditions see (a)). The solid lines represent the result of a best fit analysis assuming a diamagnetic ground state of the [4Fe-4S]²⁺-cluster with the parameters given in Table 1S, Supporting Information.

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-substrate analogue **2** bound [4Fe-4S]²⁺-cluster in LytB as obtained by energy minimization of the whole LytB protein using the ONIOM-Method. c) Structure of the thiol-substrate analogue **3** bound cluster in LytB obtained with the same procedure as used in b). Corresponding structure files in pdb format are given in the Supporting Information.