

From structure determination to design of antibiotic drugs - riboflavin biosynthesis in *Mycobacterium tuberculosis* as a target

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Vitamin B2, commonly called riboflavin, is one of eight water-soluble B vitamins. Like its close relative, vitamin B1 (thiamine), riboflavin plays a crucial role in certain metabolic reactions, for example, in the final metabolic conversion of monosaccharides, where reduction-equivalents and chemical energy in the form of ATP are produced via the Embden–Meyerhoff pathway. Higher animals, including humans, are dependent on riboflavin uptake through their diet. However, most of the known microorganisms and a number of pathogenic enterobacteria are absolutely dependent on the endogenous synthesis of riboflavin because they are unable to take up the vitamin from the environment. Because the enzymes involved in riboflavin biosynthesis pathways are not present in the human or animal host, they are promising candidates for the inhibition of bacterial growth.

Mycobacterium tuberculosis is one of the human pathogens responsible for causing eight million cases of new infections and two million human deaths every year in both developing and industrialized countries [1].



Fig 1: *Mycobacterium tuberculosis*, First discovered in 1882 by Robert Koch, *M. tuberculosis* has an unusual, waxy coating on the cell surface (primarily mycolic acid), which makes the cells impervious to Gram staining; acid-fast techniques are used instead.

Treatment of the active forms of the disease has become increasingly difficult because of the growing antibiotic resistance of *Mycobacterium tuberculosis*. The elucidation of the complete genomes of *Mycobacterium tuberculosis* and the related *Mycobacterium leprae* has provided powerful tools for the development of novel drugs that are urgently required [2–4]. Both *Mycobacterium tuberculosis* and *Mycobacterium leprae* comprise complete sets of genes required for the biosynthesis of riboflavin (vitamin B2). As the genome of *Mycobacterium leprae* has undergone a dramatic process of gene fragmentation, the fact that all riboflavin biosynthesis genes were retained in apparently functional form indicates that the biosynthetic pathway is of vital importance for the intracellular lifestyle of the pathogen. By extrapolation of this argument, it appears likely that the riboflavin pathway genes are also essential for *Mycobacterium tuberculosis*.

The biosynthesis of riboflavin has been studied extensively over recent years. Two enzymes, lumazine synthase (EC 2.5.1.9; LS) and riboflavin synthase (RS), catalyzing the penultimate and the last step of riboflavin biosynthesis, respectively, are the main targets of our interest.

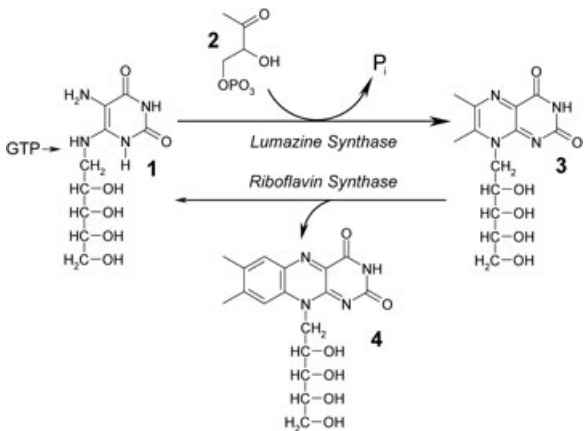


Fig 2: Terminal reactions catalyzed by lumazine synthase and riboflavin synthase in the pathway of riboflavin biosynthesis

It has been shown that in *Bacillus subtilis*, these two enzymes form a complex comprised of an inner core consisting of three α -subunits (RS) encapsulated by an icosahedral shell containing 60 β -subunits (LS) [5-7].

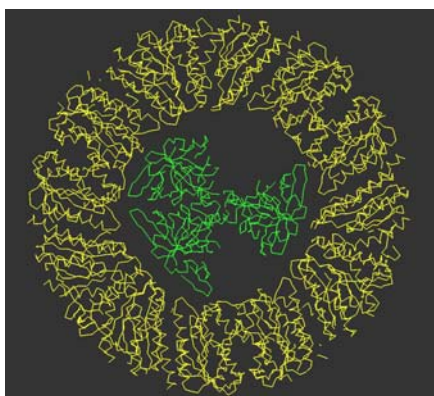


Fig 3: Computer-generated C _{α} carbon model of the icosahedral complex of lumazine synthase (60 β -subunits, yellow) and riboflavin synthase (3 α -subunits, green), a cross section through the particle center is shown.

The β -subunits catalyze the turnover of 3,4-dihydroxy-2-butanone-4-phosphate (2) and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1) to 6,7-dimethyl-8-(d-ribityl)-lumazine (3), whereas the α -subunits catalyze the formation of one riboflavin molecule (4) from two molecules of (3), respectively (Fig. 2).

The isolation and purification of LSs from different organisms has revealed the pentameric nature of this enzyme, which can be found in two different oligomeric states. In *Bacillus subtilis*, *Aquifex aeolicus* and *Spinacia oleracea*, the protein exists as an icosahedral capsid formed from 60 identical subunits (12 pentamers) [7-9]. LSs from

Saccharomyces cerevisiae, *Schizosaccharomyces pombe*, *Brucella abortus* and *Magnaporthe grisea* are homopentameric enzymes [9–12]. Recently, we have solved the structure of LS from *Mycobacterium tuberculosis*, which has shown the homopentameric state as well [13], Fig 4. The structure was solved by molecular replacement with yeast lumazine synthase used as search structure.

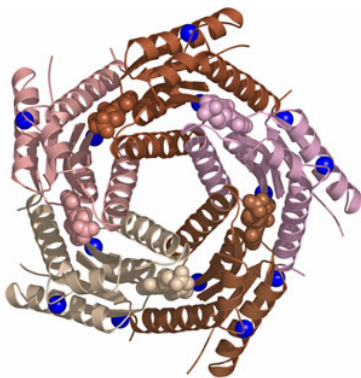


Fig 4: Ribbon model of the pentameric assembly of lumazine synthase from *Mycobacterium tuberculosis*, view along the fivefold local symmetry axis, blue spheres represent bound potassium ions, the active sites located between subunits are occupied by bound 6-(1,3,7-trihydro-9-D-ribityl-2,4,8-purinetrione-7-yl) hexane 1-phosphate (TS68), figure was generated with PYMOL.

The LS monomer shows some folding similarity to bacterial flavodoxins [14] and is constructed from a central four-stranded β -sheet flanked on both sides by two and three α -helices, respectively. In spite of the fact that riboflavin biosynthesis was studied for several decades, the chemical nature of the second LS substrate, the four-carbon precursor of the pyrazine ring, remained unknown for a long time. The elucidation of the structure of this compound by Volk and Bacher in 1991 [15] allowed detailed studies of lumazine synthase catalysis. In order to investigate the catalytic mechanism of the formation of 6,7-dimethyl-8-(d-ribityl)-lumazine, Cushman and coworkers have designed and synthesized several series of inhibitors that mimic the substrate, the intermediates and the product of the reaction [16–22] catalysed by LS. The first detailed description of the active site of LS was provided by the X-ray structure of *Bacillus subtilis* LS in complex with the substrate analogue 5-nitro-6-d-ribitylamino-2,4- (1H,3H) pyrimidinedione [23].

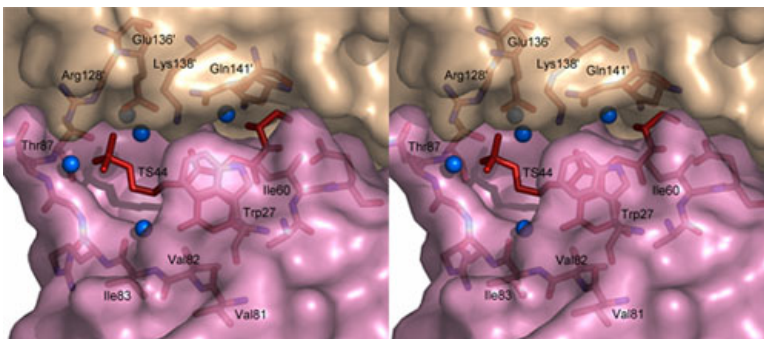


Fig 5: Stereo view of the active site surface of lumazine synthase from *Mycobacterium tuberculosis* with bound purinetrione inhibitor TS44, blue spheres: water molecules

It has been shown that the lumazine synthase active site is located at the interface of two neighbouring subunits and, furthermore, that it is built by highly conserved hydrophobic and positively charged residues from both subunits, Fig 5.

Lumazine synthase inhibitors can be considered as potential lead compounds for the design of therapeutically useful antibiotics. Recently, a new series of compounds based on the purinetrione aromatic system was designed [22,24]. Somewhat later it was found that those compounds demonstrated the highest binding affinity and specificity to LS from *M. tuberculosis* in comparison with the LSs from other bacteria.

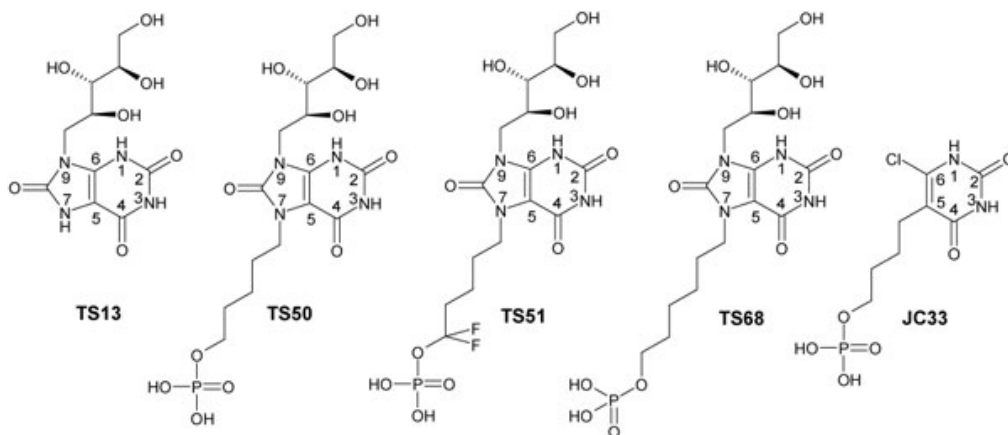


Fig 6: Designed purinetrione inhibitors of lumazine synthase and riboflavin synthase from *Mycobacterium tuberculosis*

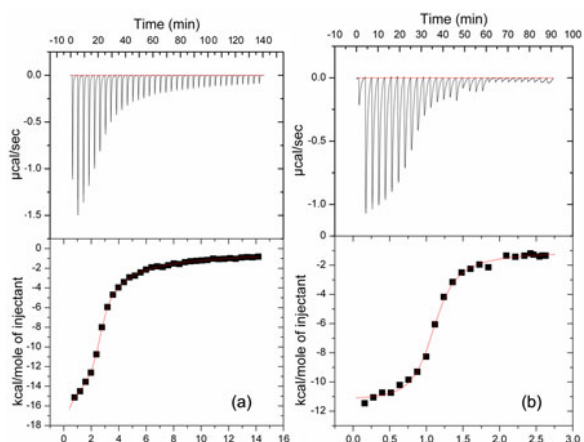


Fig 7: Isothermal titration calorimetry diagrams from *M. tuberculosis* lumazine synthase titrated with 3-(1,3,7-trihydro-9-D-ribityl-2,6,8-purinetrione-7-yl)propane 1-phosphate (TS44) (a) and 3-(1,3,7-trihydro-9-D-ribityl-2,6,8-purinetrione-7-yl)butane 1-phosphate (TS70) (b)

Two structures of *M. tuberculosis* LS in complex with two ribityl-purinetrione compounds (TS44 and TS70) bearing an alkyl phosphate group were solved recently[13], Fig 5. In order to provide structural information for the design of optimized LS inhibitors, we have undertaken the structure determination of *M. tuberculosis* LS complexes with

four differently modified purinetrione compounds. Binding constants and other thermodynamic binding parameters were determined by isothermal titration calorimetry (ITC) experiments (see E. Morgunova et al (2006) FEBS J. 273, 4790-4804)

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