Biosynthesis of riboflavin in Archaea
6,7-Dimethyl-8-ribityllumazine synthase of Methanococcus jannaschii

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Heterologous expression of the putative open reading frame MJ0303 of Methanococcus jannaschii provided a recombinant protein catalysing the formation of the riboflavin precursor, 6,7-dimethyl-8-ribityllumazine, by condensation of 5-amino-6-ribitylaminol-2,4(1H,3H)-pyrimidinedione and 3,4-dihydroxy-2-butanoate 4-phosphate. Steady state kinetic analysis at 37 °C and pH 7.0 indicated a catalytic rate of 11 nmol·mg⁻¹·min⁻¹; Km values for 5-amino-6-ribitylaminol-2,4(1H,3H)-pyrimidinedione and 3,4-dihydroxybutanone 4-phosphate were 12.5 and 52 μM, respectively. The enzyme sediments at an apparent velocity of about 12 S.

Sedimentation equilibrium analysis indicated a molecular mass around 1 MDa but was hampered by nonideal solute behaviour. Negative-stained electron micrographs showed predominantly spherical particles with a diameter of about 150 Å. The data suggest that the enzyme from M. jannaschii can form capsids with icosahedral 532 symmetry consisting of 60 subunits.

Keywords: Archaea; Methanococcus jannaschii; riboflavin biosynthesis; lumazine synthase; quaternary structure.

Experimental procedures

Materials

5-Amino-6-ribitylaminol-2,4(1H,3H)-pyrimidinedione (structure 2) was freshly prepared from 5-nitro-6-ribitylaminol-2,
4(1H,3H)-pyrimidinedione [30,31] by catalytic hydrogenation [32]. 3,4-Dihydroxy-2-butanoate 4-phosphate (structure 4) was freshly prepared from ribose 5-phosphate by treatment with pentose phosphate isomerase and 3,4-dihydroxy-2-butanoate 4-phosphate synthase [19]. Recombinant 3,4-dihydroxy-2-butanoate 4-phosphate synthase of *Escherichia coli* was prepared using published procedures [33]. Oligonucleotides were custom-synthesized by MWG Biotech, Ebersberg, Germany.

**Bacterial strains**

Microbial strains and plasmids used in this study are summarized in Table 1.

**Construction of an expression plasmid**

PCR amplification using *M. jannaschii* cDNA as a template and the oligonucleotides, MJ-RibE-1 and MJ-RibE-2 (Table 2) as primers produced a DNA fragment that served as a template for a second round of PCR amplification using the oligonucleotides, MJ-RibE-2 and MJ-RibE-3 as primers. The resulting product was purified with the purification kit from Qiagen, digested with the restriction endonucleases *Eco*RI and *Bam*HI, and ligated into the expression-vector pNCO113 (Table 1) digested with the same enzymes. The resulting plasmid, pNCO-MJ-RibE, was transformed into *Escherichia coli* XL1-Blue cells (Table 1) [35].

**Construction of an expression plasmid for modified lumazine synthase of *Bacillus subtilis***

The coding region of the *ribH* gene of *B. subtilis* was amplified by PCR using the plasmid, p602-BS-RibH [36] as the template and the oligonucleotides, BS-RibH-AN-G6 and BS-RibH-2 as primers (Table 2). The resulting product was cleaved with the restriction enzymes *Eco*RI and *Bam*HI and ligated into the plasmid, pNCO113 (Table 1) that had been treated with the same enzymes. The resulting plasmid, pNCO-BS-RibH-AN-G6, was transformed into *Escherichia coli* XL1-Blue cells (Table 1) [35].

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**Table 1. Bacterial strains and plasmids.**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> strain XL-1-Blue</td>
<td><em>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac(F’ proAB, lacF’ZAM15, Tn10(tet’))</em></td>
<td>[35]</td>
</tr>
<tr>
<td>Plasmids for the RibE gene of <em>M. jannaschii</em> and the RibH gene of <em>B. subtilis</em></td>
<td>Expression vector</td>
<td>[34]</td>
</tr>
<tr>
<td>pNCO113</td>
<td>Expression vector</td>
<td>[34]</td>
</tr>
<tr>
<td>pNCO-MJ-RibE</td>
<td>RibE gene wild type</td>
<td>This study</td>
</tr>
<tr>
<td>pNCO-BS-RibH-AN-G6</td>
<td>RibH gene truncated at the N-terminus</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 2. Oligonucleotides used for construction of expression plasmids.** Recognition sites are emboldened.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Endonuclease</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ-RibE-1</td>
<td></td>
<td>5'-GGAGAAATTAACATGGTATTTGATGTTAATCCCTG-3'</td>
</tr>
<tr>
<td>MJ-RibE-2</td>
<td><em>Bam</em>HI</td>
<td>5'-TTCTTTAGGAGGATCCATTTTAAACAAATTT-3'</td>
</tr>
<tr>
<td>MJ-RibE-3</td>
<td><em>Eco</em>RI</td>
<td>5'-ACACAGAAATCTTAAAGAGGAAATTAACTAGTG-3'</td>
</tr>
<tr>
<td>BS-RibH-AN-G6</td>
<td><em>Eco</em>RI, <em>Nco</em>I</td>
<td>5'-ATATAGAGAATTTCATTTAATGGGAAATTACGAAAGTTACAG-3'</td>
</tr>
<tr>
<td>BS-RibH-2</td>
<td><em>Bam</em>HI</td>
<td>5'-TTTTTTGGAGATCTATTTCGAAAGACGGGCTTTAG-3'</td>
</tr>
</tbody>
</table>
pNCO-RibH-AN-G6, was transformed into *E. coli* XL1-Blue cells.

**DNA sequencing**

Sequencing was performed by the dideoxy chain termination method [37] using a model 377A DNA sequencer from Applied Biosystems (Foster City, CA, UK). Plasmid DNA was isolated from cultures (5 mL) of XL-1 Blue strains grown overnight in LB medium containing ampicillin (150 mg L\(^{-1}\)) using Nucleobond AX20 columns (Macherey-Nagel, Düren, Germany).

**Purification of *M. jannaschii* 6,7-dimethyl-8-ribityllumazine synthase**

The frozen cell mass of the recombinant *E. coli* strain XL1-Blue carrying the plasmid, pNCO-MJ-RibE, was thawed in 20 mM potassium phosphate, pH 7.0. The suspension was ultrasonically treated and centrifuged. The supernatant was placed on a column of hydroxyapatite (2.5 × 10 cm, Amersham Pharmacia Biotech, Freiburg, Germany) that had been equilibrated with 20 mM potassium phosphate, pH 7.1. The column was developed with a linear gradient of 0.02–1 M potassium phosphate, pH 7.1 (total volume, 400 mL). Fractions were combined and ammonium sulfate was added to a final concentration of 2.46 M. The precipitate was harvested and dissolved in 100 mM potassium phosphate, pH 7.0. The solution was placed on top of a Sephacryl S-400 column (2.6 × 60 cm, Amersham Pharmacia Biotech, Freiburg, Germany) that had been equilibrated with 100 mM potassium phosphate, pH 7.0. Fractions were combined and concentrated by ultrafiltration.

**Purification of the lumazine synthases of *B. subtilis* and *A. aeolicus***

Purification of the mutant enzyme of *B. subtilis* and the wildtype lumazine synthase of *A. aeolicus* was performed as described [17,38].

**SDS/PAGE**

SDS/PAGE using 16% polyacrylamide gels was performed as described [39]. Molecular mass standards were supplied by Sigma.

**Peptide sequencing**

Sequence determination was performed by the automated Edman method using a 471-A Protein Sequencer (Perkin Elmer).

**Assay of 6,7-dimethyl-8-ribityllumazine synthase activity**

Reaction mixtures contained 100 mM potassium phosphate, pH 7.0, 5 mM EDTA, 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (structure 2, Fig. 1) (freshly prepared) and 3,4-dihydroxy-2-butanone 4-phosphate (structure 4) as required, and protein. The reaction was monitored photometrically at 410 nm.
are in the range of 200–250 nmol·mg⁻¹·min⁻¹ (Table 3). The catalytic activity of lumazine synthase from spinach at 37 °C is 275 nmol·mg⁻¹·min⁻¹. Not surprisingly, the catalytic activity of enzyme from the thermophilic archaean at 37 °C is low in comparison with mesophilic organisms. At a temperature of 70 °C, the catalytic rate of the enzyme is 90 nmol·mg⁻¹·min⁻¹. Steady state kinetic experiments in the temperature range of 10–80 °C gave a linear Arrhenius Plot with a $E_A$ of 63.7 kJ·mol⁻¹ and an Arrhenius constant of $A = 2.9 \times 10^8$ s⁻¹ (Fig. 2, Table 4).

Sedimentation equilibrium analysis of *M jannaschii* produced an approximate mass of 1.1 MDa suggesting an icosahedral 60-mer structure analogous to those found in *B. subtilis, A. aeolicus* and spinach, but the deviations of the experimental data from the calculated sedimentation profile of an ideal solute (residuals in the top part of Fig. 3) are relatively large. This could be explained by nonideal solute behaviour or by an equilibrium state involving different oligomeric forms.

Electron micrographs of negative-stained lumazine synthase of *M. jannaschii* show roughly spherical particles with diameters around 15 nm (Fig. 4C). The images of the particles resemble closely those of icosahedral lumazine synthases from *B. subtilis, E. coli* and *A. aeolicus* (Fig. 4A,B,D). It should be noted that smaller oligomers, if present, are likely to have less characteristic shapes and may elude detection in electron micrographs.

**Table 3. Properties of lumazine synthases.**

<table>
<thead>
<tr>
<th>Source</th>
<th>$K_m$ (μM)</th>
<th>$K_m$ (μM)</th>
<th>$v_{max}$ (37 °C) (nmol mg⁻¹·min⁻¹)</th>
<th>Sedimentation velocity (S)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. jannaschii</em></td>
<td>52</td>
<td>12.5</td>
<td>11</td>
<td>~ 12</td>
<td>This study</td>
</tr>
<tr>
<td><em>A. aeolicus</em></td>
<td>26</td>
<td>10.0</td>
<td>31</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>55</td>
<td>9.0</td>
<td>242</td>
<td>26.5</td>
<td>[47]</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>62</td>
<td>4.2</td>
<td>197</td>
<td>26.8</td>
<td>[14]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>90</td>
<td>4.0</td>
<td>257</td>
<td>5.5</td>
<td>[14]</td>
</tr>
<tr>
<td><em>S. pombe</em></td>
<td>67</td>
<td>5.0</td>
<td>217</td>
<td>5.0</td>
<td>[48]</td>
</tr>
<tr>
<td><em>S. oleracea</em></td>
<td>26</td>
<td>20.0</td>
<td>275</td>
<td>–</td>
<td>[49]</td>
</tr>
</tbody>
</table>

$^a$ $K_m$ for 3,4-dihydroxy-2-butane 4-phosphate, $^b$ $K_m$ for 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione.

Compared with the lumazine synthase from *B. subtilis*, the enzyme from *M. jannaschii* has a shortened N-terminus (Fig. 5). In the lumazine synthase of *B. subtilis*, the first six amino acid residues form a β-strand contact with the central β-sheet of an adjacent subunit which was considered to be important for the association of the icosahedron. In order to prove the importance of the N-terminal sequence in the *B. subtilis* enzyme an N-terminal deletion mutant was produced as described in the Experimental procedures section. The mutant protein failed to fold in a soluble conformation when more than five amino acid residues were removed from the N-terminal domain (data not shown).

Boundary sedimentation of lumazine synthase from *M. jannaschii* afforded a sedimentation constant of about 12 S, whereas the sedimentation constants of 60-meric icosahedral lumazine synthases from various other organisms were invariably found in the range of 26 S (Table 3). Notably, the sedimenting boundary of the *M. jannaschii* enzyme is broader than that expected for a monodisperse, ideal solute. It is therefore not possible to determine the sedimentation rate with high accuracy.

In order to illustrate the characteristic difference in the sedimentation behaviour of the enzymes from *M. jannaschii* and *B. subtilis*, Fig. 6 shows a boundary sedimentation experiment with a mixture of the two proteins. In the upper part of that figure, the *B. subtilis* enzyme is seen to sediment as a relatively sharp boundary with an apparent velocity of 26 S. By comparison, the *M. jannaschii* enzyme observed in the lower part is characterized by a relatively slow-sedimenting, broad boundary.

**Discussion**

Lumazine synthase of the thermophilic Archaea show only relatively low similarity with those of eubacteria (Figs 5 and 7). In negatively stained electron micrographs, the enzyme from *M. jannaschii, E. coli, A. aeolicus* and *B. subtilis* all appear as essentially spherical particles with diameters around 15 nm (Fig. 4) [43]. In sedimentation equilibrium studies, these proteins have apparent molecular masses of 0.9–1 MDa, which identifies them as homooligomeric aggregates. However, the sedimentation equilibrium data of the *M. jannaschii* enzyme deviate significantly from the prediction for a homodisperse solute with ideal solute behaviour (Fig. 3).

The enzymes from *B. subtilis, A. aeolicus*, and spinach have all been shown by X-ray crystallography to consist...
of 60 identical subunits [15–17]. The particles have icosahedral 532 symmetry and form approximately spherical capsids with a central, approximately spherical cavity with a diameter of about 5 nm. In the case of lumazine synthase from Bacillaceae, the capsids can enclose a homotrimeric riboflavin synthase module [12,16,44,45]. That enzyme complex can catalyse both terminal reaction steps of the riboflavin biosynthesis, thus producing riboflavin from one molecule of structure 2 and two molecules of structure 4. The unusual molecular topology of that enzyme complex is associated with kinetic anomalies resulting from substrate channeling between the different protein modules [46].

Whereas the electron microscopic observations and the sedimentation equilibrium data suggest a similar molecular structure (i.e., a 60-meric icosahedral capsid architecture) for the M. jannaschii enzyme, the boundary sedimentation data are at odds with that model. The icosahedral lumazine synthases of E. coli and B. subtilis all sediment at a rate of about 26 S and show close to ideal solute behaviour. In

Table 4. Activation parameters for lumazine synthases from different organisms.

<table>
<thead>
<tr>
<th>Origin</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J K$^{-1}$mol$^{-1}$)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. jannaschii</td>
<td>63.7 ± 3.1</td>
<td>91 ± 6.6</td>
<td>61 ± 3.1</td>
<td>-96.8 ± 10.1</td>
<td>This study</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>74.6 ± 1.1</td>
<td>83 ± 1.0</td>
<td>76 ± 1.0</td>
<td>-22.4 ± 3.6</td>
<td>[50]</td>
</tr>
<tr>
<td>A. aeolicus</td>
<td>74.3 ± 1.1</td>
<td>88 ± 2.3</td>
<td>72 ± 1.1</td>
<td>-53.8 ± 3.4</td>
<td>This study</td>
</tr>
<tr>
<td>S. oleracea</td>
<td>87.1 ± 1.7</td>
<td>82 ± 0.4</td>
<td>84 ± 1.7</td>
<td>7.0 ± 5.6</td>
<td>[51]</td>
</tr>
<tr>
<td>M. grisea</td>
<td>90.0 ± 2.9</td>
<td>80 ± 0.4</td>
<td>83 ± 2.9</td>
<td>9.8 ± 9.8</td>
<td>[51]</td>
</tr>
<tr>
<td>E. coli</td>
<td>87.9 ± 4.2</td>
<td>82 ± 0.4</td>
<td>85 ± 4.2</td>
<td>9.8 ± 14.0</td>
<td>[51]</td>
</tr>
<tr>
<td>A. aeolicus</td>
<td>74.3 ± 1.1</td>
<td>88 ± 2.3</td>
<td>72 ± 1.1</td>
<td>-53.8 ± 3.4</td>
<td>This study</td>
</tr>
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<td>85 ± 4.2</td>
<td>9.8 ± 14.0</td>
<td>[51]</td>
</tr>
</tbody>
</table>

Fig. 3. Sedimentation equilibrium centrifugation of lumazine synthase from M. jannaschii. A solution containing 0.3 mg protein per mL of 50 mM potassium phosphate, pH 7.0, was centrifuged at 2000 g and 4 °C for 72 h. The line was calculated for an ideal solute with a relative mass of about 1 MDa and a partial specific volume of 0.752 mL g$^{-1}$. Residuals are shown in the top section.

Fig. 4. Electron micrographs of recombinant lumazine synthases from B. subtilis (A), E. coli (B), M. jannaschii (C) and A. aeolicus (D). The proteins were adsorbed on carbon and negatively stained with uranyl acetate. The bars represent 100 nm.

Fig. 5. Sequence comparison of the N-terminal domains of lumazine synthases. Conserved residues are shown with inverted contrast. Prolines are shown in grey. Residues that are part of the active site are marked by an asterisk [16].
level which is at present not understood. A more detailed description of structural peculiarities of the *M. jannaschii* enzyme may have to await the determination of its three-dimensional structure by X-ray crystallography.

It is unknown whether the *M. jannaschii* enzyme associates with a different protein, similar to the riboflavin synthase–lumazine synthase complex of Bacillaceae.

The kinetic properties of the *M. jannaschii* are remarkably different from those of the orthologs of eubacteria and euarkyots. At 37 °C, the catalytic rate is only about 5% when compared to mesophilic enzymes (Table 3). Even at a temperature of 70 °C, the specific activity is relatively low, with a value of 90 nmol·mg⁻¹·min⁻¹. By comparison, lumazine synthase from the hyperthermophilic, *A. aeolicus*, has catalytic rates of 31 and 425 nmol·mg⁻¹·min⁻¹ at temperatures of 37 and 70 °C (Fig. 2, Table 3).

The activation parameters of the *M. jannaschii* enzyme are strikingly different from those reported for other lumazine synthases. Enzymes from eubacteria and euarkyots have activation energies ranging from about 74–90 kJ·mol⁻¹, more than 10 kJ·mol⁻¹ in excess of the value for the enzyme from *M. jannaschii* (Table 4). On the other hand, the *M. jannaschii* enzyme has a large negative activation entropy (–973 K⁻¹·mol⁻¹), whereas the activation entropies of the other enzymes in Table 4 are close to zero, except for *A. aeolicus*.

The folding topology of all lumazine synthase studied at atomic resolution is characterized by parallel β-sheets flanked on both sides by α-helices. The N-terminus typically participates in the β-sheet of the adjacent subunit. The N-terminal part of the *M. jannaschii* enzyme is significantly shorter as compared to the orthologs from eubacteria, fungi and plants and could hardly serve as a link to the β-sheet of the adjacent subunit (Fig. 5). Remarkably, the pentameric lumazine synthase of *S. cerevisiae* tolerates the deletion of 17 amino acid residues at the N-terminus [13]. On the other hand, the icosahedral lumazine synthase of *B. subtilis* fails to fold correctly when more than five amino acid residues are deleted of the N-terminus. It is also noteworthy that the N-terminal segments of the pentameric, but not those of the icosahedral lumazine synthases, comprise proline residues. The *M. jannaschii* enzyme differs from both groups of lumazine synthases with respect to the N-terminus and the sedimentation behaviour.

Coenzyme biosynthesis pathways need to produce only relatively small amounts of the final product. Although the excess production of riboflavin has been observed in certain ascomycetes such as *Ashbya gossypii* and *Eremothecium ashbyii*, the amount of riboflavin produced by most microorganisms and by plants is low. The production of excess amounts could reduce the overall fitness by the wasting of resources. Hence, it is not surprising that the enzymes of riboflavin biosynthesis typically have low catalytic activities – in the low nmol·mg⁻¹·min⁻¹ range. These low activities may reflect the virtual absence of selective pressure conducive to the evolution of more efficient catalysis. This is particularly striking in case of the reaction catalysed by lumazine synthase which has been found to proceed with remarkably high velocity in mM substrate mixtures at pH 7.0 and room temperature [47]. The acceleration of that reaction by lumazine synthase from
M. jannaschii is unimpressive at best, with a catalytic rate in the range of 11 nmol mg⁻¹ min⁻¹ corresponding to a turnover number of around 0.17 per enzyme subunit per minute. In light of these arguments, the complex molecular structures of many well-studied lumazine synthases appears even more remarkable. Apparently, an amazingly complex molecular machinery is required in order to achieve the slight catalytic acceleration in the formation of 6,7-dimethyl-8-ribityllumazine that suits the metabolic requirements of the microorganisms.

Acknowledgements

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