doi:10.1016/j.jmb.2004.08.016 J. Mol. Biol. (2004) 343, 267–278

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Evolution of Vitamin B_2 Biosynthesis. A Novel Class of Riboflavin Synthase in Archaea†

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The open reading frame MJ1184 of *Methanococcus jannaschii* with similarity to riboflavin synthase of *Methanothermobacter thermoautotrophicus* was cloned into an expression vector but was poorly expressed in an *Escherichia coli* host strain. However, a synthetic open reading frame that was optimized for expression in *E. coli* directed the synthesis of abundant amounts of a protein with an apparent subunit mass of 17.5 kDa. The protein was purified to apparent homogeneity. Hydrodynamic studies indicated a relative mass of 88 kDa suggesting a homopentamer structure. The enzyme was shown to catalyze the formation of riboflavin from 6,7-dimethyl-8-ribityllumazine at a rate of 24 nmol mg^{-1} min^{-1} at 40 °C. Divalent metal ions, preferably manganese or magnesium, are required for maximum activity. In contrast to pentameric archaeal type riboflavin synthases, orthologs from plants, fungi and eubacteria are trimeric proteins characterized by an internal sequence repeat with similar folding patterns. In these organisms the reaction is achieved by binding the two substrate molecules in an antiparallel orientation. With the enzyme of *M. jannaschii*, ^{13}C NMR spectroscopy with ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine samples as substrates showed that the regiochemistry of the dismutation reaction is the same as observed in eubacteria and eukaryotes, however, in a non-pseudo-c_2 symmetric environment. Whereas the riboflavin synthases of *M. jannaschii* and *M. thermoautotrophicus* are devoid of similarity with those of eubacteria and eukaryotes, they have significant sequence similarity with 6,7-dimethyl-8-ribityllumazine synthases catalyzing the penultimate step of riboflavin biosynthesis. 6,7-Dimethyl-8-ribityllumazine synthase and the archaeal riboflavin synthase appear to have diverged early in the evolution of Archaea from a common ancestor. Some Archaea have eubacterial type riboflavin synthases which may have been acquired by lateral gene transfer.

Keywords: Archaea; *Methanococcus jannaschii*; riboflavin biosynthesis; novel type of riboflavin synthase; synthetic gene

Introduction

The biosynthesis of vitamin B_2 (riboflavin) in eubacteria and fungi has been studied in considerable detail.[1,2] Briefly, GTP cyclohydrolase II affords 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone.[3–5] Reduction of the ribose side-chain, deamination and dephosphorylation afford 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**2**) which is converted into 6,7-dimethyl-8-ribityllumazine (**5**) by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (**4**) obtained from ribulose 5-phosphate (**3**) by a mechanistically complex rearrangement reaction (Figure 1).[6–11]

The final step in the biosynthesis of the vitamin involves the dismutation of 6,7-dimethyl-8-ribityllumazine (**5**) catalyzed by riboflavin synthase.[12–15]

† This paper is dedicated to the memory of Dr Justus Henatsch, Europa Fachhochschule Fresenius Idstein.

Abbreviations used: LS, lumazine synthase; RS, riboflavin synthase.

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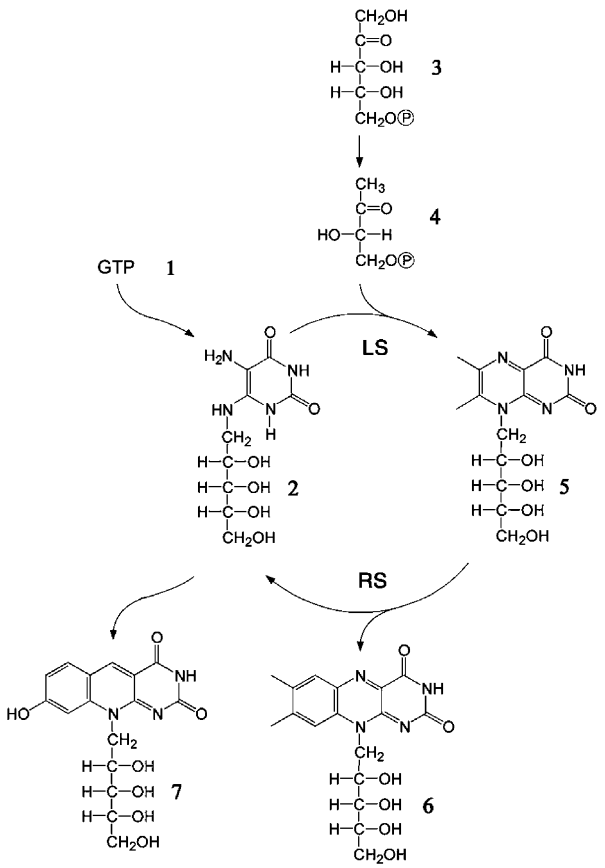

Figure 1. Biosynthesis of flavins. 1, GTP; 2, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; 3, ribulose 5-phosphate; 4, 3,4-dihydroxy-2-butanone 4-phosphate; 5, 6,7-dimethyl-8-ribityllumazine; 6, riboflavin; 7, 7,8-didemethyl-8-hydroxy-5-deazariboflavin.

The mechanistically unusual reaction involves the transfer of a four-carbon fragment between two identical substrate molecules. The second product, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, is recycled in the biosynthetic pathway by 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase). A pentacyclic intermediate of the reaction catalyzed by riboflavin synthase of *Escherichia coli* has been reported recently[16] whose structure is in line with the regiochemical course of the reaction involving a head to tail arrangement of the two four-carbon units from which the xylene ring of riboflavin is assembled.[17–19] Cofactor F_{420} found in methanogenic bacteria, is structurally similar to flavins.

The biosynthesis of riboflavin in Archaea has not been studied in detail. An *in vivo* study with *Methanothermobacter thermoautotrophicus* using [U-$^{13}C_2$]acetate and [1-$^{13}C_1$]pyruvate as tracers indicated that the xylene ring of the vitamin is assembled from two four-carbon units.[20] It was also shown that Compound **2** serves as an intermediate in the biosynthetic pathways of riboflavin as well as the 5-deazaflavin (**7**) derivative, coenzyme F_{420}.[21] A gene, coding for the 7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase was identified recently.[22] Moreover, Archaea were shown to form lumazine synthases and 3,4-dihydroxy-2-butanone 4-phosphate synthases closely resembling those of eubacteria, fungi and plants.[7,10,23]

The only known riboflavin synthase of archaeal origin, i.e. that of *M. thermoautotrophicus*, is a peptide of 156 amino acid residues devoid of similarity with the riboflavin synthases of eubacteria, fungi and plants[24] whose subunits comprise more than 200 amino acid residues and fold into two domains with closely similar topology due to their intramolecular sequence similarity.[14,25,26]

Here, we describe the efficient expression of riboflavin synthase of *Methanococcus jannaschii* using a synthetic gene and we show that riboflavin synthases of Archaea are devoid of sequence similarity to eubacterial or eukaryotic orthologs. On the contrary, they are paralogs of 6,7-dimethyl-8-ribityllumazine synthases.

Results

The hypothetical open reading frame MJ1184 of *M. jannaschii* predicting a protein of 156 amino acid residues with 66% similarity to riboflavin synthase of *M. thermoautotrophicus* (Figure 2) was amplified by PCR and was cloned into the expression vector pNCO113. A recombinant *E. coli* strain carrying the resulting plasmid failed to produce significant amounts of the cognate protein.

We therefore constructed a synthetic gene that was optimized for expression of the *M. jannaschii* gene in *E. coli* by stepwise PCR elongation using six oligonucleotide pairs as primers (Figure 3). The synthetic DNA segment was cloned into the

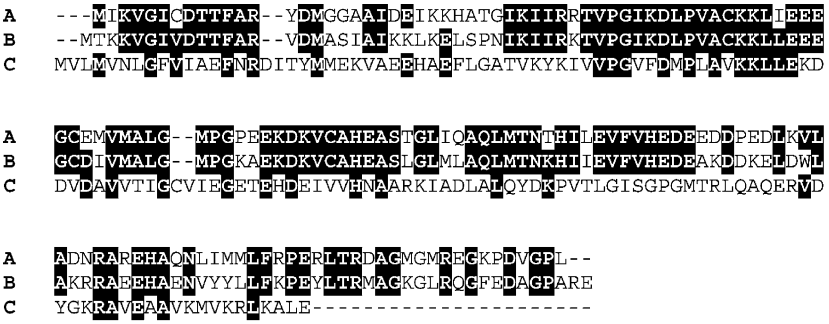

Figure 2. Sequence comparison of lumazine synthase and archaeal type riboflavin synthase. A, Riboflavin synthase of *Methanothermobacter thermoautotrophicus delta H* (accession no. Q59587); B, riboflavin synthase of *Methanococcus jannaschii* (accession no. Q58584); C, lumazine synthase of *Methanococcus jannaschii* (accession no. Q57751). Identical residues are shaded in black.

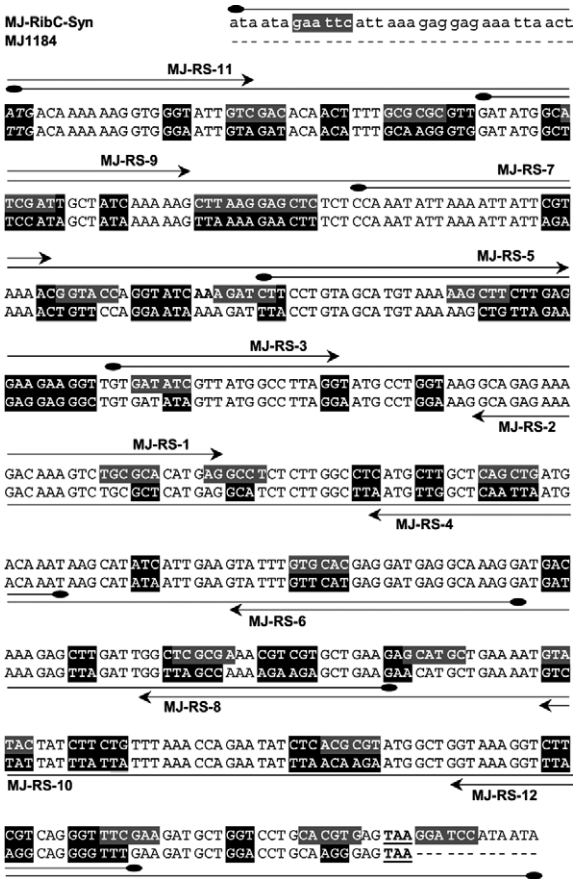

Figure 3. Construction of a synthetic gene for riboflavin synthase of *M. jannaschii*. Alignment of the wild-type DNA sequence (MJ1184) and the synthetic DNA sequence (MJ-RibC-syn) with 5′ and 3′ overhangs including the synthetic EcoRI and BamHI sites. Changed codons are shaded in black. New single restriction sites are shaded in grey. Oligonucleotides used as forward primers are drawn above and reverse primers below the aligned DNA sequences. The start codons of both sequences are in italics and the stop codons are shown in bold and underlined type.

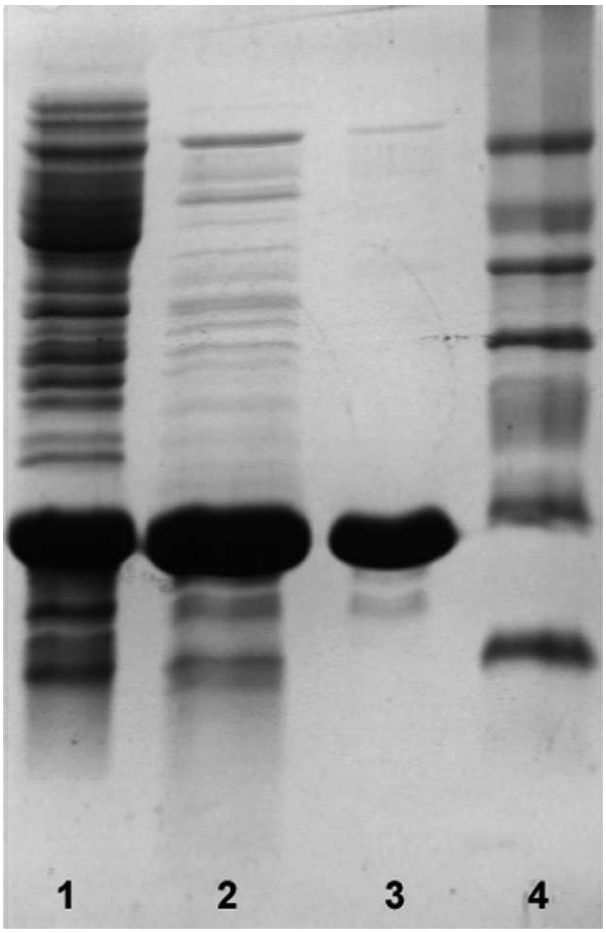

Figure 4. Purification of recombinant riboflavin synthase from *M. jannaschii* as shown by SDS-PAGE. Lane 1, crude lysate of *E. coli* M15-pREP4-pNCO-MJ-RS-WT; lane 2, cell extract after heat treatment; lane 3, protein fraction after gel filtration (Superdex 200); lane 4, SDS-PAGE size marker proteins.

plasmid pNCO113, and its sequence was verified by dideoxy termination sequencing. The synthetic gene contains 21 new singular restriction sites spaced at distances of 1–48 bp. Sixty codons (38%) were adapted to the preferred *E. coli* codon usage. The sequence has been deposited in GenBank (accession number AY442958).

A plasmid harboring the synthetic gene under the control of a phage T5 promoter and a *lac* operator directed the abundant synthesis of a protein with an approximate mass of 17.5 kDa in a recombinant *E. coli* strain. The recombinant protein was purified by heat treatment followed by size exclusion chromatography. The purified protein appeared homogeneous as judged by SDS-PAGE (Figure 4). Partial Edman degradation afforded the N-terminal sequence TKKVGIVDTTFARV as predicted from the nucleotide sequence. Notably, the N-terminal methionine had been removed by post-translational processing in the heterologous host bacteria. The subunit mass as determined by electrospray mass spectrometry was 17,364 Da in good agreement with a calculated mass of 17,363 Da.

The recombinant protein catalyzed the formation of riboflavin from 6,7-dimethyl-8-ribityllumazine at a rate of 24 nmol mg^{-1} min^{-1} at pH 7.0 and at 40 °C. Divalent cations, preferentially manganese or magnesium, were required for maximum catalytic activity. The residual catalytic activity of the purified enzyme without added divalent cations was 2 nmol mg^{-1} min^{-1}. That residual activity was not diminished any further by the addition of EDTA. Steady state kinetic analysis indicates positive cooperativity with a Hill coefficient of 2.1 (data not shown).

Kinetic properties of riboflavin synthases of different microorganisms are summarized in Table 1. The enzyme of *M. jannaschii* is similar to the enzyme of *M. thermoautotrophicus* with regard to catalytic activity.

The enzyme sediments as a single, symmetrical

Table 1. Specific activity of riboflavin synthases of different organisms

Organism	v_{max} (nmol mg^{-1} min^{-1})	Temperature (°C)	Source
M. jannaschii	24	40	This study
M. thermoautotrophicus	32[a]	65	24
	45[b]		
E. coli	21	20	15
B. subtilis	33	37	70
S. pombe	158	37	Unpublished
S. cerevisiae	232	37	71
A. thaliana	197	37	Unpublished

[a] Recombinant.
[b] Wild-type.

boundary with an apparent velocity of 4.3 S at 20 °C (Figure 5). Sedimentation equilibrium afforded a relative mass of 88.3 kDa which is close to the mass predicted for a homopentamer (87.5 kDa) (Figure 6).

The reaction catalyzed by riboflavin synthase implicates the transfer of a four-carbon fragment between two molecules of 6,7-dimethyl-8-ribityllumazine. Thus, the eight carbon atoms of the xylene moiety of riboflavin are all derived from the carbon atoms 6α, 6, 7 and 7α of the substrate. In that process, each methyl group of the acceptor substrate looses two H-atoms and becomes part of the carboxylic ring system. In studies with riboflavin synthase from Baker's yeast using deuterium-labeled 6,7-dimethyl-8-ribityllumazine as substrate, Beach & Plaut could show early on by ^{1}H NMR spectroscopy that the 6α methyl group of the donor substrate ends up as the 8α methyl group of the product, riboflavin,[17] thus suggesting that the two substrate molecules must be arranged with antiparallel orientation at the active site of the enzyme. This finding was later confirmed by work with riboflavin synthase of *Bacillus subtilis* using ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine as substrate.[19] It is also well in line with modeling studies based on the X-ray structures of riboflavin synthase from *E. coli* and *Schizosaccharomyces pombe*.[14,25]

Since the riboflavin synthases of Archaea are devoid of sequence similarity with those of eubacteria and eukarya, it was in order to check the regiochemistry of the catalytic process in *M. jannaschii*. For that purpose, ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine was treated with riboflavin synthase of *M. jannaschii*. On the basis of the known regiochemistry in eubacteria and yeast, the labeling patterns of riboflavin derived from ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine can be predicted (Figure 7). For example, the formation of [7,9a-$^{13}C_2$]- (6a), [6,8α-$^{13}C_2$]- (6b), and [7α,9-$^{13}C_2$]-riboflavin (6c) can be predicted *via* [7-$^{13}C_1$]- (5a), [6α-$^{13}C_1$]- (5b), and [7α-$^{13}C_1$]-labeled 6,7-dimethyl-8-ribityllumazine (5c), respectively. Figure 8(A) shows ^{13}C signals of riboflavin obtained from

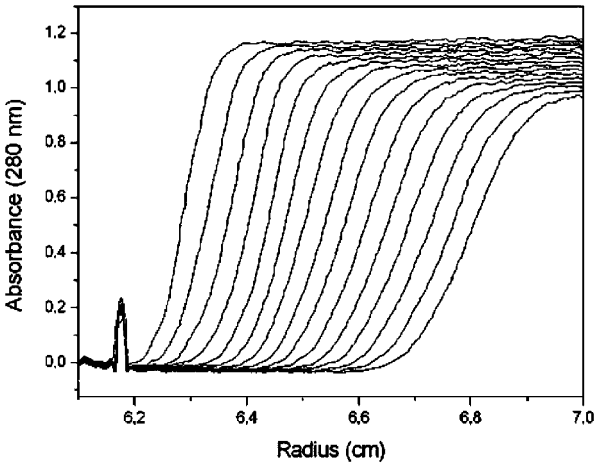

Figure 5. Boundary sedimentation of riboflavin synthase of *M. jannaschii*. Conditions used were: 20 °C, 288,000*g*; 50 mM Tris hydrochloride, 100 mM KCl (pH 7.0); initial protein concentration, 25 mg ml^{-1}. The sample was scanned at intervals of five minutes.

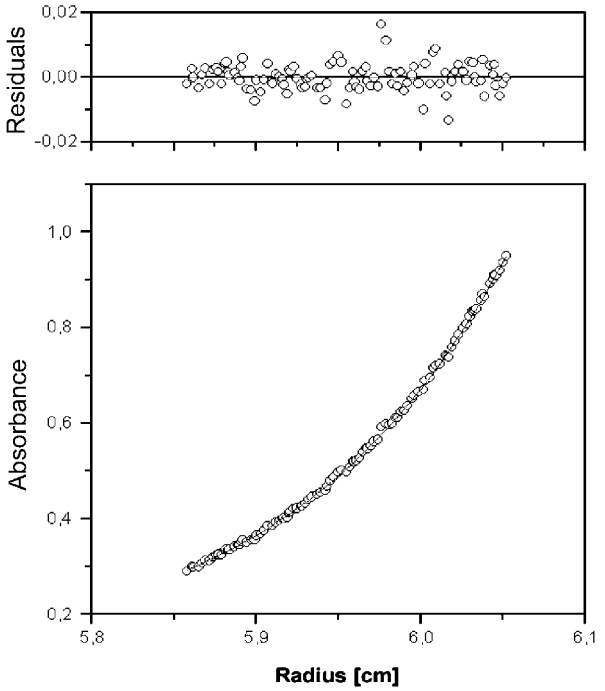

Figure 6. Sedimentation equilibrium of riboflavin synthase of *M. jannaschii*. Conditions used were: 10 °C, 11,000 rpm, 50 mM Tris hydrochloride, 100 mM KCl, (pH 7.0); initial protein concentration, 0.6 mg ml^{-1}. Residuals are shown at the top.

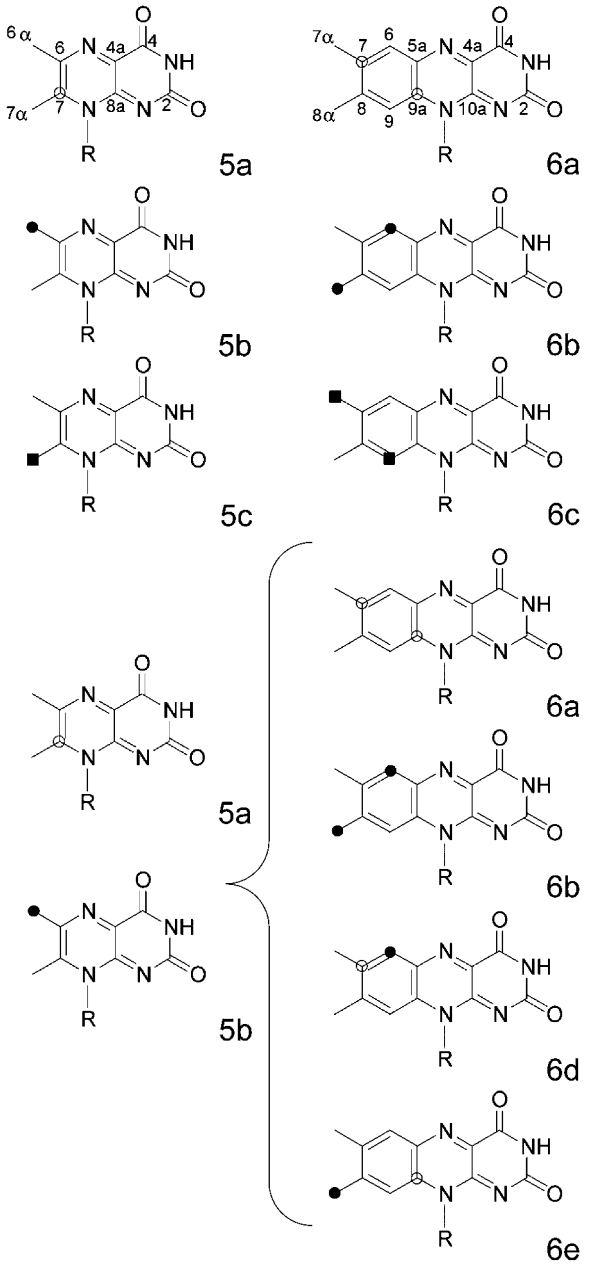

Figure 7. Isotopomers of 6,7-dimethyl-8-ribityllumazine (5a–c) and riboflavin obtained from 5a–c (6a–c) and an equimolar mixture of 5a and 5b (6a,b,d,e).

[7-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine with riboflavin synthase of *M. jannaschii*. The doublets at 139.2 ppm and 132.0 ppm are assigned as C-7 and C-9a, respectively, on the basis of a detailed analysis of the ^{13}C NMR spectrum of riboflavin which has been reported elsewhere.[27] The fine splitting of the signals can be explained by long-range carbon–carbon coupling in the doubly ^{13}C-labeled product. The other 15 carbon atoms of the enzyme product have intensities below the level of detection due to the absence of ^{13}C labeling.

Figure 8(B) and (C) shows ^{13}C signals of riboflavin obtained from [6α-$^{13}C_1$]- and [7α-$^{13}C_1$]-6,7-dimethyl-8-ribityllumazine, respectively. The signals appear as broad singlets or doublets due to $^{13}C^{13}C$ coupling *via* three bonds with coupling constants of 2–3 Hz. The signals are assigned to carbon atoms 6 and 8α in the experiment with [6α-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine and to carbon atoms 9 and 7α in the experiment with [7α-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine. These observations are in line with the predictions based on the regiochemical course of eubacterial and eukaryal riboflavin synthases.

A mixture of two isotopomeric substrate samples, [6α-$^{13}C_1$]- (5b) and [7-$^{13}C_1$]- 6,7-dimethyl-8-ribityllumazine (5a), should afford a total of four different riboflavin isotopomers (6a, 6b, 6d, and 6e), since each of the substrate isotopomers can serve either as the four-carbon donor or as the four-carbon acceptor in every possible combination (Figure 7). In line with that expectation, the signal of C-7 at 139.2 ppm appears as two superimposed doublets (Figure 8(D)). The outer lines are due to $^{13}C^{13}C$ coupling *via* one-bond coupling between C-6 and C-7 in the product isotopomer 6d. The doublet at the center of the signal group represents C-7 coupled to C-9a *via* three bonds in the isotopomer 6a (Figure 7). Similarly, the signal of C6 at 130.5 ppm comprises a doublet due to coupling between C-6 and C-7 in isotopomer 6d and a central singlet from isotopomer 6b. In summary, it can be concluded that the regiochemistry of riboflavin synthase of *M. jannaschii* is identical with the corresponding reactions in eubacteria and yeast.

A BLAST search with the *M. jannaschii* riboflavin synthase sequence revealed significant sequence similarity with 6,7-dimethyl-8-ribityl-lumazine synthases (Figure 9). Nethertheless, riboflavin synthase of *M. jannaschii* has no detectable lumazine synthase activity (<100 pmol mg^{-1} min^{-1}). As illustrated in Figure 2, riboflavin synthase and 6,7-dimethyl-8-ribityl-lumazine synthase of *M. jannaschii* show 34% identity and 59% similarity (screening matrix BLOSUM 62). A more detailed analysis including putative orthologs and/or paralogs in the genomes of 16 completely sequenced microorganisms[28] retrieved nine putative genes homologous to riboflavin synthase of *M. jannaschii*. The similarity between the cognate protein sequences is illustrated by the dendrogram in Figure 10. Eight sequences from Archaebacteria showed close similarity to the riboflavin synthases of *M. jannaschii* and *M. thermoautotrophicus*. Tentatively, these sequences are all believed to specify riboflavin synthases of the archaebacterial type. On the other hand, 12 archaebacterial sequences are similar to putative 6,7-dimethyl-8-ribityllumazine synthases from archaebacteria, eubacteria, yeasts, and plants, including the lumazine synthases of *M. jannaschii*, *B. subtilis*, *E. coli*, *Aquifex aeolicus*, *Brucella abortus*, *Saccharomyces cerevisiae*, *S. pombe* and *Spinacia oleracea* which have all been studied at the protein level.[14,23,29–35] Notably, all archaeal sequences in that group form a cluster with the *M. jannaschii* protein.[23] It is also worth noting that

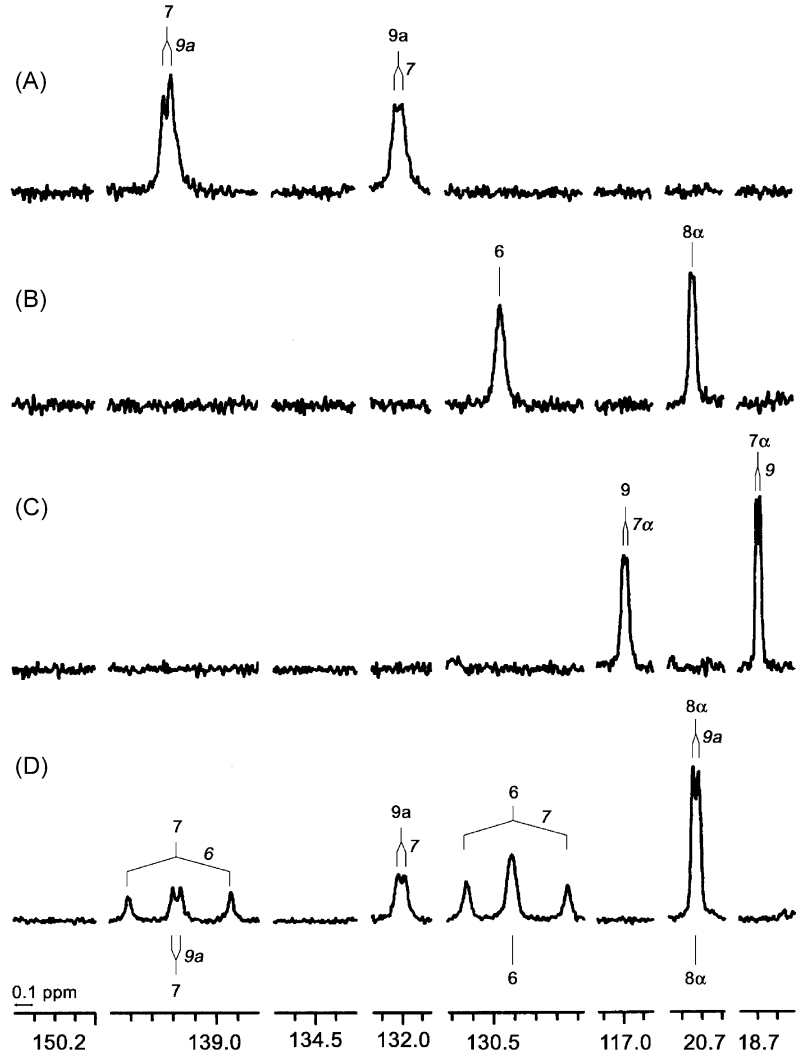

Figure 8. ^{13}C NMR signals of riboflavin isotopomers obtained by the catalytic activity of riboflavin synthase of *M. jannaschii* with (A) [7-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine; (B) [6α-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine; (C) [7α-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine; (D) equimolar mixture of [7-$^{13}C_1$]- and [6α-$^{13}C_1$]6,7-dimethyl-8-ribityllumazine as substrates.

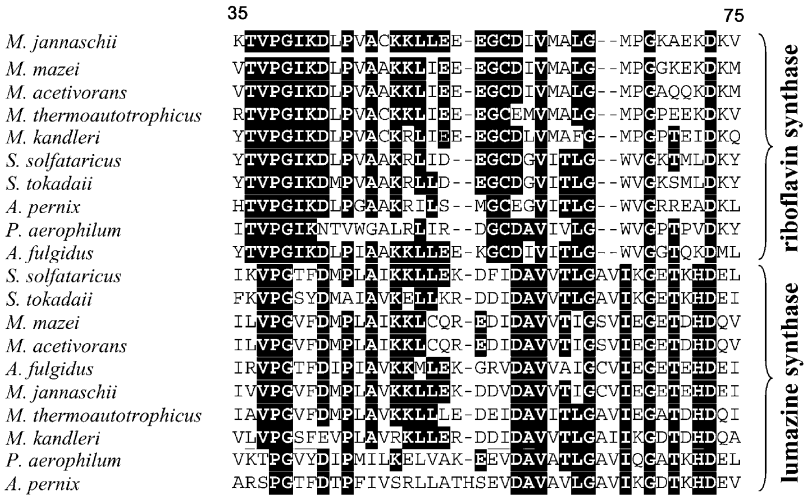

Figure 9. Sequence comparison (highly conserved sequence from position 35 to 75 of riboflavin synthase of *M. jannaschii*; first line) of archaeal type riboflavin synthase (RS) and lumazine synthase (LS). *Methanosarcina mazei Goe I* (RS, accession no. NP_632269; LS, accession no. NP_632268); *Methanococcus jannaschii* (RS, accession no. Q58584; LS, accession no. Q57751); *Methanopyrus kandleri AV19* (RS, accession no. NP_613646; LS, accession no. Q8TYL5); *Methanosarcina acetivorans C2A* (RS, accession no. NP_616743; LS, accession no. NP_616744); *Methanothermobacter thermoautotrophicus delta H* (RS, accession no. Q59587; LS, accession no. NP_276506); *Pyrobaculum aerophilum* (RS, accession no. NP_560637; LS, accession no. Q8ZTE3); *Aeropyrum pernix* (RS, accession no. Q9YDC5; LS, accession no. Q9YC88); *Sulfolobus solfataricus* (RS, accession no. NP_341940; LS, accession no. Q980B5); *Sulfolobus tokadaii* (RS, accession no. NP_376265; LS, accession no. Q975M5); *Archaeoglobus fulgidus DSM 4304* (RS, accession no. NP_070245; LS, accession no. NP_070953). Identical residues are shaded in black.

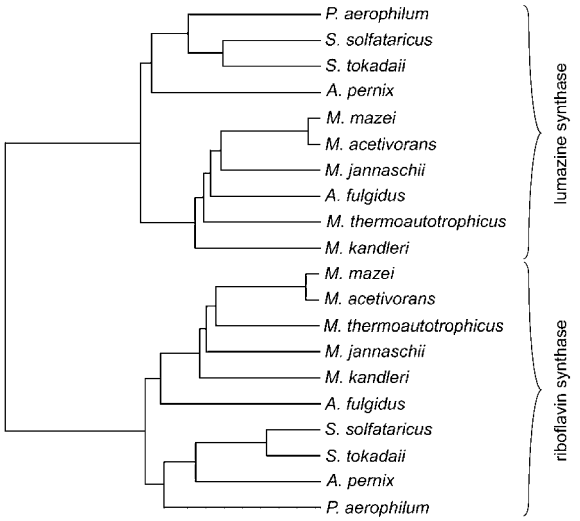

Figure 10. Phylogenetic tree of riboflavin synthases compared with lumazine synthases from archae bacteria. For details see the legend to Figure 9.

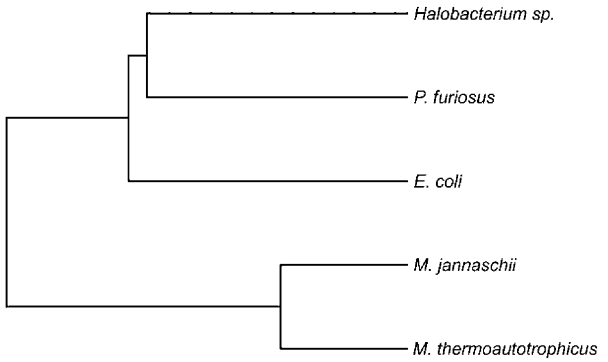

Figure 11. Phylogenetic tree of riboflavin synthases. *Methanothermobacter thermoautotrophicus delta H* (accession no. Q59587), *Methanococcus jannaschii* (accession no. Q58584), *Halobacterium sp. NRC-1* (accession no. NP_281179), *Pyrococcus furiosus DSM 3638* (accession no. NP_577790) and *Escherichia coli* (accession no. P29015).

Gram-negative bacteria form a subcluster by themselves; the same is true for the group of Gram-positive eubacteria as well as for the groups of fungi and plants, respectively.

Two archaebacterial species (*Pyrococcus furious* and *Halobacterium NRC-1*) carry putative genes with close similarity to the 6,7-dimethyl-8-ribityllumazine synthases of *M. jannaschii* but do not contain any putative orthologs of the *M. jannaschii* riboflavin synthase. However, the genomes of these microorganisms comprise genes specifying putative proteins with considerable similarity to the riboflavin synthases of eubacteria, fungi and plants (Table 2; Figure 11). Notably, the predicted protein sequences from *P. furious* and *Halobacterium NRC-1* show the intramolecular sequence similarity which is characteristic of the subunits of the homotrimeric riboflavin synthases of eubacteria and fungi. More specifically, the hypothetical N-terminal and C-terminal domains of the proteins from *Pyrococcus furiosus* and *Halobacterium NRC-1* share 20.4% and 25.2% identical amino acid residues, respectively.

Four of the completely sequenced archaebacterial species (*Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Thermoplasma acidophilum*, and *Thermoplasma volcanicum*) were devoid of any genes with similarity to 6,7-dimethyl-8-ribityllumazine synthase. Moreover, these organisms are also devoid of putative orthologs of 3,4-dihydroxy-2-butanone 4-phosphate synthase, yet another enzyme involved in the latter stages of the riboflavin biosynthetic pathway, thus suggesting that they do not synthesize riboflavin at all.

Discussion

The coenzymes derived from riboflavin (vitamin B_2) are characterized by an extraordinary chemical versatility. They can catalyze redox processes involving one- and two-electron transitions but also a variety of non-redox reactions such as photorepair of thymidine dimers in photodamaged DNA and the dehydration of non-activated organic substrates.[36,37] More recently, they have also been shown to act as chromophores in blue light photoreceptors in plants and fungi.[38–43]

On structural grounds, flavins and flavoenzymes should have been able to cooperate with non-protein enzymes in a hypothetical RNA world. Most notably, FAD would have provided optimum

Table 2. Distance matrix between riboflavin synthases of *M. jannaschii*, *M. thermoautotrophicus*, *P. furiosus*, *Halobacterium sp. NRC-I* and *E. coli*

Microorganism		A	B	C	D	E
M. jannaschii (156 aa)[a]	A	100				
M. thermoautotrophicus (153 aa)	B	65.38[b] (102)[c]	100			
P. furiosus (183 aa)	C	14.21 (26)	13.11 (24)	100		
Halobacterium sp. NRC-I (211 aa)	D	13.15 (28)	8.02 (17)	35.85 (76)	100	
E. coli (213 aa)	E	13.15 (28)	13.15 (28)	33.80 (72)	29.77 (64)	100

[a] Sequence length.
[b] Identity (%).
[c] Identity (residues).

conditions for base pairing with catalytic RNA species. It has been shown that artificial RNA sequences (aptamers) can bind FMN with very high affinity.[44,45] It has also been shown that the regulation of the riboflavin biosynthesis operon in Bacillaceae is based on the interaction of FMN with the mRNA specified by the *rib* operon resulting in premature transcription termination.[46]

A hypothetical RNA world would most probably have required catalytic redox processes, and flavin derivatives would have been excellent candidates due to the fact that the isoalloxazine chromophore can arise by a highly unusual reaction in the absence of any catalyst.[47] Thus, Wood, Plaut and their respective co-workers demonstrated the formation of riboflavin upon boiling of aqueous solutions of the biosynthetic precursor of riboflavin, 6,7-dimethyl-8-ribityllumazine, under anaerobic conditions.[48–50] It has also been shown that the lumazine derivative **5** can be formed under relatively mild conditions in the absence of catalysts from 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**2**) and ribulose bisphosphate (**3**).[47] Hence, flavins would have been excellent candidates for redox cofactors that could be obtained by spontaneous, prebiotic processes.

Since flavocoenzymes are apparently indispensable in all organisms, they must be obtained by biosynthesis or from the environment. Riboflavin biosynthesis has been shown to proceed in plants and in autotrophic microorganisms. Orthologs of 6,7-dimethyl-8-ribityllumazine synthases have been found in all organisms studied which were also known to biosynthesize riboflavin. The enzymes are c_5 symmetric homopentamers or 532 symmetric icosahedral capsids consisting of 60 identical subunits; the latter particles are best described as dodecamers of pentamers.[23,31–35,51,52] The subunit folds of all 6,7-dimethyl-8-ribityl-lumazine synthases studied so far are closely similar β sheets flanked on both sides by pairs of α helices.

The riboflavin synthases of eubacteria and yeasts have been shown to be homotrimers with unusual properties. Each subunit folds into two domains with closely similar folding topology.[14,53–55] Each of the six domains of the homotrimeric enzyme can bind one 6,7-dimethyl-8-ribityllumazine molecule in a shallow surface cavity, and the catalytic sites are built up from the respective substrate binding sites of one N-terminal domain and the C-terminal domain of an adjacent subunit. Unexpectedly, the *E. coli* enzyme is devoid of trigonal symmetry and may only form one active site at a given time.[25] Notably, homotrimeric riboflavin synthase molecules can be enclosed in the central cavity of the icosahedral 6,7-dimethyl-8-ribityllumazine synthases of Bacillaceae.[56,57]

Whereas the riboflavin synthases of the Archaebacteria, *M. jannaschii* and *M. thermoautotrophicus*, have no detectable sequence similarity whatsoever with the homotrimeric riboflavin synthases described above, they have considerable sequence similarity with 6,7-dimethyl-8-ribityllumazine synthases. Moreover, they share with the lumazine synthases the homopentamer structure as shown by hydrodynamic analysis.

As shown above, 12 completely sequenced Archaebacteria have paralog pairs which can be easily assigned as lumazine synthases and riboflavin synthases, respectively, on the basis of sequence similarity. On the other hand, ten of those Archaebacteria were found to be devoid of trimeric eubacterial/eukaryotic type riboflavin synthases.

The pentameric riboflavin synthases branch off the lumazine synthases at a very early stage of evolution (Figure 10). 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.[29,51] Quite obviously, the active site cavity is large enough to accommodate a riboflavin molecule. However, the mechanistic and stereochemical constraints described here also suggest that the pentameric lumazine synthase type riboflavin synthases can accommodate two molecules 6,7-dimethyl-8-ribityllumazine with essentially antiparallel orientation at the active site. Structure determination of an archaeal riboflavin synthase can be expected to provide answers to these obvious questions.

The redox properties of the riboflavin synthase substrate, 6,7-dimethyl-8-ribityllumazine, are rather similar to those of riboflavin. The lumazine derivative can easily undergo reversible reduction, but a more detailed study of its redox properties has not been performed. In principle, lumazine derivatives should be able to cooperate with enzymes as redox cofactors in a similar way as flavocoenzymes. No such enzymes have been reported up to now. However, 6,7-dimethyl 8-ribityllumazine is known to serve as the cofactor of certain luminescent proteins in marine bacteria which act as optical transponders for bioluminescence emission.[58,59] More specifically, these lumazine proteins acquire energy from luciferase by radiation-less transition; the role of lumazine proteins in the bioluminescence process has been shown to implicate modulation of the emission wavelength and enhancement of the quantum yield.[60,61] However, since the pentameric riboflavin synthases branch off the lumazine synthases at a very early level in archaeal development and at a very early level in general, the genetic data do not afford information with regard to the utilization of 6,7-dimethyl-8-ribityllumazine as an early coenzyme analog.

The dendrogram of lumazine synthases and pentameric riboflavin synthases is remarkably well in line with the currently accepted evolution of Archaebacteria, notably the division between Euryarchaeota and Crenarchaeota (Figure 10).[28] The presence of eubacterial type riboflavin synthases in two archaebacterial species may be explained by lateral gene transfer.

Materials and Methods

Materials

6,7-Dimethyl-8-ribityllumazine was synthesized by published procedures.[62] The preparation of [6α-$^{13}C_1$]-, [7-$^{13}C_1$]- and [7α-$^{13}C_1$]-6,7-dimethyl-8-ribityllumazine will be reported elsewhere. Restriction enzymes and Vent DNA polymerase were purchased from New England Biolabs, Schwalbach, Germany. Bacteriophage T4 DNA ligase was from Gibco BRL, Eggenstein, Germany. Taq Polymerase was from Finnzyme, Epsoo, Finland. DNA fragments were purified with the QIAquick PCR Purification Kit from Qiagen, Hilden, Germany. Oligonucleotides were custom-synthesized by MWG Biotech, Ebersberg, Germany.

Gene synthesis

The overlapping oligonucleotides MJ-RS-1 and MJ-RS-2 were annealed, and a double strand DNA segment of 104 bp was obtained by DNA polymerase treatment. In a sequence of five PCR amplifications using that oligonucleotide as template, the oligonucleotides listed in Table 3 were used pairwise (starting with MJ-RS-3 and MJ-RS-4) for the elongation of each prior amplificate (Figure 3). The final 517 bp amplificate was digested with EcoRI and BamHI and was ligated into the plasmid pNCO113 which had been treated with the same restriction enzymes. The resulting plasmid designated pNCO-MJ-RS-WT was transformed into *E. coli* XL1-Blue cells by published procedures (Table 4).[63] Transformants were selected on LB agar plates supplemented with ampicillin (170 mg l^{-1}). The plasmid was reisolated and transformed into *E. coli* M15 [pREP4] cells[64] carrying the pREP4 repressor plasmid for the overexpression of *lac* repressor protein. Kanamycin (20 mg l^{-1}) and ampicillin (170 mg l^{-1}) were added to secure the maintenance of both plasmids in the host strain.

Assay of protein concentration

Protein concentration was estimated by a modified Bradford procedure.[65]

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis

Experiments were performed with 16% (w/v) polyacrylamide gels by published procedures.[66] Molecular weight standard were supplied by Sigma (Munich, Germany).

Protein purification

The recombinant *E. coli* strain M15 [pREP4]-MJ-RS-WT was grown in LB medium containing ampicillin (170 mg l^{-1}) and kanamycin (20 mg l^{-1}) at 37 °C with shaking overnight. Erlenmeyer flasks containing 500 ml of medium were then inoculated at a ratio of 1 : 50 and were incubated at 37 °C with shaking. At an absorbance of 0.6 (600 nm), isopropylthiogalactoside was added to a final concentration of 2 mM, and incubation was continued for four hours. Cells were harvested by centrifugation and stored at −20 °C.

Frozen cell mass (5 g) was thawed in 50 mM Tris hydrochloride (pH 7). The suspension was ultrasonically treated and centrifuged. The supernatant was brought to 80 °C. After five minutes, the mixture was cooled to 10 °C and centrifuged. The supernatant was placed on top of a Superdex-200 column (2.6 cm×60 cm) which was then developed with 50 mM Tris hydrochloride (pH 7), containing 70 mM potassium chloride. Fractions were combined and concentrated by ultrafiltration using Amicon 3 kDa membranes.

Table 3. Oligonucleotides used for the construction of the synthetic gene coding for the riboflavin synthase of *M. jannaschii*

Designation	Sequence (5′ to 3′)
MJ-RS-1	tgtgatatcgttatggccttaggtatgcctggtaaggcagagaaagacaaagtctgcgcacatgag
MJ-RS-2	atttgtcatcagctgagcaagcatgaggccaagagaggcctcatgtgcgcagactttgtctttctctgc
MJ-RS-3	cttcctgtagcatgtaaaaagctgcttgaggaagaaggttgtgatatcgttatggccttagg
MJ-RS-4	cctttgcctcatcctcgtgcacaaatacttcaatgatatgcttatttgtcatcagctgagcaagcatg
MJ-RS-5	ccaaatattaaaattattcgtaaaacggtaccaggtatcaaagatcttcctgtagcatgtaaaaagctg
MJ-RS-6	cttcagcacgacgtttcgcgagccaatcaagctctttgtcatcctttgcctcatcctcgtgcacaaatac
MJ-RS-7	gatatggcatcgattgctatcaaaaagcttaaggagctctctccaaatattaaaattattcgtaaaac
MJ-RS-8	attctggtttaaacagaagatagtatacattttcagcatgctcttcagcacgacgtttcgcgagcc
MJ-RS-9	atgacaaaaaaggtgggtattgtcgacacaacttttgcgcgcgttgatatggcatcgattgctatcaaaaag
MJ-RS-10	cgaaaccctgacgaagacctttaccagccatacgcgtgagatattctggtttaaacagaagatagtatac
MJ-RS-11	ataatagaattcattaaagaggagaaattaactatgacaaaaaaggtgggtattgtc
MJ-RS-12	tattattataagcttactcacgtgcaggaccagcatcttcgaaaccctgacgaagacctttacc

Table 4. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source
E. coli strains		
XL1-Blue	recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F′, proAB, lacI^{q}ZΔM15, Tn*10*(tet^r)]	63
M15[pREP4]	lac, ara, gal, mtl, recA$^+$, uvr$^+$, Str^R, (pREP4: Kan^R, lacI)	64
Expression plasmids		
pNCO113	*E. coli* expression vector	6,64
pNCO-MJ-RS-WT	Expression vector for the overexpression of riboflavin synthase of *M. jannaschii* in *E. coli*	This study

Protein sequencing

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

Assay of riboflavin synthase activity

Assay mixtures contained 50 mM Tris hydrochloride (pH 7.0), 5 mM $MgCl_2$, and 500 μM 6,7-dimethyl-8-ribityllumazine. The reaction was initiated by the addition of protein. The mixtures were incubated for 30 minutes at 40 °C, and the reaction was then terminated by the addition of trichloroacetic acid to a final concentration of 0.3 M. The mixtures were centrifuged, and the supernatant was analyzed photometrically The absorbance coefficient for riboflavin is 9100 $M^{-1}\,cm^{-1}$ at 470 nm.[67]

Assay of lumazine synthase activity

Assay mixtures contained 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 16 mM 5-amino-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (**2**), 10 mM 3,4-dihydroxy-2-butanone 4-phosphate (**4**) and protein in a total volume of 600 μl. Absorbance was monitored photometrically at 410 nm at 37 °C. Blank values obtained without enzyme were subtracted.

Analytical ultracentrifugation

Experiments were performed with an Optima XL analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) equipped with absorbance and interference optics at 10 °C and 11,000 rpm. Aluminum double sector cells with sapphire windows were used throughout. Protein samples (0.58 mg ml^{-1}) were dialyzed against 50 mM Tris hydrochloride (pH 7.0), containing 100 mM potassium chloride. A partial specific volume of 0.745 $cm^3\,g^{-1}$ was estimated for riboflavin synthase of *M. jannaschii* on the basis of the amino acid composition.[68]

NMR spectroscopy

^{13}C NMR spectra were recorded at 125.7 MHz using a DRX500 AVANCE spectrometer from Bruker Instruments (Karlsruhe, Germany). Experimental setup and data processing were performed according to standard Bruker software (XWINNMR).

Electrospray mass spectrometry

Experiments were performed with a triple quadrupol ion spray mass spectrometer API365 (SciEx, Thornhill, Ontario, Canada).[69]

Acknowledgements

We thank Sebastian Schwamb and Thomas Wojtulewicz for skillful technical assistance. We thank Fritz Wendling for expert help with the preparation of the manuscript and Richard Feicht for skillful help for protein preparation. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and the Hans-Fischer-Gesellschaft.

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Edited by D. Rees

(Received 1 April 2004; received in revised form 5 August 2004; accepted 8 August 2004)