FEBS Letters 587 (2013) 3968–3972

FEBS Letters

journal homepage: www.FEBSLetters.org

Structure of the (*E*)-4-hydroxy-3-methyl-but-2-enyl-diphosphate reductase from *Plasmodium falciparum*

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ARTICLE INFO

Article history:
Received 6 September 2013
Revised 15 October 2013
Accepted 20 October 2013
Available online 1 November 2013

Edited by Stuart Ferguson

Keywords:
Isoprenoid biosynthesis
LytB
X-ray structure
Drug design
Plasmodium falciparum

ABSTRACT

Terpenoid precursor biosynthesis occurs in human and many pathogenic organisms via the mevalonate and 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathways, respectively. We determined the X-ray structure of the Fe/S containing (*E*)-4-hydroxy-3-methyl-but-2-enyl-diphosphate reductase (LytB) of the pathogenic protozoa *Plasmodium falciparum* which catalyzes the terminal step of the MEP pathway. The cloverleaf fold and the active site of *P. falciparum* LytB corresponds to those of the *Aquifex aeolicus* and *Escherichia coli* enzymes. Its distinct electron donor [2Fe–2S] ferredoxin was modeled to its binding site by docking calculations. The presented structural data provide a platform for a rational search of anti-malarian drugs.
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1. Introduction

Malaria is considered as one of the world's leading causes of morbidity and mortality with annually over 500 million symptomatic infections and 1 million lethal cases [1]. Infections to humans are caused by parasitic protozoa of the genus *Plasmodium* that is subdivided into the four pathogenic species *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. *P. falciparum*, the causative agent of malignant tertian malaria (malaria tropica), is the most dangerous among them and responsible for the majority of the lethal cases [1]. As *P. falciparum* increasingly developes resistances against commonly used drugs for therapeutic and prophylactic purposes [2] identification of novel targets for finding anti-malarial agents is of prime importance.

Terpenoids such as dolichols, quinones, carotenoids, vitamins and sterols, are involved in vital metabolic processes in all kingdoms of life. Despite their great diversity with over 35 000 distinct compounds, all terpenoids are derived from the two 5-carbon isoprene precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Interestingly, nature has evolved two distinct pathways for their biosynthesis: the mevalonate and the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathways. Animals, fungi, archaea and some bacteria use the well-known mevalonate pathway, the vast majority of bacteria and some parasitic protozoa of the phylum Apicomplexa, the MEP pathway [3–5]. Since the MEP pathway is not used by humans, it is considered as an attractive target for the development of new antimicrobial agents, in particular, drugs against the parasitic protozoa *Plasmodium*, responsible for the malaria disease [6–9]. Enzymes of the MEP pathways have been thoroughly explored in the last 20 years with respect to their molecular and functional properties [10]. The terminal enzyme of the MEP pathway, (*E*)-4-hydroxy-3-methyl-but-2-enyl-diphosphate reductase (LytB/IspH) converts (*E*)-4-hydroxy-3-methyl-but-2-enyl-diphosphate (HMBPP) into IPP and DMAPP (Fig. 1) [11]. LytB was characterized as [4Fe–4S] carrying monomeric or dimeric enzyme with of a molecular mass of 30–35 kDa per monomer. X-ray structures were recently determined for the *Aquifex aeolicus* and *Escherichia coli* enzymes; the latter in complex with substrate and products. On this basis a structure-based mechanism could be postulated and inhibition studies initiated [12–15].

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

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http://dx.doi.org/10.1016/j.febslet.2013.10.029

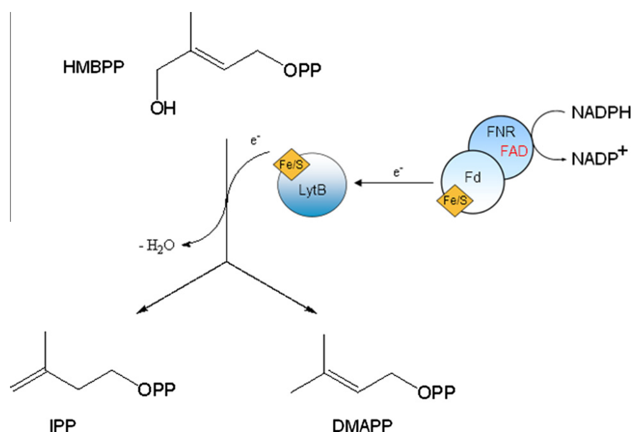

Fig. 1. Reaction of LytB which converts HMBPP into IPP or DMAPP. Electrons to the [4Fe–4S] cluster of LytB are supplied in *P. falciparum* by the ferredoxin/ferredoxin-$NADP^+$ reductase system. NADPH transfers a hydride to FAD that conducts two times one electron to a [2Fe–2S] cluster of ferredoxin and further to the [4Fe–4S] cluster of LytB.

In this report we describe the structure, and the potential substrate and electron donor binding sites of LytB of *P. falciparum*, the direct target for drug developments against the major pathogenic malaria parasite.

2. Materials and methods

2.1. Enzyme production

TOP 10 *E. coli* cells (Invitrogen) were transformed with the pASKPfalLytB vector containing His_6-tagged LytB and grown in LB-broth medium (Roth) supplemented with 150 μg ml^{-1} ampicillin and 300 μM $FeCl_3$ at 37 °C [16]. The cells were harvested by centrifugation (17700×g, 15 min, 4 °C) and stored at −30 °C until further use. All subsequent steps were carried out under oxygen exclusion. The cell pellet (30 g) was resuspended in 300 ml 30 mM Tris–HCl (pH 7.5), 150 mM NaCl, 15 mM imidazole and lysed by ultrasonic treatment at 0 °C. Purification was performed using Co-TALON, Source 15S and Superdex 200 columns as described in detail in the Supplementary data. LytB was stored at a concentration of ca. 12 mg ml^{-1} in 30 mM Tris–HCl (pH 7.5) and 150 mM NaCl.

2.2. Crystallization and X-ray structure analysis

Crystallization was performed at 18 °C in an anaerobic tent (95% N_2, 5% H_2) using the sitting drop vapour diffusion method. Best crystals grew by mixing 2.0 μl enzyme solution (12 mg ml^{-1} LytB, 30 mM Tris–HCl, 150 mM NaCl, pH 7.5) and 1.0 μl reservoir solution composed of 30% w/v PEG 4000, 100 mM sodium citrate (pH 5.6) and 100 mM ammonium sulfate. Data were collected at the Swiss-Light source beamline PXII and processed with XDS [17]. The structure was determined with the multiple anomalous dispersion method using the iron of the Fe/S cluster as anomalous scatterer. The iron positions were detected with SHELXD [18] and the phases calculated with SHARP [19]. After phase improvement with SOLOMON [20] a polypeptide model could be built in COOT [21] using the known LytB structures [12,13]. The structure was refined with REFMAC5 [22]. Crystal parameters, data collection and refinement statistics are listed in Table 1. Figs. 1–3 were produced with PYMOL (Schrödinger, LLC]. The atomic coordinates and the structure factor of LytB have been deposited in the Protein Data Bank, www.pdb.org with the ID code 4N7B.

2.3. Docking calculations

Fully unbiased protein–protein docking calculations on the basis of the determined LytB structure and the known ferredoxin structure from *P. falciparum* (PDB: 1iue) were performed with the molecular simulations software ICM-Pro (Molsoft LLC, La Jolla, CA) [23]. We calculated the size of the surface area buried at the interface, the number of hydrophobic contacts, binding energies, and overall electrostatic complementarities and reduced the generated number of LytB–ferredoxin complexes by defining a maximal distance between the redox centers involved in inter-protein electron transfer. A number of scripts were written to calculate interatomic distances, angles and torsion angles, as well as to evaluate the binding energies of the generated complexes. The most promising docking complexes were chosen and further refined by energy minimization and side chain optimization procedures. The software ICM-ODA (optimal docking area) [24] was used to determine the surface section of LytB that is most suitable to form an interface with another protein.

3. Results and discussion

3.1. Structure of LytB of P. falciparum

Recombinantly produced His_6-tagged LytB enzyme from *P. falciparum* was crystallized under anaerobic conditions. Its structure was determined by the multiple anomalous dispersion method and refined to final R/R_{free} factors of 17.5/19.5% at 2.2 Å resolution (Table 1).

In accordance to previous solution data [16] *P. falciparum* LytB was found like *E. coli* LytB as a monomer in contrast to the dimeric *A. aeolicus* LytB. *P. falciparum* LytB is essentially composed of three related α/β-domains (domains A–C) arranged in a cloverleaf form with the Fe/S-cluster in its center (Fig. 2; Supplementary Fig. 1A). This architecture corresponds to that of *A. aeolicus* and *E. coli* LytB [12,13]. In contrast to the *A. aeolicus* enzyme, the *P. falciparum* and *E. coli* enzymes contain a C-terminal extension fused to helix α4c. After a turn it linearly spans over the entire domain C and is partly associated as a strand to the central four-stranded parallel β-sheet. Subsequently, the C-terminal extension passes the backside of the Fe/S cluster and continues to the top of domain A. In contrast to *E. coli* LytB the terminal residues of the *P. plasmodium* enzyme are folded as an α-helix (α1D) which is attached to helix α1A (Fig. 2A). Superposition between the different LytB structures with DALI [25] results in an rms deviation of 3.0 Å between *P. falciparum* and *A. aeolicus* LytB (sequence identity 32%) and of 1.4 Å between *P. falciparum* and *E. coli* LytB (sequence identity 40%).

This higher similarity between the latter enzymes is mainly due to a large-scale conformational change of the variable domain C (Fig. 2B). *A. aeolicus* LytB is crystallized in an open state [12] whereas *P. falciparum* and *E. coli* LytB in a closed state [13]. Open-to-close transition implicates a rotation of domain C towards the active site crevice. Interestingly, domain C of *E. coli* LytB is rotated ca. 10° further down to the active site crevice than domain C of *P. falciparum* LytB resulting in displacements at its top up to 5 Å. Further significant deviations up to 5 Å between the known LytB structures are only found in the loops following helices α2B, α3B and α4C. The loop following helix α1A is conformationally distinct between *A. aeolicus* and *P. falciparum*/*E. coli* LytB. These differences do, however, not affect the active site structure.

3.2. The Fe/S-cluster and the catalytic site

The *P. falciparum* LytB crystal structure exhibits an inactive [3Fe–4S] cluster already observed in *A. aeolicus* and most *E. coli* LytB structures [12,13]. The three iron atoms are ligated to three

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