Evolution of Vitamin B\textsubscript{2} Biosynthesis: 6,7-Dimethyl-8-Ribityllumazine Synthases of \textit{Brucella}

Vanessa Zylberman,\textsuperscript{1} Sebastián Klinke,\textsuperscript{1} Ilka Haase,\textsuperscript{2} Adelbert Bacher,\textsuperscript{2} Markus Fischer,\textsuperscript{2,*} and Fernando Alberto Goldbaum\textsuperscript{1,**}

Fundación Instituto Leloir, Buenos Aires, Argentina, \textsuperscript{1} and Lehrstuhl für Organische Chemie und Biochemie Technische Universität München, Lichtenbergstraße 4, D-85748 Garching, Germany\textsuperscript{2}

Received 7 February 2006/Accepted 7 June 2006

The penultimate step in the biosynthesis of riboflavin (vitamin B\textsubscript{2}) involves the condensation of 3,4-dihydroxy-2-butanoate 4-phosphate with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione, which is catalyzed by 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase). Pathogenic \textit{Brucella} species adapted to an intracellular lifestyle have two genes involved in riboflavin synthesis, \textit{ribH1} and \textit{ribH2}, which are located on different chromosomes. The \textit{ribH2} gene was shown previously to specify a lumazine synthase (type II lumazine synthase) with an unusual decameric structure and a very high \textit{K}_m for 3,4-dihydroxy-2-butanoate 4-phosphate. Moreover, the protein was found to be an immunodominant \textit{Brucella} antigen and was able to generate strong humoral as well as cellular immunity against \textit{Brucella abortus} in mice. We have now cloned and expressed the \textit{ribH1} gene, which is located inside a small riboflavin operon, together with two other putative riboflavin biosynthesis genes and the \textit{musB} gene, specifying an antitermination factor. The \textit{RibH1} protein (type I lumazine synthase) is a homopentamer catalyzing the formation of 6,7-dimethyl-8-ribityllumazine at a rate of 18 nmol mg\textsuperscript{-1} min\textsuperscript{-1}. Sequence comparison of lumazine synthases from archaea, bacteria, plants, and fungi suggests a family of proteins comprising archaeal lumazine and riboflavin synthases, type I lumazine synthases, and the eubacterial type II lumazine synthases.

Vitamin B\textsubscript{2} (riboflavin) (compound 6 [Fig. 1]) is the precursor of flavin mononucleotide and flavin adenine dinucleotide, essential cofactors for a wide variety of redox enzymes. Moreover, they are involved in numerous other physiological processes involving light sensing, bioluminescence, circadian time keeping, and DNA repair (for a review, see reference 39). The vitamin is biosynthesized by plants, fungi, and certain microorganisms but must be obtained from dietary sources and/or the intestinal flora by animals.

The pathways of riboflavin biosynthesis in microorganisms and plants have been reviewed recently (9, 10). The final steps are catalyzed by 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase [compound VI]) and riboflavin synthase (compound VII). More specifically, lumazine synthase catalyzes the condensation of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (substrate 2) with 3,4-dihydroxy-2-butanone 4-phosphate (substrate 4), resulting in the pteridone derivative, substrate 5 (Fig. 1).

Lumazine synthases from a variety of eubacteria (including \textit{Escherichia coli}, \textit{Bacillus subtilis}, \textit{Mycobacterium tuberculosis}, and the hyperthermophile \textit{Aquifex aeolicus}), archaea (\textit{Methanococcus jannaschii}), fungi (\textit{Saccharomyces cerevisiae}, \textit{Schizosaccharomyces pombe}, and \textit{Magnaporthe grisea}), and a plant (spinach) have been studied in some detail (11, 16, 22, 32, 34, 35, 40–42, 44, 56, 57). The enzymes from fungi and from \textit{M. tuberculosis} are C5-symmetric homopentamers, and the lumazine synthases of plants, most eubacteria, and archaea are 532-symmetric, hollow capsules, which are best described as dodecamers of pentamers. The subunit folds of these enzymes and the topology of the pentamer moieties are closely similar. The topologically equivalent active sites (5 in the case of the pentameric enzymes and 60 in the case of the icosahedral enzymes) are invariably located at interfaces between adjacent subunits in the pentamer moieties. Recently, it was found that pentameric riboflavin synthases of archaea are closely related to 6,7-dimethyl-8-ribityllumazine synthases (15, 25, 45).

Brucellosis is a disease of humans and livestock that is caused by closely related \textit{Brucella} species adapted to intracellular life within the cells of a variety of mammals; the main pathogenic species for domestic animals are \textit{Brucella abortus, Brucella melitensis}, and \textit{Brucella suis}. Goldbaum and coworkers have shown that an 18-kDa \textit{B. abortus} antigen with sequence similarity to lumazine synthases is a serological marker of active disease in human brucellosis patients (17, 19). Immunization with the protein has been shown to induce both cellular and humoral immune responses in mice. Moreover, the generation of protective immunity has also been observed in this model (2). Hence, the 18-kDa protein is of considerable immunologic interest and has been suggested to be a general carrier for the engineering of subunit vaccines (36). In preliminary enzymatic studies, this protein was shown to catalyze the formation of 6,7-dimethyl-8-ribityllumazine (compound 5), albeit at a low rate. Recently, the catalytic properties of this protein have been analyzed in closer detail (31). The three-dimensional structure of the 18-kDa antigen has been studied in considerable detail by Goldbaum and coworkers, and the protein has been reported to be a homodecamer which is best
Materials. 5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (substrate 2) and 6,7-dimethyl-8-ribityllumazine (substrate 5) were synthesized according to the recently published methods (47) (GATC Biotech, Konstanz, Germany) and were transformed into E. coli BL21(DE3) competent cells (50) (Stratagene, La Jolla, CA), resulting in the recombinant E. coli strain BL21(DE3)-pT7-7-BARibH1.

Bacterial culture. E. coli BL21(DE3)-pT7-7-BARibH1 was grown to an optical density (at 600 nm) of 1.0 in LB medium containing 100 μg of ampicillin per ml at 37°C with shaking (150 rpm). An aliquot (5 ml) of this culture was diluted into 500 ml of medium, and incubation with shaking was continued to an optical density of 1.0. Isopropyl-β-thiogalactoside was added to a final concentration of 1 mM, and the suspension was incubated for 4 h at 37°C with shaking (150 rpm).

Phylogenetic analysis. Sequences used for the phylogenetic analysis of lumazine synthase proteins were obtained from the HMM library, the genome assignment server Superfamily 1.69, and the NCBI database. The amino acid sequences were aligned with the ClustalX (version 1.81) program for multiple sequence alignment (53). Phylogenetic analysis of this alignment was inferred with the maximum-likelihood heuristic algorithm implemented by PHYML, version 3.2.024 (33).

RESULTS

Genomic organization of B. abortus lumazine synthase genes. The sequences of the complete genomes of B. abortus, B. suis, and B. melitensis were recently published (4, 23, 43) and show that the 18-kDa lumazine synthase antigen (type II lumazine synthase) is specified by the ribH2 gene, which is located on chromosome II. In all Brucella species analyzed, open reading frames (ORFs) with similarity to formate dehydrogenase (BMEI0588, BAB2-0534) and a sugar binding periplasmic precursor (BMEI0590) were found, and ORFs with unknown function surround the ribH2 gene (Fig. 2). This group of genes does not appear to have a common transcriptional control, as judged by analysis of the ORF orientation and promoter localization. Moreover, analysis of the 5′ untranslated region suggests that the ribH2 gene is under the regulatory control of a putative RFN element that is believed to sense the flavin mononucleotide concentration (54, 55).

Surprisingly, closer study of the three sequenced Brucella genomes revealed the presence of a second gene locus with sequence similarity to lumazine synthase genes. This gene is

FIG. 1. Biosynthesis of riboflavin in eubacteria. I, GTP cyclohydrolase II; II, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione 5′-phosphate deaminase; III, 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate reductase; IV, hypothetical phosphatase; V, 3,4-dihydroxy-2-butanoate 4-phosphate synthase; VI, 6,7-dimethyl-8-ribityllumazine synthase; VII, riboflavin synthase; I, GTP; 2, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione; 3, ribulose 5′-phosphate; 4, 3,4-dihydroxy-2-butanoate 4-phosphate synthase; 5, 6,7-dimethyl-8-ribityllumazine; 6, riboflavin.
FIG. 2. Topology of riboflavin biosynthesis operons. Sequences were derived from fully sequenced genomes available from GenBank. Orientations of the genes involved in riboflavin biosynthesis or adjacent to those genes are given as arrows (not drawn to scale). Organisms (accession numbers) are as follows: *Brucella meliloti* 16 M chromosome I (NC_003317), *Brucella suis* 1330 chromosome I (NC_004310), *Brucella abortus* biovar 1 strain 9-941 chromosome I (NC_006932), *Sinorhizobium meliloti* 86-1380 chromosome I (NC_003317), *Rhodopseudomonas palustris* CGA009 (NC_005296), *Bradyrhizobium japonicum* USDA 110 (NC_004463), *Caulobacter crescentus* CB15 (NC_002696), *Xanthomonas axonopodis* pv. citri strain 306 (NC_003919), *Xanthomonas oryzae* KACC10331 (NC_006834), *Xylella fastidiosa* Temecula1 (NC_004556), *Pseudomonas syringae* pv. tomato strain DC3000 (NC_004578), *Mesorhizobium loti* USDA 110 (NC_004643), *Xanthomonas oryzae* KACC10331 (NC_004310).  

![Image](http://jb.asm.org/)

The amino acid sequence identity between the type I and...
type II lumazine synthases of B. abortus is 21% (Fig. 3). Interestingly, both lumazine synthases have higher sequence similarities with other lumazine synthases, e.g., B. subtilis (type I, 33%; type II, 24%) and E. coli (type I, 37%; type II, 29%) than with each other.

**Quaternary structure of the type I lumazine synthase of B. abortus.** For a detailed characterization, the ribH1 gene of B. abortus was cloned into an expression plasmid under the control of a T7 RNA polymerase promoter and lacZ operator. In a recombinant E. coli strain, the expression construct directed the formation of a 17-kDa protein, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The recombinant protein was purified to apparent homogeneity by anion-exchange chromatography, followed by gel permeation chromatography. Edman degradation of the N terminus of the recombinant protein resulted in the sequence MEFLM SKHEADA, in agreement with the translated ORF (Fig. 3), thus confirming the integrity of the protein sequence at the N terminus.

The type I lumazine synthase of B. abortus sediments at an apparent velocity of 5.9S at 20°C (Fig. 4A). In comparison, it should be noted that pentameric lumazine synthases from the yeasts S. cerevisiae and S. pombe have similar apparent sedimentation coefficients, of 5.5S and 5.0S, respectively (11, 42). Sedimentation equilibrium experiments indicated a molecular mass of 88 kDa, with an ideal monodisperse model used for calculation (Fig. 4B). The calculated subunit molecular mass of 17,599 Da indicates a pentameric mass of 88 kDa, in excellent agreement with the experimental data.

**Functional characterization of the type I lumazine synthase of B. abortus.** Enzymatic studies show that the type I lumazine synthase catalyzes the formation of 6,7-dimethyl-8-ribityllumazine (substrate 5) at a rate of about 18 ± 2 nmol mg⁻¹ min⁻¹ at 37°C (Table 1). Notably, this value is about 1 order of magnitude below the catalytic rates of lumazine synthases from other mesophilic microorganisms and from spinach (Table 1). The kinetic data for the substrate, 3,4-dihydroxy-2-butanoate-4-phosphate (substrate 4), were fitted to the Hill equation (equation 1).

\[
V = \frac{V_{\text{max}} \cdot S^n}{K^* + S^n} \tag{1}
\]

where \( V \) is the reaction velocity, \( V_{\text{max}} \) is the maximum reaction velocity, \( S \) is the substrate concentration, \( K \) is the reaction constant, and \( n \) is the Hill coefficient, resulting in a \( K \) value of 125 ± 10 μM for substrate 4.

Steady-state kinetic experiments showed a decreasing reaction velocity at high concentrations of the substrate 5-amino-6-ribitylaminol-2,4(1H,3H)-pyrimidinedione. The data could be
fungal and yeast lumazine synthases (S. cerevisiae, S. pombe, and M. grisea).

**DISCUSSION**

Genomic analysis of *Brucella* identified two related ORFs (*ribH1* and *ribH2*) coding for two proteins designated type I and type II lumazine synthases, respectively. The similarity between the type I and type II lumazine synthases, which are located on two different chromosomes, is quite low (Fig. 3). Hence, we must assume that either the separation of both lumazine synthase genes occurred very early in evolution or one of the *ribH* genes was acquired prior to *α*-proteobacterial speciation by lateral gene transfer. At this time, there is no experimental evidence that would permit a decision.

Recent studies have shown the existence of a family of pentameric lumazine synthase-like riboflavin synthases (Figs. 5 and 6). These enzymes have been found exclusively in archaea, which have also been shown to be devoid of riboflavin synthases of the trimeric eubacterium/yeast/plant type. The cavity harboring the active site of the pentameric riboflavin synthase of *M. jannaschii* is similar to that of the lumazine synthases. The binding mode of the acceptor lumazine molecule in pentameric riboflavin synthase closely resembles the pyrimidine-dione-binding site of the lumazine synthases (Fig. 6). Notably, these archaeal riboflavin synthases are evolutionarily old and have no detectable lumazine synthase activity (13, 15, 45). Completely sequenced archaeal genomes typically comprise sets of two similar genes, coding for a lumazine synthase-like riboflavin synthase and a regular lumazine synthase. Interestingly, present-day lumazine synthases have retained the capacity to bind riboflavin. For example, the lumazine synthase of the yeast *S. pombe* is yellow colored due to the presence of a tightly bound riboflavin (11).

The situation in the archaea is comparable to that in the eubacteria, with type I and type II lumazine synthases as we describe in the present work (Fig. 5). However, there is no

**TABLE 1. Properties of lumazine synthases**

<table>
<thead>
<tr>
<th>Origin</th>
<th>$K_m$ (µM)*</th>
<th>$V_{max}$ at 37°C (nmol mg⁻¹ min⁻¹)</th>
<th>Sedimentation velocity (S)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>55 ± 5</td>
<td>242 ± 6</td>
<td>26.5</td>
<td>29</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>62</td>
<td>197</td>
<td>26.8</td>
<td>42</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>90</td>
<td>257</td>
<td>5.5</td>
<td>42</td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td>67</td>
<td>217</td>
<td>5.0</td>
<td>11</td>
</tr>
<tr>
<td><em>S. oleracea</em></td>
<td>26 ± 3</td>
<td>275</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td><em>B. abortus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I lumazine synthase</td>
<td>125 ± 10*</td>
<td>18 ± 2</td>
<td>5.9</td>
<td>This study</td>
</tr>
<tr>
<td>Type II lumazine synthase</td>
<td>4,000</td>
<td>20 ± 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Substrate 4, 3,4-dihydroxy-2-butanone 4-phosphate; substrate 2, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione. * A Hill coefficient ($n = 2 ± 0.3$).

**TABLE 2. Summary of genomic, structural, and biochemical knowledge about *B. abortus* lumazine synthases**

<table>
<thead>
<tr>
<th>Previous protein name</th>
<th>Gene/protein name</th>
<th>Chromosome location</th>
<th>Quaternary arrangement</th>
<th>Lumazine synthase activity</th>
<th>Source and/or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-kDa antigen</td>
<td><em>ribH1</em> type I lumazine synthase</td>
<td>I</td>
<td>Pentameric</td>
<td>+</td>
<td>31; this study</td>
</tr>
<tr>
<td></td>
<td><em>ribH2</em> type II lumazine synthase</td>
<td>II</td>
<td>Decameric</td>
<td>–</td>
<td>17-19, 58</td>
</tr>
</tbody>
</table>

best approximated with the model represented by equation 2, indicating a $K_m$ of 90 ± 16 µM and an inhibition constant of 370 ± 70 µM (Table 1):

$$V = \frac{V_{max} \cdot [1]}{K_m + [1] \cdot \left(1 + \frac{[1]}{K_s}\right)}$$

where $V$ is the reaction velocity, $V_{max}$ is the maximum reaction velocity, $S$ is the concentration of substrate 2, $K_m$ is the Michaelis-Menten constant, and $K_s$ is the substrate inhibition constant.

As shown in Table 1, the affinity for 3,4-dihydroxy-2-butanone 4-phosphate (substrate 4) is in the same range as the values reported for other lumazine synthases. In contrast, the $K_m$ value for substrate 2 is about 10 times higher than that observed for other orthologs.

Table 2 summarizes the genomic localization and the structural and biochemical characterization of *B. abortus* type I and type II lumazine synthases.

**Structure-based phylogenetic analysis of lumazine synthases.** We conducted a phylogenetic analysis comparing atypical, duplicated genes of archaea and bacteria with structurally characterized pentameric and icosaheal lumazine synthases. From this analysis, depicted in Fig. 5, a completely new picture emerges, showing that in addition to eubacterial type I lumazine synthases and archaeal lumazine synthases, this folding gave origin to at least two new evolutionarily related functions: the archaeal riboflavin synthases, which are lumazine synthase-like riboflavin synthases with no detectable lumazine synthase activity (13, 15, 45), and the eubacterial type II lumazine synthases.

Eubacteria that harbor a *ribH2* gene have a type I lumazine synthase of apparent pentameric arrangement, with the exception of that of *Pseudomonas syringae*, which is clustered with the icosaheal lumazine synthases. In addition, pentameric type I lumazine synthases from some of the *α*-Proteobacteria (*Brucella*, *Rhizobium*, and *Rhodobacter*) diverge from pentameric fungal and yeast lumazine synthases (S. cerevisiae, S. pombe, and M. grisea).
evidence that either type I or type II lumazine synthase has any riboflavin synthase activity (results not shown).

It should be noted that the eubacterial type I lumazine synthases that have been reported in the literature have low catalytic activities, in the range of about 200 to 300 nmol mg$^{-1}$ min$^{-1}$ when assayed near the optimum growth temperature of the cognate species (Table 1). By comparison with these data, the activity of the $B. abortus$ type I lumazine synthase is more than 10-fold lower.

The generally low catalytic activity of lumazine synthases does not result from a particularly large free energy barrier of the reaction catalyzed. Quite to the contrary, the condensation of 3,4-dihydroxy-2-butanol 4-phosphate (substrate 4) and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (substrate 2) is characterized by such a low energy barrier that it can proceed at an appreciable rate at room temperature in aqueous solution and at neutral pH (30). Thus, it is mandatory that blank assays without enzyme be run in order to correct for the contribution of the uncatalyzed reaction in lumazine synthase activity measurements (30). In the case of the lumazine synthase of $B. abortus$, with its particularly low catalytic rate, correction for the contribution of the uncatalyzed reaction becomes most important.

The low catalytic activities of the lumazine synthases are in no way unique among the enzymes of the riboflavin pathway. All activity values for the entire riboflavin pathways in $E. coli$, $B. subtilis$, and yeast are in the range of nanomoles per milligram per minute (5–7, 11, 12, 14, 20, 24, 28, 38, 46, 48). Since, at least in the case of lumazine synthase and riboflavin synthase, the inherent free energy barriers of the catalyzed reactions cannot be the reason for these low rates, we must assume, for lack of other arguments, that the selective pressure controlling this pathway favors the evolution of catalysts with low reaction rates. In fact, riboflavin is required in only small quantities and is readily synthesized by the cell from folic acid in the case of tryptophan auxotrophs (21).
FIG. 6. Structural comparison of type I and type II lumazine synthases and archaebial riboflavin synthase. (Top) Superposition structure superposition of riboflavin synthase from \textit{M. jannaschii} (MjaRS; PDB entry code 2B9N [45]), type I lumazine synthase from \textit{S. pombe} (SpoLS; PDB entry code 1KYZ [16]), and type II lumazine synthase from \textit{B. abortus} (BabRibH2; PDB entry code IT13 [31]). The active sites are formed by two adjacent monomers of SpoLS with bound riboflavin (green; residues E17 to D112 and S113 to L158), BabRibH2 with bound 5-nitro-6-(o-ribitylaminio)-2,4(1H,3H)-pyrimidinedione (gray; residues S12 to E106 and T107 to L156), and MjaRS with bound 6,7-dioxo-8-ribityllumazine, resembling the acceptor lumazine molecule (red; residues T2 to M90 and T91 to Y135). Secondary structure element labeling refers to SpoLS. The five topologically equivalent active sites of pentameric lumazine synthases are located at the interfaces between adjacent monomers of the pentamer; two of them (A and B) are shown. Ligands are drawn in the respective colors. (Bottom) Enlarged ligand-binding sites of all three enzymes, with Trp27 from SpoLS, Trp22 from BabRibH2, and Phe12 from MjaRS.

amounts, and excess production would unnecessarily deplete the precursor pools. Still, it remains an open question why the catalytic activity of the \textit{B. abortus} type I enzyme is at the lower end of all documented lumazine synthases.

An earlier study showed that the decameric arrangement of type II \textit{B. abortus} lumazine synthase is related to a very high \(K_p\) for 3,4-dihydroxy-2-butanoate 4-phosphate (substrate 4) (31). The in vitro concentration of 3,4-dihydroxy-2-butanoate 4-phosphate is unknown. However, unless we assume that it is in the same numerical range as the \(K_m\) of the type II enzyme, we must assume that the bulk of the 6,7-dimethyl-8-ribityllumazine (substrate 6) would be generated by the enzyme with the lower \(K_m\) value, i.e., the type I lumazine synthase, whereas the type II enzyme could at best supply a minor amount of the overall riboflavin production.

Thus, the question of which selective pressures could have prevented the loss of the ribH2 gene (in case both lumazine synthase genes are evolutionarily old in \textit{Brucella}-related organisms) or could have favored its more recent acquisition by horizontal gene transfer is still open. Interestingly, the type II lumazine synthase is an immunodominant antigen of \textit{B. abortus}, and there is unpublished evidence that links this protein to \textit{Brucella} virulence, suggesting that the type II lumazine synthase has evolved for a new, yet-unknown function.

ACKNOWLEDGMENTS

This work was supported by a Howard Hughes Medical Institute international grant (to F.A.G.) and by a grant from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyt) República Argentina. F.A.G. is a member of the research career of CONICET, and V.Z. and S.K. are recipients of a fellowship from CONICET. This work was supported by the Fonds der Chemischen Industrie and the Hans-Fischer-Gesellschaft eV. M.F. and F.A.G. acknowledge support for exchange visits between laboratories by the BMBF and SECTY (project ARG 04/Z06).

We acknowledge R. Ugalde, D. Comerici, and Ines Marchesini for genomic \textit{B. abortus} DNA and for early genomic analysis and Diana Posadas for helping us in the phylogenetic analysis.

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