REVIEW

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Biosynthesis of flavocoenzymes

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The biosynthesis of one riboflavin molecule requires one molecule of GTP and two molecules of ribulose 5-phosphate. The imidazole ring of GTP is hydrolytically opened, yielding a 2,5-diaminopyrimidine that is converted to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione by a sequence of deamination, side chain reduction, and dephosphorylation. Condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione with 3,4-dihydroxy-2-butanone 4-phosphate obtained from ribulose 5-phosphate affords 6,7-dimethyl-8-ribityllumazine. Dismutation of the lumazine derivative yields riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, which is recycled in the biosynthetic pathway. The enzymes of the riboflavin pathway are potential targets for antibacterial agents.

1 Introduction

Flavocoenzymes are essential cofactors for the catalysis of a wide variety of redox reactions (for review *cf.* ref. 1). Moreover, they are involved in numerous other physiological processes involving light sensing, bioluminescence, circadian time-keeping and DNA repair.[2–21]

Vitamin B_2 (riboflavin) (Fig. 1, **10**) is the universal precursor of flavocoenzymes. It is biosynthesised by plants and many microorganisms but must be obtained from dietary sources by animals. The compound is manufactured in relatively large quantity (about 3000 metric tons per year) for use as a vitamin in human and animal nutrition and as a food colorant. Biotechnological aspects were an important driving force for studies on its biosynthesis which extended over a period of more than five decades. The production of the vitamin by fermentation has by now essentially replaced chemical synthesis (for review see ref. 22).

The biosynthesis of riboflavin has been reviewed repeatedly and the reader is directed to these articles for details of earlier work.[23–38] This review will predominantly describe recent developments in the area.

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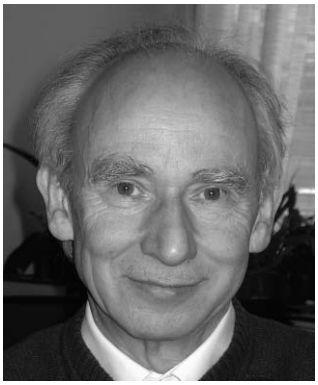
Markus Fischer



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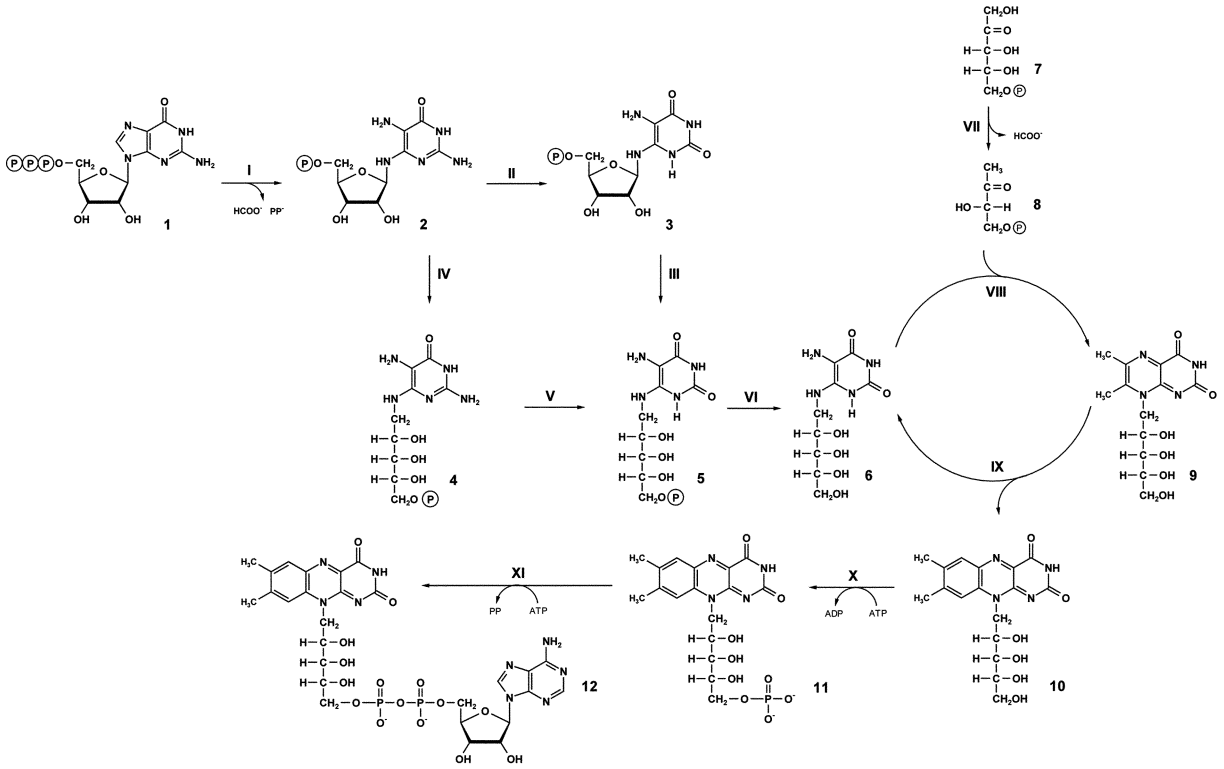

Fig. 1 Biosynthesis of riboflavin and flavocoenzymes.

The pathway of riboflavin biosynthesis summarised in Fig. 1 starts off from one molecule of GTP (**1**) and two molecules of ribulose 5-phosphate (**7**).[36,39–42] GTP cyclohydrolase II (Fig. 1, step **I**) catalyses the release of formate from the imidazole ring and the release of pyrophosphate from the side chain of the nucleotide precursor affording 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (**2**).[40,42] Hydrolytic release of the position 2 amino group, reduction of the ribose side chain and dephosphorylation afford 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**6**). The sequential order of deamination and side chain reduction varies in different taxonomic groups as described below.[43–48] 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione phosphate (**5**) needs to be dephosphorylated prior to the enzymatic formation of 6,7-dimethyl-8-ribityllumazine (**9**).[49,50]

The pyrimidine derivative **6** affords 6,7-dimethyl-8-ribityllumazine (**9**) by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (**8**) which is obtained from ribulose 5-phosphate (**7**) by a skeletal rearrangement.[49,51–54]

The final step of the biosynthetic pathway involves an unusual dismutation of the pteridine derivative **9** affording riboflavin (**10**) and the pyrimidine **6**.[35,55–59]

Although the early intermediates of the riboflavin pathway are 5′ phosphoric acid esters, the product of the biosynthetic pathway is unphosphorylated riboflavin. Hence, phosphorylation of riboflavin by flavokinase (Fig. 1, step **X**) is invariably required in prototrophic as well as in auxotrophic species in order to obtain riboflavin phosphate (FMN, **11**) and flavin adenine dinucleotide (FAD, **12**). The literature on riboflavin kinase and FAD Synthetase (Fig. 1, steps **X** and **XI**) has been reviewed earlier in some detail.[60]

Certain flavoenzmyes contain covalently linked flavocoenzymes. At least in the case of nicotine oxidase, the covalent linkage occurs by autoxidation.[61–63]

Recent developments in these areas are described in more detail below. A description of the numerous functions of flavins as cofactors of redox proteins is beyond the scope of this article. This field has been the subject of a three volume monograph.[1]

2 GTP cyclohydrolase II

GTP cyclohydrolase II (Fig. 1, step **I**) was originally discovered in cell extract of *Escherichia coli* and was shown to convert GTP (**1**) into a mixture of 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinedione phosphate (**2**), formate and inorganic pyrophosphate.[40,42] More recently, it was shown that the enzyme can also convert GTP into GMP, albeit at a lower rate.[64]

The hypothetical reaction mechanism shown in Fig. 2 suggests the initial formation of a covalent enzyme/intermediate adduct under release of pyrophosphate.[64] Hydrolytic cleavage of two carbon nitrogen bonds in the covalent adduct could then afford formate. Cleavage of the hypothetical phosphodiester bond, before or after hydrolytic opening of the imidazole ring, could result in formation of the pyrimidine **2**, the first committed intermediate of the riboflavin pathway. On the other hand, earlier cleavage of the phosphodiester motif, at the level of the hypothetical intermediate **13**, could afford GMP; in line with that hypothesis, GMP is unable to serve as substrate. 2,5-Diamino-6-ribosylamino-4(3*H*)-pyrimidinedione 5′-triphosphate can be converted into the riboflavin precursor **2** but does not fulfil the criteria of a kinetically competent intermediate.

GTP cyclohydrolase II of *E. coli* has been shown to contain one zinc ion per subunit. The replacement of any of the cystein residues 54, 65, or 67 yields catalytically inactive mutant proteins devoid of zinc.[65] Although the 3-dimensional structure of GTP cyclohydrolase II remains to be determined, these data strongly suggest the involvement of zinc in the hydrolytic opening of the imidazole ring of GTP.

Surprisingly, presteady state kinetic analysis showed that the hydrolytic release of pyrophosphate, the first step in the enzyme reaction, is the rate-determining step. Since all subsequent partial reactions are rapid by comparison with pyrophosphate release, they could not be resolved individually by kinetic analysis.[66]

The mechanistic peculiarities of GTP cyclohydrolase II call for a brief discussion of GTP cyclohydrolase I, the first committed enzyme in the biosynthesis of tetrahydrofolate and

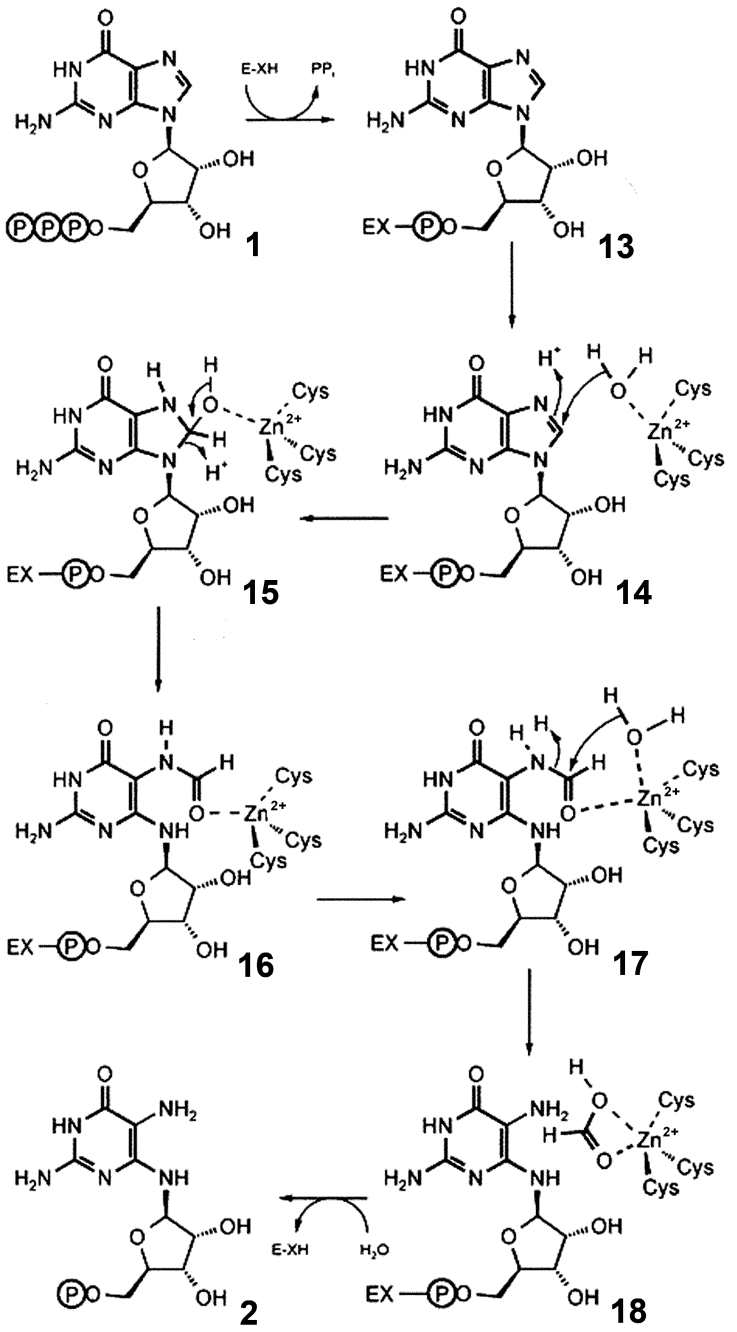

Fig. 2 Hypothetical mechanism for release of formate by GTP cyclohydrolase II.[65] Reprinted from *Eur. J. Biochem.*, **269**, 2002, Kaiser *et al.*, Biosynthesis of vitamin B2. An essential zinc ion at the catalytic site of GTP cyclohydrolase II, 5264–5270, Copyright (2002), with permission from Elsevier.

tetrahydropterin (Fig. 3) which converts GTP (**1**) into the bicyclical dihydroneopterin 3′-triphosphate (**25**) by a sequence of two hydrolytic steps (conducive to the release of C-8 as formate, in close parallel to GTP cyclohydrolase II) followed by an Amadori rearrangement of the carbohydrate side chain and, ultimately, ring closure. The mechanism comprises the following steps: (i) nucleophilic attack by a zinc-complexed water molecule to the position C-8 of the imidazole ring of the substrate (transition from GTP to intermediate **19**); (ii) hydrolytic opening of the imidazole ring (transition from **19** to **21**) with transient formation of a Schiff base (intermediate **20**); (iii) hydration of the formyl group of the 2-amino-5-formylamino-6-(ribosylamino)-4(3*H*)-pyrimidone 5′-triphosphate (transition from **21** to **22**); (iv) release of formate (transition from **23** to **24**); (v) Amadori rearrangement of the ribose ring and (vi) closure of the dihydropyrazine ring by intramolecular condensation affords the product dihydroneopterin 3′-triphosphate (**25**).[67–74]

In close parallel with GTP cyclohydrolase II, one zinc ion chelated by two thiol groups (Cys110 and 181 of the *E. coli* enzyme) and one histidine (His113 of the *E. coli* enzyme) is essential for hydrolytic cleavage of the two carbon nitrogen bonds resulting in the release of formate. An additional histidine (His112 of the *E. coli* enzyme) contacts the zinc ion *via* an interspersed water molecule and is believed to act as the nucleophile for hydrolytic attack of the imidazole ring.[74–77]

The formation of the dihydropyrazine ring is slow by comparison with the opening of the imidazole ring.[69,70] Thus, in both GTP cyclohydrolases, the hydrolytic cleavage of the imidazole ring is not the rate limiting step, although one would intuitively expect a relatively high free energy barrier.

Whereas the three-dimensional structure of GTP cyclohydrolase I has been studied in considerable detail,[74–76,78–80] that of GTP cyclohydrolase II remains to be determined. Numerous GTP cyclohydrolase II genes from different organisms have been

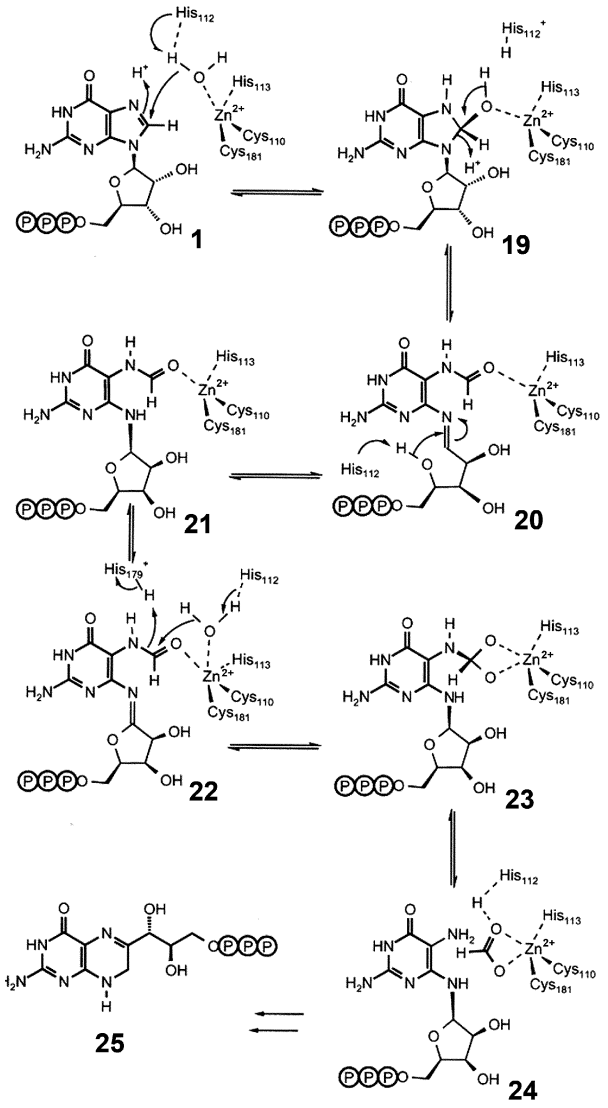

Fig. 3 Reaction catalysed by GTP cyclohydrolase I.[74] Reprinted from *J. Mol. Biol.*, **326**(2), 2003, Rebelo *et al.*, Biosynthesis of Pteridines. Reaction Mechanism of GTP Cyclohydrolase I, 503–516, Copyright (2003), with permission from Elsevier.

cloned and characterised.[81–86] The enzyme of *E. coli* was shown to form a homodimer.[40,85]

Plants and many eubacteria specify fusion proteins combining a GTP cyclohydrolase II domain (Fig. 1, step **I**) with a 3,4-dihydroxy-2-butanone 4-phosphate synthase domain (Fig. 1, step **VII**). These enzymes catalyse both initial steps of the convergent riboflavin pathway.[87–89]

3 Formation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione

5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**6**) was first established as an intermediate in the biosynthesis of riboflavin by work with riboflavin deficient mutants of the yeast, *Saccharomyces cerevisiae* and the ascomycete *Ashbya gossypii*.[90,91] It is formed in three steps from the product of GTP cyclohydrolase II (Fig. 1, step **I**). By comparison with the other reaction steps of the biosynthetic pathway, these steps are less well known.

Early *in vivo* work with riboflavin deficient yeast mutants indicated that the ribosyl moiety of **2** is reductively transformed (Fig. 1, step **IV**) into the ribityl side chain of **4**.[92,93] Deamination of **4** (Fig. 1, step **V**) affords the ribitylaminopyrimidinedione derivative **5**.[45,94] On the other hand, the deamination step was shown to precede the side chain reduction in eubacteria[48] and in higher plants.[46]

Many eubacteria specify bifunctional proteins combining deaminase (Fig. 1, step **II**) and reductase (Fig. 1, step **III**) domains. The deaminase and reductase domains of the bifunctional *ribG* gene of *B. subtilis* could be individually expressed as catalytically active proteins. More specifically, the N-terminal 1–147 amino acids were sufficient for deaminase activity, and the C-terminal 113–361 amino acids were sufficient for reductase activity.[48]

All available data indicate that the product of the early biosynthetic steps is the 5′-phosphate **5** which is produced from **3** in eubacteria and plants and from **4** in fungi and archaea.[45,94–99] On the other hand, lumazine synthase requires the dephosphorylated derivative **6** as substrate.[49,50] Hence, it appears that **5** must be dephosphorylated (Fig. 1, step **VI**) in order to become available as a substrate for lumazine synthase (Fig. 1, step **VIII**). However, a specific enzyme for catalysis of that reaction has not been identified hitherto. Similarly, in the tetrahydrofolate pathway, it is unknown how the dephosphorylation of dihydroneopterin triphosphate (**25**) is achieved. Conceivably, **5** could be dephosphorylated by a phosphatase (Fig. 1, step **VI**) with relatively low substrate specificity, but at least in yeasts, that enzyme should be able to discriminate between the isosteric compounds **4** and **5** in order to avoid preliminary termination of the biosynthetic reaction cascade *via* dephosphorylation of **4**.[94] It should also be noted that certain recombinant riboflavin producer strains of *Bacillus subtilis* can form very large amounts of the vitamin, apparently without any need for overexpression of the hypothetical phosphatase.[88,100]

4 3,4-Dihydroxy-2-butanone 4-phosphate synthase

A green fluorescent compound was found in the fifties by Masuda in the culture of a flavinogenic strain of the ascomycete, *Eremothetium ashbyii*, and was identified as 6,7-dimethyl-8-ribityllumazine (**9**).[101–103] Subsequent work by Plaut and his coworkers showed that this compound serves as the direct biosynthetic precursor of riboflavin (**10**).[55,56,104]

The structure of **9** suggested the requirement for an appropriate 4-carbon compound for the formation of the pyrazine ring from the purine precursor, but that compound remained elusive for an extended period (for review of the early work in that area, *cf.* Refs. 25, 28, 105).

In vivo studies with ^{13}C-labeled compounds ultimately identified the precursor of the 4-carbon compound as a carbohydrate at the biosynthetic level of a pentose or pentulose.[43,53,106–110] These *in vivo* studies showed that the formation of the specific 4-carbon intermediate of riboflavin biosynthesis proceeds by a skeletal rearrangement of the pentose/pentulose precursor under loss of carbon atom 4.

Work with riboflavin deficient mutants of the yeast, *Candida guilliermondii*, culminated in the isolation of an enzyme which converts ribulose 5-phosphate (Fig. 1, **VII**) into 3,4-dihydroxy-2-butanone 4-phosphate (**8**).[52,54,111,112] The enzyme catalyses the elimination of carbon 4 of the substrate as formate. Work with the purified enzyme confirmed the skeletal rearrangement that had been postulated on basis of the *in vivo* studies.[52–54,113]

A hypothetical mechanism for that complex reaction is summarised in Fig. 4.[114] It is believed that the initial reaction step consists in the formation of the endiol **26**. The elimination of the position 1 hydroxy group followed by keto–enol tautomerisation could afford the diketone **29** which could then undergo a sigmatropic rearrangement affording the branched aldose **30** that could fragment under formation of the endiol **32**. The reaction could be terminated by an enzyme-catalysed keto–enol tautomerisation.[113,114]

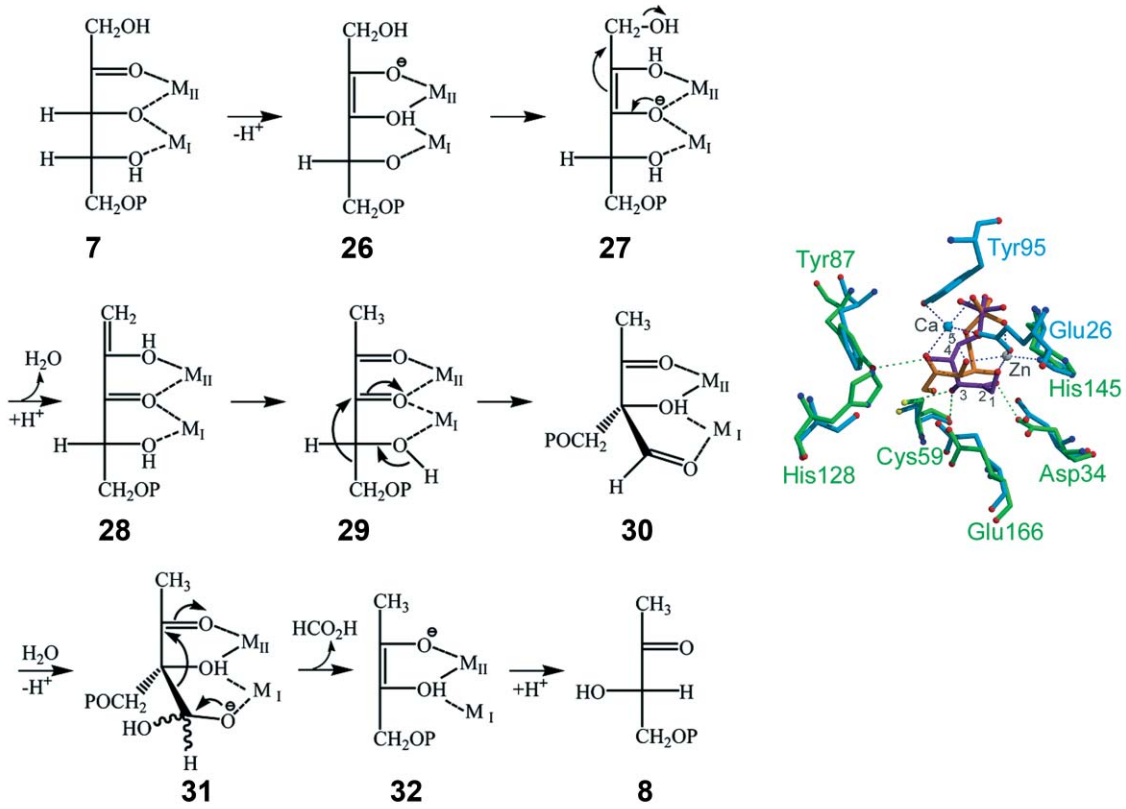

Fig. 4 Left: Hypothetical reaction mechanism of 3,4-dihydroxy-2-butanone 4-phosphate synthase (Fig. 1, step **VII**). Ribulose 5-phosphate (**7**) is bound to two divalent metal ions (MI, zinc; MII, calcium). Right: Comparison of the *C. albicans* VII (green) active site to that of *M. jannaschii* (blue): superposition of the active sites shows two different conformations for bound **7** (*C. albicans*: purple; *M. jannaschii*: orange). For *M. jannaschii* VII interactions with the bound metal ions are shown (Ca: blue; Zn: grey).[114,128,134] Reprinted from *J. Mol. Biol.*, **341**(4), 2004, Echt *et al.*, Potential Anti-infective Targets in Pathogenic Yeasts: Structure and Properties of 3,4-Dihydroxy-2-butanone 4-phosphate Synthase of *Candida albicans*, 1085–1096, Copyright (2004), with permission from Elsevier.

An endiol derived from ribulose 1,5-bisphosphate with close structural similarity to **27** is assumed to serve as the CO_2 acceptor in the reaction catalysed by ribulose bisphosphate carboxylase.[115–117] The diketo compound **29**, assumed to serve as intermediate in the reaction catalysed by 3,4-dihydroxy-2-butanone 4-phosphate synthase, was proposed to be a side product formed from ribulose 1,5-bisphosphate in mutants of ribulose bisphosphate carboxylase.[118–120]

The hypothetical intermediate **29** of the 3,4-dihydroxy-2-butanone 4-phosphate synthase reaction is also structurally similar to 1-deoxy-D-xylulose 5-phosphate, which was found to serve as an intermediate in the recently discovered non-mevalonate pathway of isoprenoid biosynthesis. In that pathway, a sigmatropic migration is supposed to yield a branched carbohydrate, which is subsequently reduced under formation of 2-C-methyl-D-erythritol 4-phosphate (for reviews, see refs. 121, 122).

Apparently, the final reprotonation of the endiol **32** in the 3,4-dihydroxy-2-butanone 4-phosphate synthase reaction proceeds under enzymatic catalysis since only the L(*S*)-enantiomer of **8** is obtained.[52,54] The stereochemistry of the rearrangement reaction has been studied in some detail.[123] Studies with stereospecifically labeled 5-[$^2H_1$]ribulose 5-phosphates showed that the rearrangement proceeds under retention of the configuration at C-4 of **8**, which is well in line with a 1,2-sigmatropic rearrangement.

The riboflavin precursor **9** could be obtained by reaction of **6** with ribulose 1,5-bisphosphate in the absence of an enzyme catalyst.[124,125] Ribulose bisphosphate was used in these experiments because the 1-phosphate group is superior to the position 1 hydroxyl group of **7** as a leaving group. Using ^{13}C-labeled starting materials, the uncatalysed reaction was shown to proceed with elimination of C-3 of the carbohydrate precursor as opposed to the elimination of C-4 in the enzyme-catalysed reaction.

3,4-Dihydroxy-2-butanone 4-phosphate synthase (Fig. 1, **VII**) was initially isolated from the yeast, *C. guilliermondii.* Subsequently, the genes specifying that enzyme have been cloned and expressed from a variety of microorganisms and plants.[84,87,113,126–129]

The enzyme from *E. coli* is a homodimer of 47 kDa subunits.[127,130–132] On the other hand, the enzymes from *B. subtilis* and from spinach are fusion proteins with GTP cyclohydrolase II domains fused to the C-terminal end of a 3,4-dihydroxy-2-butanone 4-phosphate synthase domain. These enzymes can catalyse both initial reaction steps of the convergent riboflavin biosynthetic pathway.[87]

The structure of 3,4-dihydroxy-2-butanone 4-phosphate synthase of *E. coli* has been solved by X-ray crystallography and by NMR spectroscopy.[130,131] X-Ray structures were also reported for the enzymes from *Magnaporthe grisea*, *Methanococcus jannaschii* and *Candida albicans*[114,128,133,134] (Fig. 5). The active site could be localised by crystallographic analysis of the enzyme from *M. jannaschii* and *C. albicans* in complex with ribulose phosphate;[114,128] glycerol has also been shown to bind to the active site of the protein.[131]

The two topologically equivalent active sites of 3,4-dihydroxy-2-butanone 4-phosphate synthase are located close to the subunit interface (Fig. 6). A loop comprising several highly conserved glutamate and aspartate residues is believed to fold over the substrate once it has bound to the active site (Fig. 7). The replacement of any of the conserved acidic amino acid residues in that loop inactivates the enzyme. Several other charged amino acid residues at the active site are likewise essential for enzymatic activity.

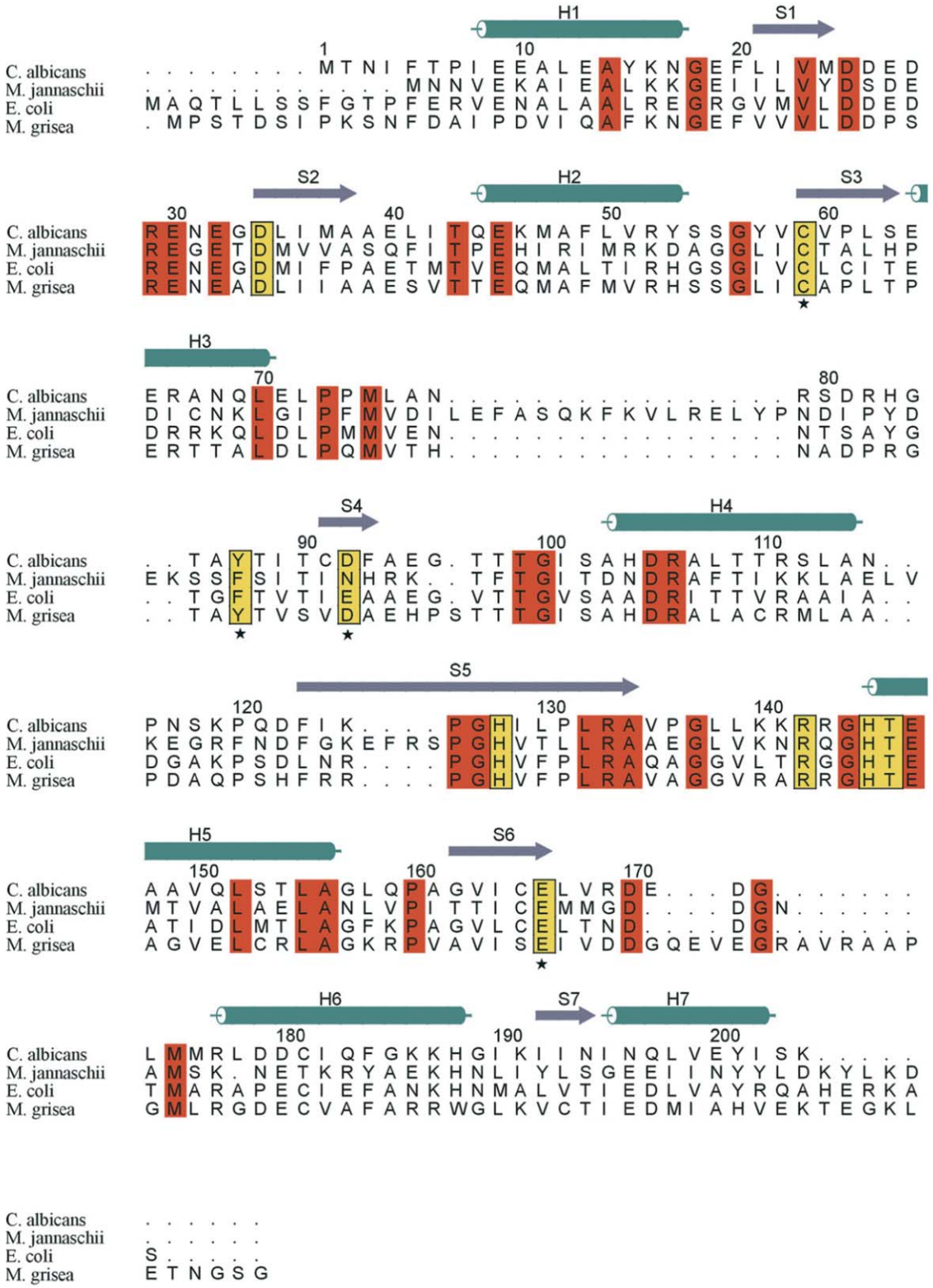

Fig. 5 Sequence alignment of 3,4-dihydroxy-2-butanone 4-phosphate synthases (Fig. 1, step **VII**) with known X-ray structure. Residues mutated in this study are marked with stars. Conserved residues are marked red. Active site residues are marked yellow. Secondary structure elements of the *C. albicans* enzyme are shown above the alignment.[114,128,133,134] Reprinted from *J. Mol. Biol.*, **341**(4), 2004, Echt *et al.*, Potential Anti-infective Targets in Pathogenic Yeasts: Structure and Properties of 3,4-Dihydroxy-2-butanone 4-phosphate Synthase of *Candida albicans*, 1085–1096, Copyright (2004), with permission from Elsevier.

5 Lumazine synthase

The condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**6**) with 3,4-dihydroxy-2-butanone 4-phosphate (**8**) is catalysed by 6,7-dimethyl-8-ribityllumazine synthase (Fig. 1, **VIII**). A hypothetical reaction mechanism is shown in Fig. 8.[51]

Based on the regiochemistry of the condensation which has been established by ^{13}C NMR spectroscopy, it is believed that the reaction is initiated by the formation of the Schiff' base **33** (Fig. 8).[51,91,135] The subsequent elimination of phosphate which is facilitated by the imine motif affords the enol type intermediate **34**. Subsequent to keto–enol tautomerization and rotation of the enol motif in the intermediate **34**, the carbonyl group in **36** could

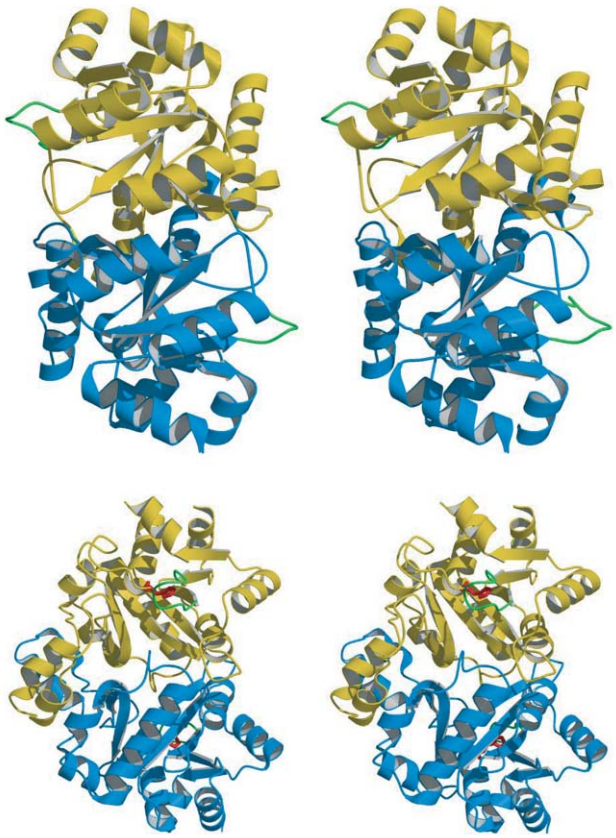

Fig. 6 Stereoview of the dimeric 3,4-dihydroxy-2-butanone 4-phosphate synthase of *M. jannaschii.* Subunits are shown in yellow and blue, respectively. The acidic active site loop is shown in light green.[114]

be attacked by the position 8 amino group, yielding the hydrated pteridine **37**. Dehydration of that unstable hydrate could afford 6,7-dimethyl-8-ribityllumazine (**9**).

The biosynthetic pathway involves the L-enantiomer of **8** which is the product of 3,4-dihydroxy-2-butanone 4-phosphate synthase (Fig. 1, step **VII**).[53,113,128] Lumazine synthase, however, has a remarkably low stereospecificity and uses the D-enantiomer almost as efficiently as the natural L-enantiomer. The velocity observed with the naturally occurring L(*S*)-enantiomer exceeds that observed with the D(*R*)-enantiomer only by a factor of 5.[51]

The carbohydrate substrate **8** of lumazine synthase is a rather unstable compound that decomposes spontaneously in neutral aqueous solutions at room temperature by elimination of phosphate to give butanedione.[51] Moreover, **8** reacts spontaneously with **6** at room temperature and neutral pH to give **9**.[136] This spontaneous condensation is incompletely regioselective, as shown by studies with 1-^{13}C-labeled **8**. These data suggest partitioning of the nonenzymatic reaction. The regiospecific product component is believed to be formed *via* the pathway shown in Fig. 8. The nonregiospecific pathway may proceed *via* elimination of phosphate from **8** and subsequent reaction of the resulting butanedione with the pyrimidine **6**.

Lumazine synthase genes have been cloned and expressed from a variety of microorganisms and plants. The enzymes are oligomers comprising 5 or 60 subunits of about 16 kDa whose structures have been studied in considerable detail by X-ray crystallography and electron microscopy.[137–150]

The representatives from fungi, *M. grisea*, *S. cerevisiae*, *Schizosaccharomyces pombe* and from the bacterium *Brucella abortus* are pentameric,[137,142,143,148,149] whereas the enzymes from *B. subtilis*, *Aquifex aeolicus*, *E. coli*, *M. jannaschii* and spinach (*Spinacia oleracea*) form capsids with icosahedral 532 symmetry and a relative mass of about 1 MDa and an outer diameter of about 15 nm.[138,139,144–148,151] The icosahedral lumazine synthases can be best described as dodecamers of pentamers (Fig. 9). In the Bacillaceae family, the central core of the capsid contains a riboflavin synthase homotrimer; historically, these unusual enzyme complexes were designated heavy riboflavin synthase.[152,153] Lumazine synthase of *B. subtilis* can be obtained as a hollow capsid in recombinant form.[154,155] Notably, the structure of the

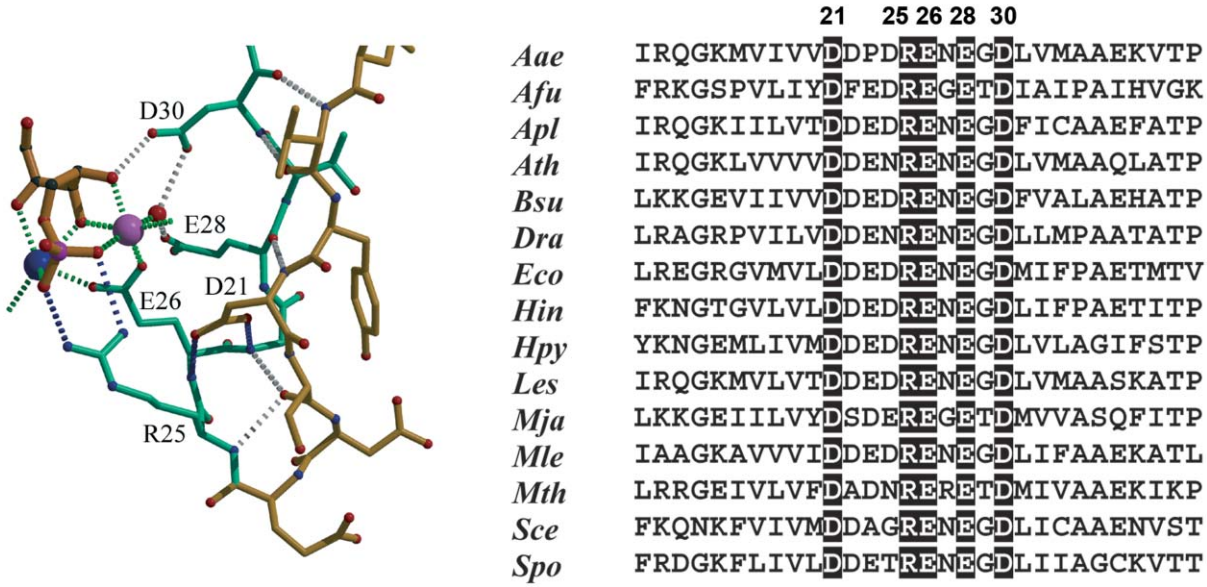

Fig. 7 Left: Structure of the acidic active site loop in the 3,4-dihydroxy-2-butanone 4-phosphate synthase of *M. jannaschii* with bound substrate ribulose-5-phosphate (Fig. 1, **7**). Right: Sequence alignment (position 11 to 40) of 3,4-dihydroxy-2-butanone 4-phosphate synthase. Aae, *Aquifex aeolicus*; Afu, *Archaeoglobus fulgidus*; Apl, *Actinobacillus pleuropneumoniae*; Ath, *Arabidopsis thaliana*; Bsu, *Bacillus subtilis*; Dra, *Deinococcus radiodurans*; Eco, *Escherichia coli*; Hin, *Haemophilus influenza*; Hpy, *Helicobacter pylori*; Les, *Lycopersicon esculentum*; Mja, *Methanoccocus jannaschii*; Mle, *Mycobacterium leprae*; Mth, *Methanothermobacter thermoautotrophicus*; Sce, *Saccharomyces cerevisiae*; Spo, *Schizosaccharomyces pombe*. Replacement of the marked amino acids yield in mutant proteins with dramatically decreased activity compared to the wild type enzyme.[113,114] Reprinted from *J. Biol. Chem.*, **377**(44), 2002, Fischer *et al.*, Biosynthesis of Riboflavin in Archaea Studies on the Mechanism of 3,4-Dihydroxy-2-butanone-4-phosphate Synthase of *Methanococcus jannaschii*, 41410–41416, Copyright (2002), with permission from The Journal of Biological Chemistry.

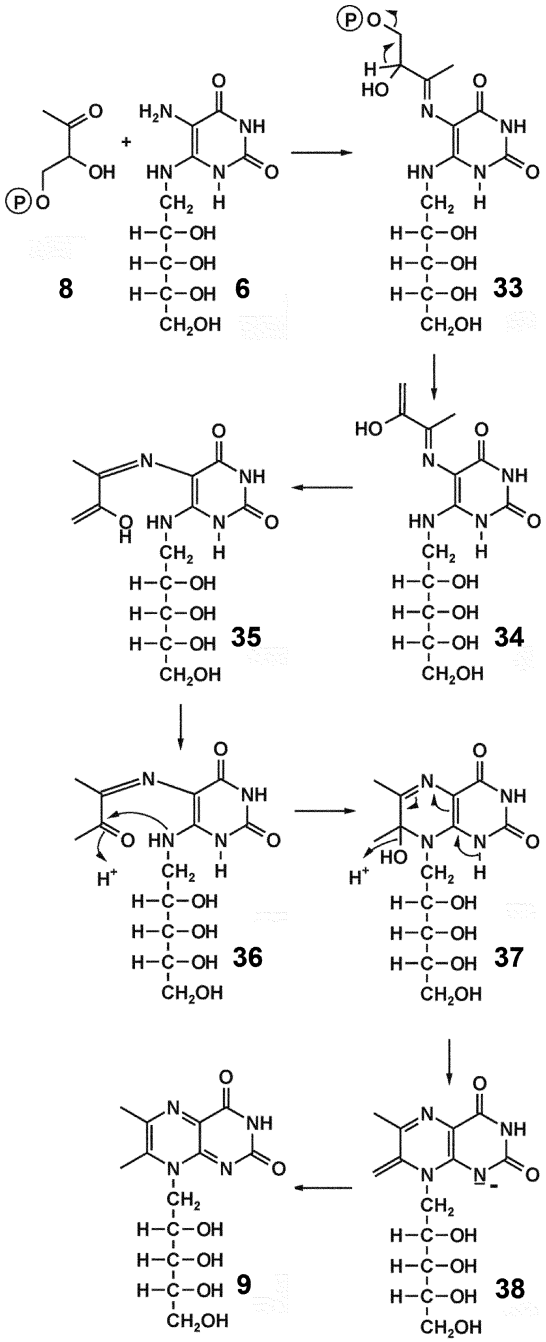

Fig. 8 Hypothetical reaction mechanism of lumazine synthase.

enzyme from the hyperthermophilic eubacterium, *A. aeolicus*, has been determined to a resolution of 1.6 Å.[146]

The pentameric enzymes of *S. cerevisiae* and *B. abortus* contain inserts of four amino acids between the helices α4 and α5 which have been hypothesised to be responsible for their inability to form an icosahedral capsid as a consequence of steric hindrance (Fig. 10).[143,149]

The *S. pombe* enzyme contains only a single added leucine residue in this location by comparison with the icosahedral enzymes studied. The purified *S. pombe* lumazine synthase was characterised by a bright yellow colour, in contrast with all other lumazine synthases which were obtained as colourless proteins. The yellow colour was caused by noncovalent binding of riboflavin together with small amounts of 6,7-dimethyl-8-ribityllumazine (Figs. 11, 12).[137,142,156]

The situation is reminiscent of earlier observations by Plaut and coworkers who obtained riboflavin synthase from baker's yeast as a complex with bound riboflavin even after extensive purification.[57] Dissociating conditions were required to remove

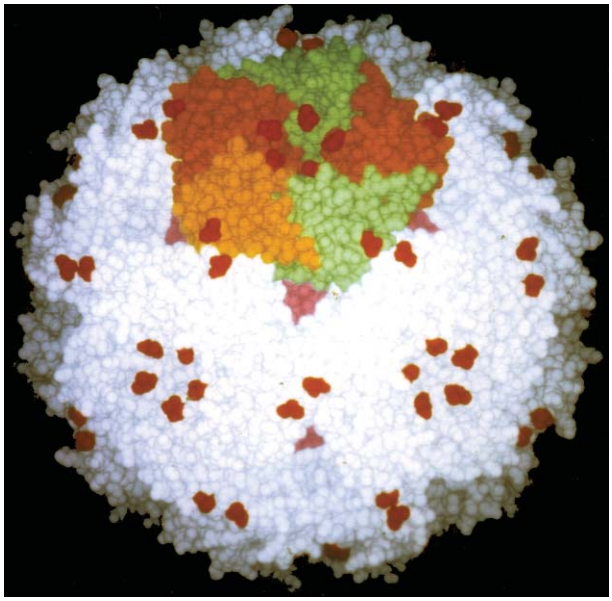

Fig. 9 Space filling model of the lumazine synthase capsid from *B. subtilis*. Subunits in one pentamer are shown in color. Selected amino acid residues are also shown in color to indicate areas where the 5-fold (red), 3-fold (violet), and 2-fold (red) symmetry axes penetrate the molecular surface.

the bound riboflavin from the *S. pombe* enzyme. This observation is well in line with the K_d value of 1.2 μM observed for riboflavin. The optical spectrum of riboflavin bound to lumazine synthase from *S. pombe* is characterised by a marked change in the relative intensities of the transition centred at 445 nm and 370 nm (Fig. 11). Moreover, significant absorbance is found in the wavelength range at least up to 550 nm.[156] This feature is much less pronounced in the W27Y mutant of lumazine synthase of *S. pombe*. One possible reason for this finding is that the phenyl ring of Y27 in the mutant is rotated such that the coplanarity of its π-system and that of riboflavin's isoalloxazine ring is reduced, whereas in the wild type enzyme the aromatic rings W27 and riboflavin are almost perfectly coplanar.[137]

The subunit fold and the pentamer topology are closely similar in all lumazine synthases studied up to now. The monomer fold is characterised by a 4-stranded β sheet which is flanked on both sides by pairs of α helices. In some cases, the N-terminal of one subunit participates in the β sheet of the adjacent subunit where it forms a fifth strand. The multiple, topologically equivalent active sites are all located at interfaces between adjacent subunits in the pentamer motif (Figs. 13, 14).

The active site topology has been analysed in considerable detail by X-ray structure analysis of the pentameric lumazine synthases of *S. cerevisiae* and *S. pombe* and the icosahedral enzyme of the hyperthermophilic eubacterium, *A. aeolicus*, in complex with various structural analogs of substrate and product and of putative intermediates.[137,142,143,145] The pyrimidine ring of the substrate **6** forms a coplanar π-complex with a phenylalanine or tryptophan side chain of lumazine synthases. X-ray data with the intermediate analog 5-(6-D-ribitylamino-2,4-dihydroxypyrimidine-5-yl)-1-pentenyl-phosphonic acid (Fig. 15, Compound **39**), which mimics the structure of the putative Schiff base intermediate **33**, show that the phosphate residue of 3,4-dihydroxy-2-butanone 4-phosphate (**8**) and of the intermediate resulting from its condensation with **6** forms an attractive ion pair contact with a highly conserved arginine residue (Fig. 15, Arg127).[143]

Surprisingly, the exchange of amino acid residues at the active site has relatively little impact on the catalytic rate. Even the replacement of the histidine residue 88 of the *B. subtilis* enzyme which is believed to participate in acid–base catalysis of

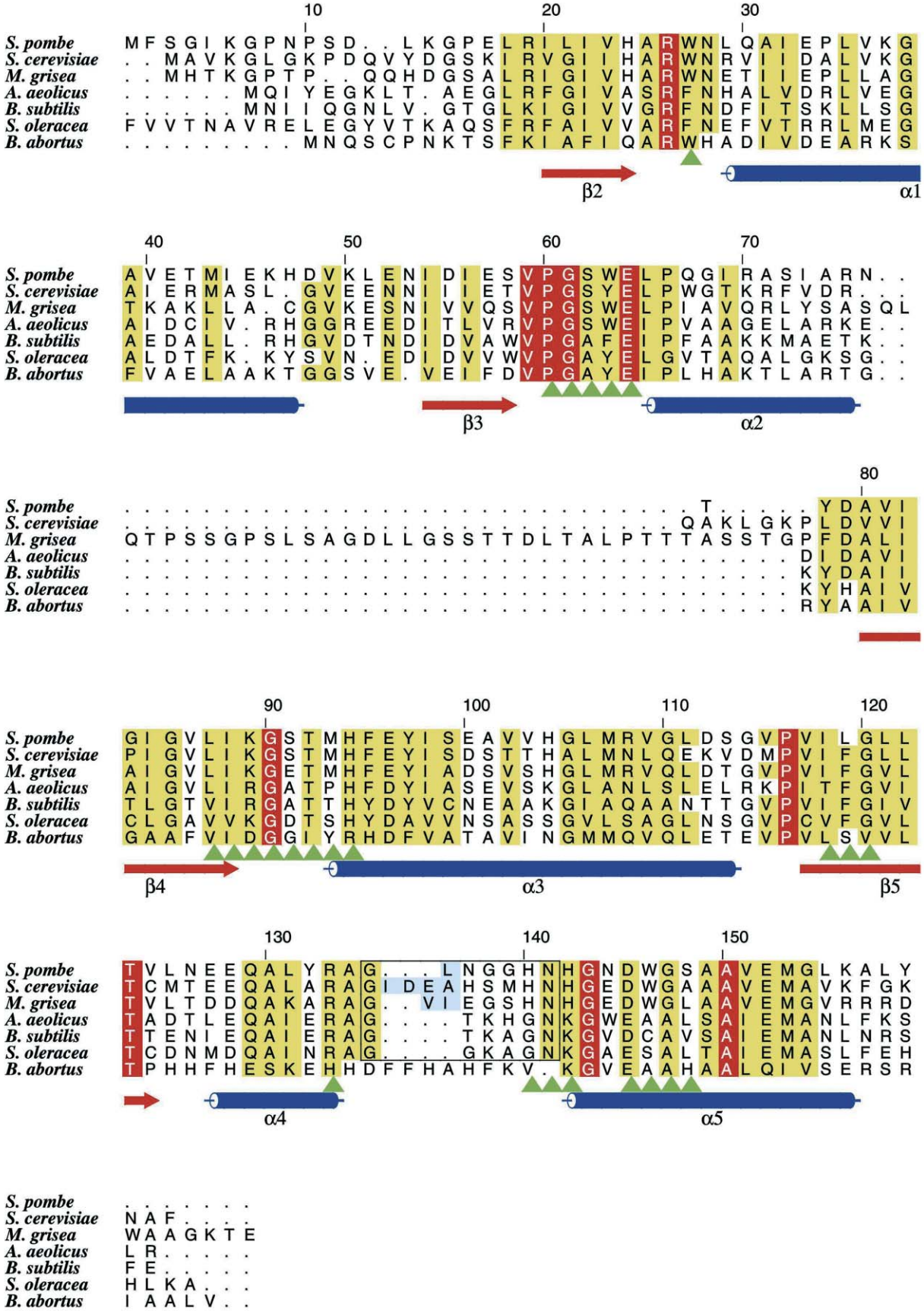

Fig. 10 Multiple sequence alignment of lumazine synthases with known crystal structure. The numbering above the alignment corresponds to wild-type enzyme from *S. pombe*. Secondary structure elements (blue, α-helices; red, β-strands) found in *S. pombe* lumazine synthase are shown below the sequences. Identical residues (red background), conserved residues (yellow background) and amino acid residues that are involved in riboflavin and phosphate ion binding (green arrows) are indicated. Insertions in the loop region (box) between the helices α4 and α5 are shown in blue.[142] Reprinted from *J. Mol. Biol.*, **318**(5), 2002, Gerhardt *et al.*, The Structural Basis of Riboflavin Binding to *Schizosaccharomyces pombe* 6,7-Dimethyl-8-ribityllumazine Synthase, 1317–1329, Copyright (2002), with permission from Elsevier.

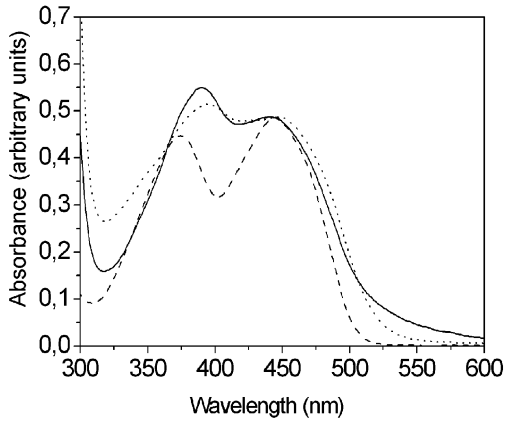

Fig. 11 Absorption spectra obtained in 50 mM potassium phosphate pH 7.0. Solid line, wild-type lumazine synthase of *S. pombe*; dotted line, W27Y mutant; dashed line, unbound riboflavin.[156] Reprinted from *Eur. J. Biochem.*, **269**, 2002, Fischer *et al.*, Biosynthesis of riboflavin. 6,7-Dimethyl-8-ribityllumazine synthase of *Schizosaccharomyces pombe*, 519–526, Copyright (2002), with permission from Blackwell Publishing.

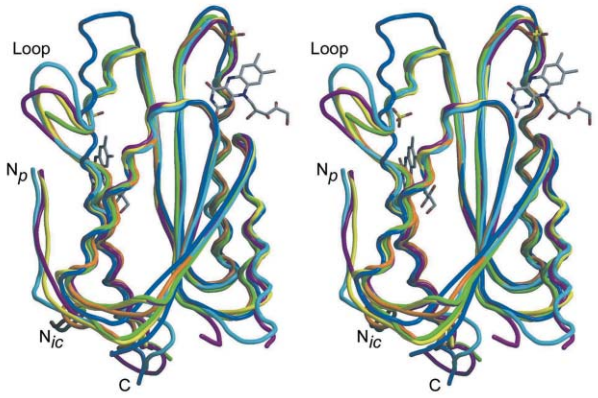

Fig. 13 Structural superposition of monomers of various lumazine synthases. Enzymes from *S. pombe* (yellow), *B. abortus* (dark blue), *S. cerevisiae* (light blue), and *M. grisea* (magenta) are pentameric, whereas lumazine synthases from *B. subtilis* (green), and spinach (orange) form icosahedral capsids. The increase of length of the loop connecting the helices α4 and α5 is in accordance with a different orientation of N termini for species forming an icosahedral capsid (N_{ic}) or a pentameric (N_p) structure. Bound riboflavin and phosphate ion are shown to mark the substrate binding site of *S. pombe* lumazine synthase.[142] Reprinted from *J. Mol. Biol.*, **318**(5), 2002, Gerhardt *et al.*, The Structural Basis of Riboflavin Binding to *Schizosaccharomyces pombe* 6,7-Dimethyl-8-ribityllumazine Synthase, 1317–1329, Copyright (2002), with permission from Elsevier.

the phosphate elimination steps has only a minor impact; a H88A mutant retains a relative activity of 10%.[155]

Presteady state kinetic analysis (Fig. 16) showed an early transient whose optical properties are well in line with the hypothetical Schiff base intermediate **33** (Fig. 8).[157,158] This transient (Fig. 16, **C**) is followed by a transient species with an absorption maximum at 445 nm (**D**) which could comprise any of the hypothetical intermediates **34** to **36** (Fig. 8). The rotation of the position 5 side chain into a conformational state suitable for ring closure or the ring closure *per se* is the rate determining step. On the basis of single wavelength stopped flow observations, it has been claimed that the dissociation of the product from the enzyme is the rate-limiting step.[159] This incorrect conclusion arose due to the inability to differentiate the strong transient band centered at 460 nm from the absorbance contribution of the product under the experimental conditions of that study.

Rate constants of partial reactions and activation parameters of lumazine synthase from *A. aeolicus* are summarised in Table 1.[157]

The maximum catalytic rate of lumazine synthases from mesophilic organisms at room temperature is about 2 per minute

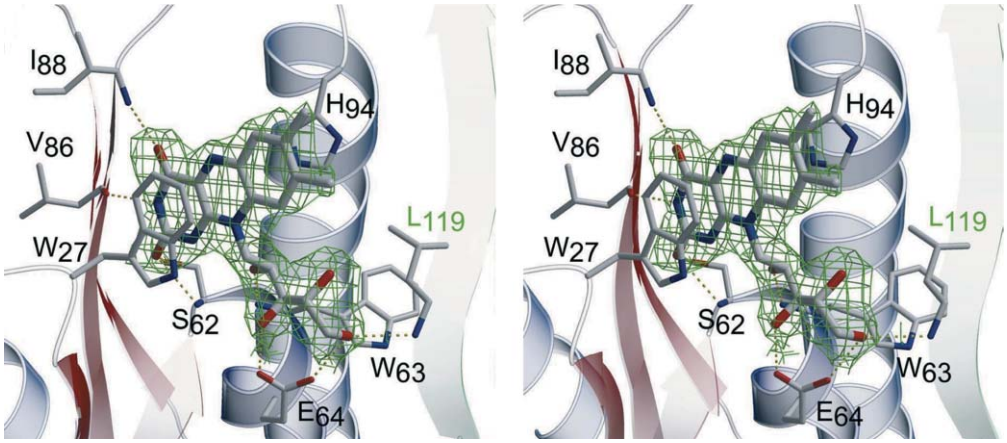

Fig. 12 Stereodrawing of the substrate binding site of lumazine synthase from *S. pombe* with bound riboflavin. Protein residues from the neighbouring subunit are shown in green.[142] Reprinted from *J. Mol. Biol.*, **318**(5), 2002, Gerhardt *et al.*, The Structural Basis of Riboflavin Binding to *Schizosaccharomyces pombe* 6,7-Dimethyl-8-ribityllumazine Synthase, 1317–1329, Copyright (2002), with permission from Elsevier.

Table 1 Rate constants of partial reactions and activation parameters of lumazine synthase from *A. aeolicus* (data from presteady state experiments)[157]

Reaction	Rate constants (25 °C)		E_a/kJ mol^{-1}	$\Delta G^{\ddagger}$/kJ mol^{-1}	$\Delta H^{\ddagger}$/kJ mol^{-1}	$-T\Delta S^{\ddagger}$/kJ mol^{-1}
A → B	k_1	0.0334 ± 0.0045 (μM^{-1} s^{-1})	57.6 ± 6.7	81.4 ± 7.4	55.1 ± 6.7	26.3 ± 0.7
B → C	k_2	0.3644 ± 0.0857 (s^{-1})	75.3 ± 5.1	75.5 ± 10.1	72.8 ± 5.1	2.7 ± 5.0
C → D	k_3	0.0172 ± 0.0016 (s^{-1})	81.0 ± 1.0	83.1 ± 3.0	78.4 ± 1.5	4.7 ± 0.9
D→ E	k_4	0.0034 ± 0.0001 (s^{-1})	76.1 ± 0.7	87.1 ± 1.4	73.5 ± 0.7	13.6 ± 0.7
A → E[a]	k	0.0027 ± 0.0002 (s^{-1})	74.3 ± 1.1	88.0 ± 2.3	72 ± 1.1	16.0 ± 1.0

[a] steady state kinetic experiment

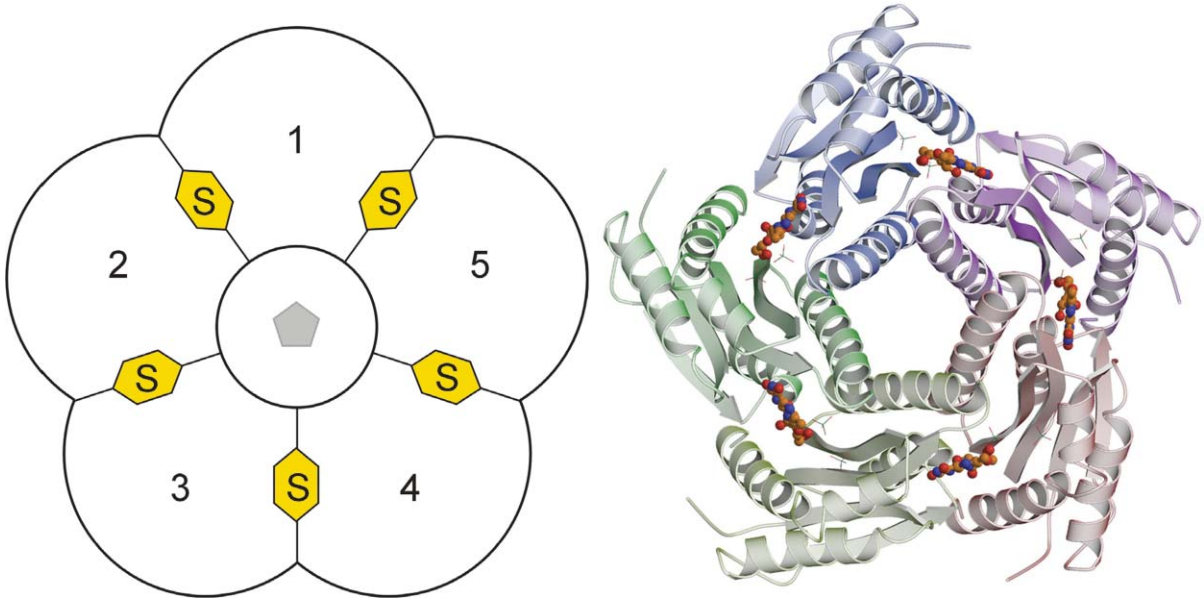

Fig. 14 Left: Schematic ligand binding at the active sites of a lumazine synthase; Right: Pentameric assembly of *S. pombe* lumazine synthase viewed along the 5-fold non-crystallographic symmetry axis. The active sites are built up by two adjacent monomers. Bound riboflavin is shown in ball and stick.

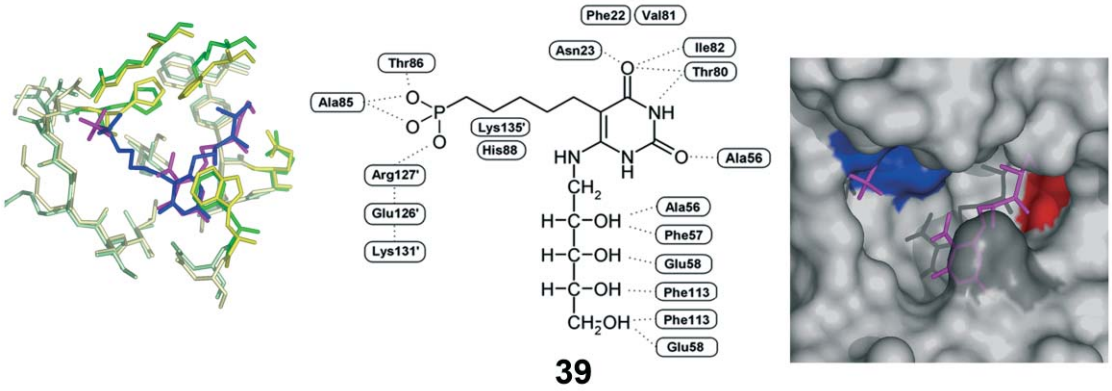

Fig. 15 Active site topology of lumazine synthase. Left: Comparison of the active sites of *B. subtilis* (LSBS) and *S. cerevisiae* (LSSC) lumazine synthase. For the alignment, only residues within a circumference of 10 Å around the pentylphosphonate inhibitor were used. Color codes are as follows: labelled residues of LSSC: yellow; all other residues in LSSC: pale yellow; labelled residues of LSSC: green; all other residues in LSSC: pale green; 5-nitro-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione and phosphate anion: blue; 5-(6-D-ribitylamino-2,4-dihydroxypyrimidine-5-yl)-1-pentenyl-phosphonic acid (**39**): pink. Center: Schematic representation of close contacts between the pentylphosphonate inhibitor and the LSBS active site after alignment. All contacts are derived from the alignment between LSBS and LSSC as described above. Ser142 and Asp138 lie in 4–8 Å distance to the ribityl side chain at the entrance to the active site. Right: Active site of *B. subtilis* lumazine synthase in complex with 5-nitro-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione. Color codes are as follows: Glu58, red; His88, Arg127, Lys131, blue; Phe22, grey.[155] Reprinted from *J. Mol. Biol.*, **326**(3), 2003, Fischer *et al.*, Enzyme Catalysis *via* Control of Activation Entropy: Site-directed Mutagenesis of 6,7-Dimethyl-8-ribityllumazine Synthase, 783–793, Copyright (2003), with permission from Elsevier.

and per active site. Since the reaction can proceed without catalysis in dilute solution at room temperature and neutral pH, the rate acceleration by the enzyme is really quite limited. Even more surprisingly, the activation energy of the uncatalysed reaction is lower than that of the enzyme catalysed reaction, at least in case of the enzymes from eubacteria and spinach (Table 2).[136,155,159] These counterintuitive findings suggest that the enzyme exerts its catalytic acceleration predominantly *via* topologic confinement of the reactants. The rate acceleration is controlled by entropic rather than enthalpic parameters.[155]

A series of fluorinated derivatives of 6,7-dimethyl-8-ribityllumazine (**9**) have been prepared and used as ^{19}F-NMR probes. The reaction of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione with hexafluorodiacetyl afforded two diastereomers of **40** and **41** (Fig. 17).[160] The presence of the fluorine atoms is responsible for the covalent hydrate structure and the existence of stable diastereomers differing by the configuration at C-7. Since the absolute configuration of the diastereomers was initially unknown, they were designated epimer A and B based on their chromatographic properties. The two epimeric

Table 2 Activation parameters of lumazine synthases from several organisms[155]

Enzyme	E_a/kJ mol^{-1}	ΔG^*/kJ mol^{-1}	ΔH^*/kJ mol^{-1}	ΔS^*/J K^{-1} mol^{-1}
B. subtilis	74.6 ± 1.1	83 ± 1	76 ± 1	−22.4 ± 3.6
S. oleracea	87.1 ± 1.7	82 ± 0.4	84 ± 1.7	7.0 ± 5.6
M. grisea	90.0 ± 2.9	80 ± 0.4	83 ± 2.9	9.8 ± 9.8
E. coli	87.9 ± 4.2	82 ± 0.4	85 ± 4.2	9.8 ± 14.0
Non-catalysed	46.3 ± 0.6	86 ± 0.5	45 ± 0.5	−127.1 ± 1.6

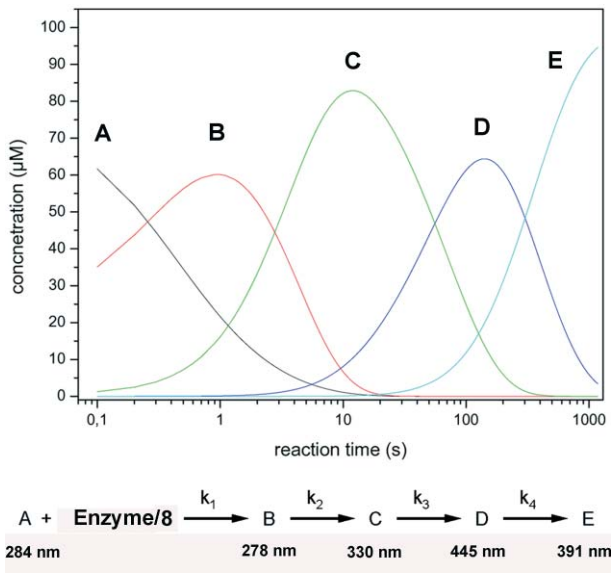

Fig. 16 Presteady state kinetics of lumazine synthase from *A. aeolicus*. Numerical deconvolution of the stopped flow experiments. Time resolved concentrations of transient species. Enzyme–**8**, enzyme pre-incubated with 3,4-dihydroxy-2-butanone 4-phosphate; **A**, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; **B, C, D**, transients; **E**, enzyme bound 6,7-dimethyl-8-ribityllumazine.[157]

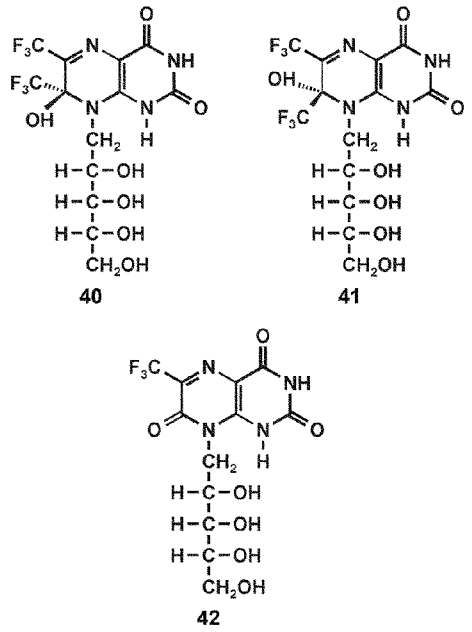

Fig. 17 Fluorinated analogues of 6,7-bis(trifluoromethyl)-8-D-ribityllumazine. **40**, Epimer A of 6,7-bis(trifluormethyl)-7-hydroxyl-8-ribityllumazine; **41**, Epimer B of 6,7-bis(trifluormethyl)-7-hydroxyl-8-ribityllumazine; **42**, 6-trifluoromethyl-7-oxo-8-ribityllumazine.

covalent hydrates of 6,7-bis(trifluoromethyl)-8-D-ribityllumazine are metabolically stable analogues of hypothetical intermediates (Fig. 8, compound **37**) proposed in the reactions catalysed by riboflavin synthase and lumazine synthase.

The distances between the trifluoromethyl groups of **40** and **41** and several side chains, including H88, have been determined by $^{15}N\{^{19}F\}$ REDOR NMR.[161] A model of the binding mode of these compounds could be obtained by superimposing ribityl and pyrimidine portions of the structures of these molecules with the ligand 5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione in the X-ray structure[138,139] followed by REDOR NMR and distance-restrained energy calculations. These studies gave evidence for the *R*-configuration at C-7 of epimer A (Fig. 17, compound **40**) and the *S*-configuration of epimer B (Fig. 17, compound **41**).

To confirm the stereochemical assignments based solely on results for epimer B, a $^{15}N\{^{19}F\}$ REDOR NMR study was performed on the complex formed from epimer A (**40**) and the uniformly ^{15}N-labeled F22W mutant of *B. subtilis* lumazine synthase. The results indicated that the fluorines of the ligands are closer to the side chain nitrogens of Arg127 and farther away from the side chain nitrogens of Lys135 in epimer B (**41**) than in epimer A (**40**). These results are consistent with the assignment of the earlier 7*R* configuration of epimer A and the 7*S* configuration of epimer B.[162]

6 Riboflavin synthase

In the final reaction step of the riboflavin pathway, 6,7-dimethyl-8-ribityllumazine (**9**) undergoes an unusual dismutation under formation of riboflavin (**10**) and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**6**) (Fig. 18). Riboflavin synthase (step **IX**) catalysing that reaction was the first enzyme of the riboflavin pathway to be discovered more than five decades ago.[101,102,104,163] The enzyme was first purified to near homogeneity (about 4000 fold) from bakers yeast.[57,59]

Genes specifying riboflavin synthase from various microorganisms and plants have been cloned and expressed in recombinant host bacteria.[164–171]

All carbon atoms of the xylene moiety of riboflavin are derived from the carbon atoms 6α, 6, 7 and 7α of 6,7-dimethyl-8-ribityllumazine (**9**). The 4-carbon moieties from the two substrate molecules are incorporated into the xylene ring of the vitamin with a head to tail orientation.[171–175] This was shown for the enzyme-catalysed reaction as well as for the uncatalysed formation of riboflavin by boiling of **9** in neutral or acidic aqueous solution.[176–178]

6,7-Dimethyl-8-ribityllumazine (**9**) has a p*K* value of about 8.4.[179] Deprotonation of **9** affords a complex mixture of anions as shown by NMR spectroscopy. Deprotonation of the unusually acidic 7-methyl group affords the exomethylene anion **43** (Fig. 19) which has been shown to be the predominant molecular species in the complex of lumazine synthase with its product. In aqueous solution, that anion species is only present at a level of about 1%, and the equilibrium is dominated by several tricyclic species arising by the addition of ribityl side chain hydroxy groups to the pyrazine carbon atom 7.[180–183] The acidic protons of the position 7 methyl group are easily exchanged with solvent water,[57,173,180] and this exchange is accelerated by riboflavin synthase.[57] On the basis of these findings, the involvement of anionic molecular species in the formation (both enzymatic and non-enzymatic) of riboflavin from **9** was proposed (Fig. 20). In support of the proposed mechanism, Plaut and coauthors could show that substrate analogs carrying a position 7 oxo-group were shown to be potent inhibitors of riboflavin synthase.[184] Ligand binding studies also showed that each subunit of riboflavin synthase can bind two substrate molecules.[36,59,185]

Studies on the interaction of riboflavin synthase of *B. subtilis* with trifluoromethyl-substituted intermediates[160,168,186,187] and ^{19}F NMR spectroscopy afforded three different bound forms suggesting binding sites of similar affinities. NOESY analysis of the kinetic network revealed that the three bound states exchange with free ligand, but not with each other, thus suggesting that the trimeric enzyme could be asymmetrical. Quantitative analysis of the NOESY spectra yielded different rate constants for the different binding sites.[168]

^{19}F NMR protein perturbation studies using wild type and mutant riboflavin synthase of *S. pombe* in complex with fluorinated intermediate analogs suggested large scale domain mobility which appears relevant for the complex reaction mechanism.[167]

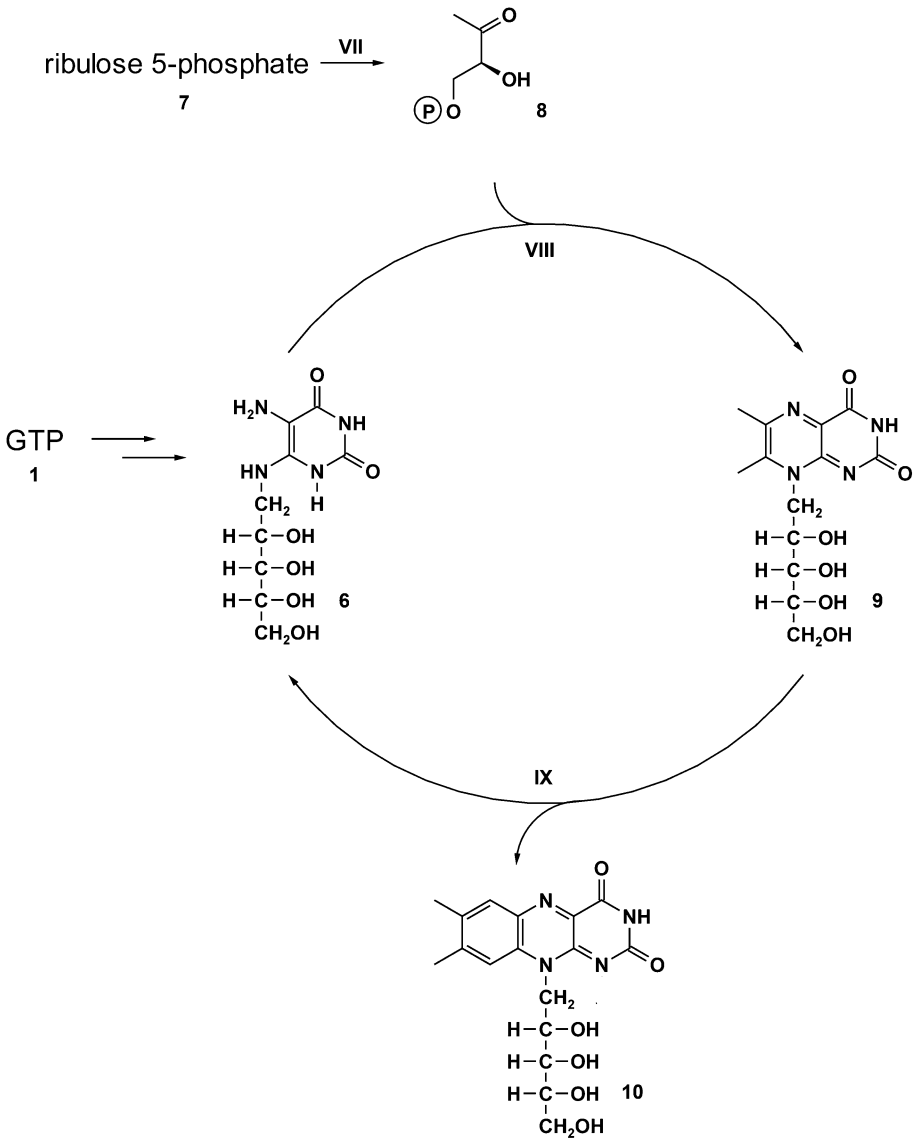

Fig. 18 Terminal step of the riboflavin biosynthesis. **1**, GTP; **6**, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; **8**, 3,4-dihydroxy-2-butanone 4-phosphate; **9**, 6,7-dimethyl-8-ribityllumazine; **10**, riboflavin; **VII**, 3,4-dihydroxy-2-butanone 4-phosphate synthase; **VIII**, 6,7-dimethyl-8-ribityl-lumazine synthase; **IX**, riboflavin synthase.

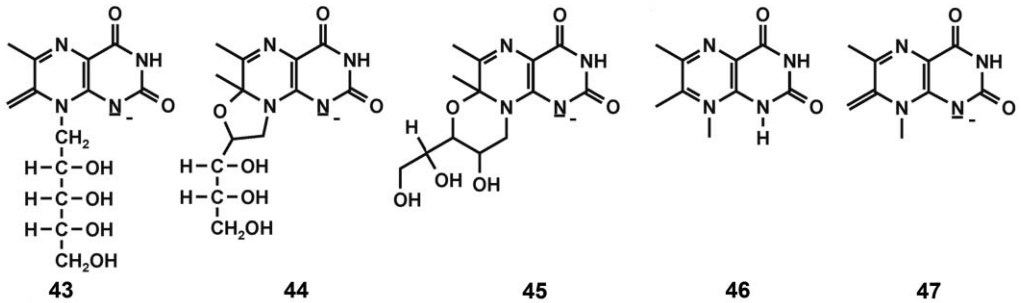

Fig. 19 Anionic species of 6,7-dimethyl-8-ribityllumazine (**9**) and 6,7,8-trimethyllumazine (**46**).[181,182,282]

Recent work with an S141A mutant of riboflavin synthase of *E. coli* afforded a pentacyclic dimer of 6,7-dimethyl-8-ribityllumazine (Fig. 20, compound **49**) after acid quench of enzyme–substrate mixtures under single turnover conditions.[188] The pentacyclic compound fulfils the requirements for a kinetically competent reaction intermediate. It can be cleaved by riboflavin synthase in two different ways affording two molecules of **9** (backward reaction) or one molecule each of riboflavin (**10**) and **6** (forward reaction). Riboflavin is formed more rapidly from **49** than from **9**.[188]

Structural comparison of the riboflavin synthases of *S. pombe* and *E. coli* suggests oligomer contact sites and delineates the catalytic site for dimerisation of the substrate and subsequent fragmentation of the pentacyclic intermediate. The pentacyclic substrate dimer was modeled into the proposed active site, and its stereochemical features were determined. The model suggests that the substrate molecule at the C-terminal domain donates a four-carbon unit to the substrate molecule bound at the N-terminal domain of an adjacent subunit in the oligomer (Figs. 20, 21).[189,190]

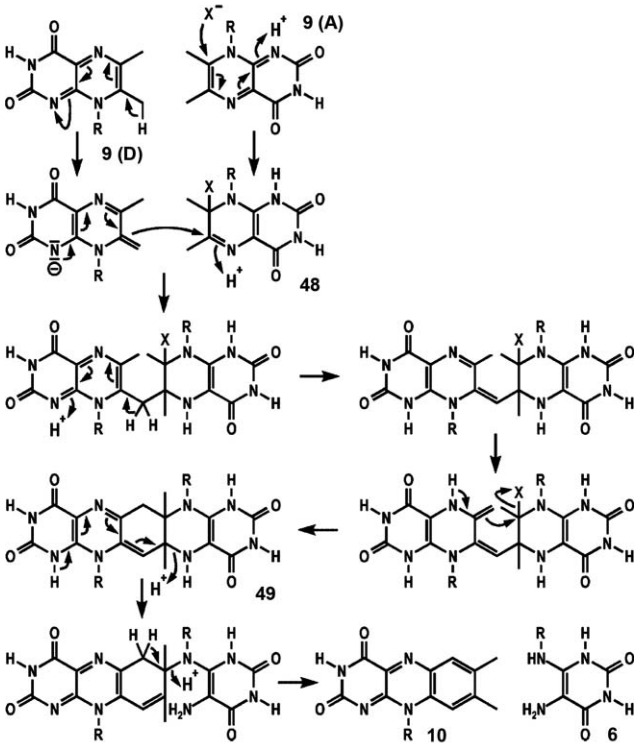

Fig. 20 Hypothetical reaction mechanism of riboflavin synthase: **9** (D), donor lumazine molecule; **9** (A), acceptor lumazine molecule; **X**, proposed nucleophile, which neutralises the carbonium centre at C-7 of **9** (A) and enables carbanion attack at C-6 of **48** by the 7-exomethylene carbon of **9** (D); **49**, pentacyclic reaction intermediate; **R**, ribityl chain.[188,190] Reprinted from *Structure*, **10**, 2002, Gerhardt *et al.*, Studies on the Reaction Mechanism of Riboflavin Synthase: X-Ray Crystal Structure of a Complex with 6-Carboxyethyl-7-Oxo-8-Ribityllumazine, 1371–1381, Copyright (2002), with permission from Elsevier.

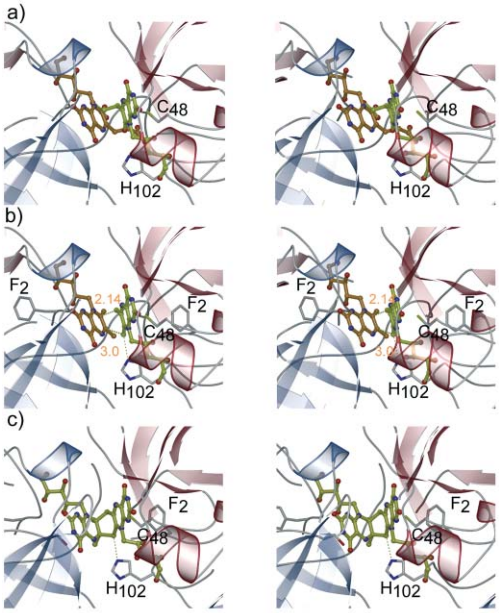

Fig. 21 Stereoview of the active site residues formed by two adjacent riboflavin synthase monomers of *S. pombe* with bound 6-carboxyethyl-7-oxo-8-ribityllumazine (**50**). a) The ligand bound to the N barrel (red) is drawn in yellow, whereas the 6-carboxyethyl-7-oxo-8-ribityllumazine in the adjacent C barrel (blue) is shown in dark yellow; b) Proposed binding of 6,7-dimethyl-8-ribityllumazine (**9**) at the active site; c) Model for the pentacyclic reaction intermediate.[190] Reprinted from *Structure*, **10**, 2002, Gerhardt *et al.*, Studies on the Reaction Mechanism of Riboflavin Synthase: X-Ray Crystal Structure of a Complex with 6-Carboxyethyl-7-Oxo-8-Ribityllumazine, 1371–1381, Copyright (2002), with permission from Elsevier.

Recently, the kinetic properties of riboflavin synthase from *E. coli* were studied under single turnover conditions. Stopped flow as well as quenched flow experiments documented the transient formation of the pentacyclic reaction intermediate (Fig. 20, compound **49**). In these experiments no other transient species were sufficiently populated to allow detection. The data are best described by a sequence of one second order and one first order reaction.[191]

The formation of riboflavin from **49** is easily explained by a sequence of two elimination reactions. However, the formation of **49** by dimerisation of **9** is still incompletely understood. The hypothetical reaction mechanism shown in Fig. 20 combines the novel findings with the reaction mechanism proposed earlier by Plaut, Wood and their coworkers.[36,173,174]

Recently, conserved amino acid residues of riboflavin synthase from *E. coli* were modified by site-directed mutagenesis. Replacement or deletion of phenylalanine residue 2 afforded catalytically inactive proteins. S41A and H102Q mutants had substantially reduced reaction velocities. Replacements of various other conserved polar residues had little impact on catalytic activity. ^{19}F NMR protein perturbation experiments using a fluorinated intermediate analog suggest that the N-terminal sequence motif MFTG is important for substrate binding.[192]

Riboflavin synthase of eubacteria and of *Arabidopsis thaliana* are homotrimers of about 75 kDa. The riboflavin synthases of plants, fungi and eubacteria show internal sequence similarity which suggested the presence of two domains with similar folding topology (Fig. 22a).[166,167,190,193] It was proposed that each domain could bind one substrate molecule, and that the active site could be formed at the interface of two domains where two substrate molecules could be brought together in an approximately C_2 symmetric arrangement that would be conducive to the antiparallel regiochemistry observed in the product (Fig. 21).

A recombinant N-terminal domain comprising amino acid residues 1–97 of *E. coli* riboflavin synthase forms a homodimer.[194] The C_2-symmetric structure of the artificial dimer is well in line with the proposed topology of the riboflavin synthase active site.[195–197]

The 3-dimensional structure of riboflavin synthases from *E. coli* and *S. pombe* have been determined by X-ray crystallography.[189,190] The backbone topology of the two domains formed by the *S. pombe* riboflavin synthase subunit show an rmsd of less than 1 Å^2 (Fig. 22b). The substrate binding sites of each respective domain could be identified by complexation with the substrate analog, 6-carboxyethyl-7-oxo-8-ribityllumazine (Fig. 22, compound **50**).[190,198] The substrate is bound with an extended conformation of the ribityl side chain by both subunits (Fig. 23).

The conditions used for crystallisation of the *S. pombe* enzyme are conducive to dissociation of the homotrimer. The two domains of the subunit are related by pseudo C_2-symmetry. However, the active site topology in a riboflavin synthase homotrimer can be gleaned from an overlay of the liganded *S. pombe* monomer with the ligand-free *E. coli* enzyme (Fig. 24). The N-terminal domain of one subunit is in close contact with the C-terminal domain of an adjacent subunit (Fig. 24).[190]

The proposed pentacyclic intermediate (**49**) fits perfectly into the cavity between the two subunits, and its hitherto unknown stereochemical features can be determined easily from that model (Fig. 21). The binding sites of the other four domains in the homotrimer are out of contact with each other (Fig. 24c). This unusual geometry could imply that the homotrimer has only one catalytic site, *i.e.* the special C_2 symmetric pair of one N-terminal and one C-terminal domain. Alternatively, the enzyme as a whole may be subject to major dynamic fluctuations which are conducive to the temporary formation of different C_2 symmetric pairs and which were shown by ^{19}F NMR spectroscopy.[167]

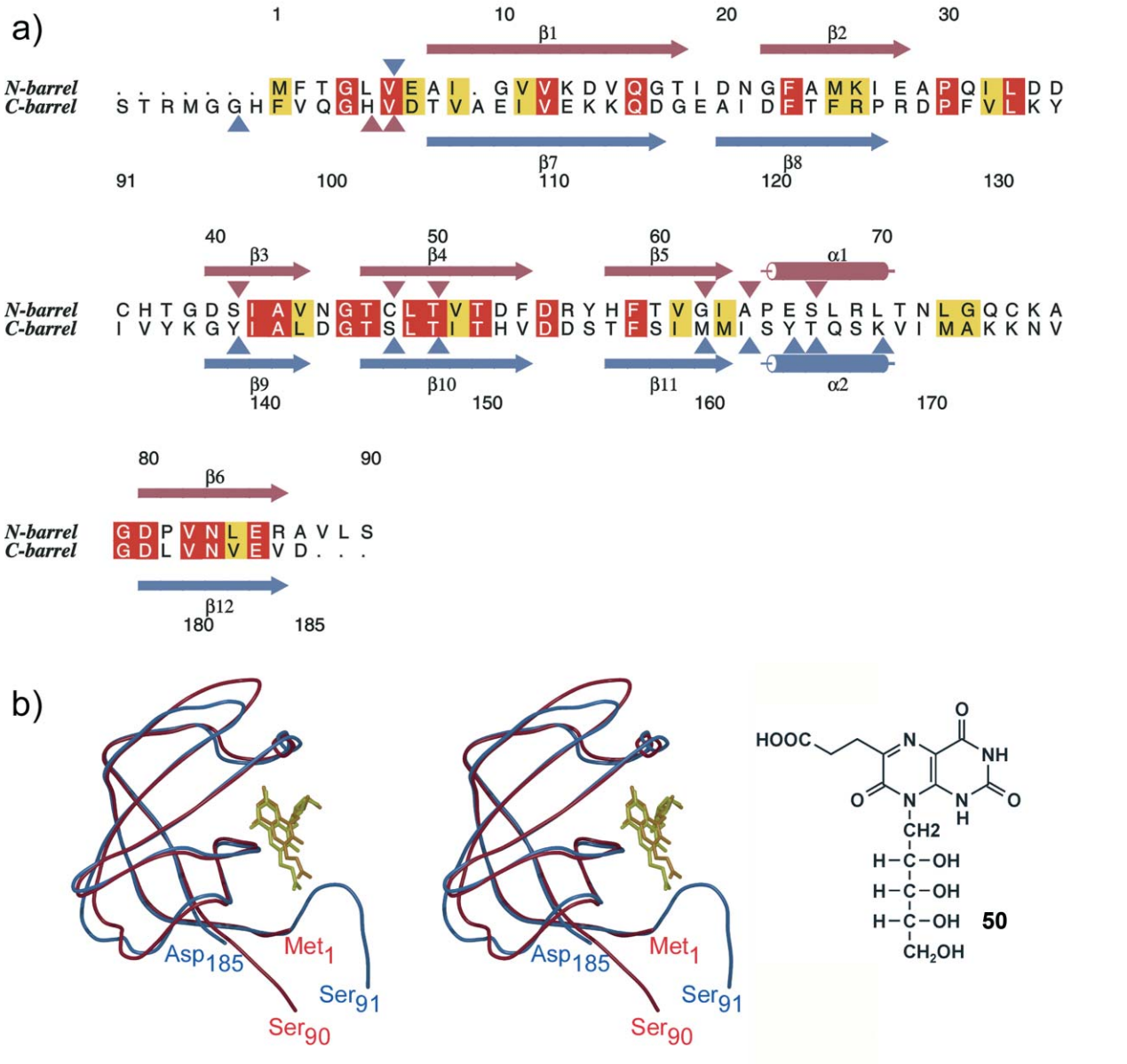

Fig. 22 Internal sequence alignment of riboflavin synthase from *S. pombe.* a) Alignment of the N barrel (red) and the C barrel (blue) domains; b) stereodiagram of an internal superposition of the two β barrels with bound substrate analog inhibitor 6-carboxyethyl-7-oxo-8-ribityllumazine (**50**).[190] Reprinted from *Structure*, **10**, 2002, Gerhardt *et al.*, Studies on the Reaction Mechanism of Riboflavin Synthase: X-Ray Crystal Structure of a Complex with 6-Carboxyethyl-7-Oxo-8-Ribityllumazine, 1371–1381, Copyright (2002), with permission from Elsevier.

7 The lumazine synthase–riboflavin synthase complex

As mentioned before, a riboflavin synthase trimer can be enclosed in the central cavity of icosahedral lumazine synthase. Fig. 25 shows a hypothetical model of the complex.

The enzyme complex catalyses the formation of one equivalent of riboflavin (**10**) from one equivalent of **6** and two equivalents of **8** (Fig. 18). Kinetic analysis under steady state conditions showed that riboflavin is formed more rapidly from the lumazine synthase substrates **6** and **8** than from 6,7-dimethyl-8-ribityllumazine (**9**). This anomalous kinetic behaviour has been attributed to substrate channeling due to the confinement of the riboflavin synthase (Fig. 1, 18, step **IX**) module inside the icosahedral capsids.[199]

Both termini of each lumazine synthase subunit are located on the outer surface of the capsid, thus both termini are accessible for recombinant modifications (such as peptides, or biotin).[200] It was shown that the functionalised lumazine synthase monomers can be assembled into capsids by *in vitro* renaturation; differently functionalised protomers can be mixed to generate multifunctional capsids.[201] Furthermore, it has been shown that the cavity of recombinant icosahedral lumazine synthase capsids can be used *in vitro* as a containment of nanocrystalline iron oxide.[202]

8 Fluorescent paralogs of riboflavin synthase

Certain luminescent marine bacteria have been found to contain monomeric paralogs of riboflavin synthase (Fig. 1, step **IX**). The proteins bind 6,7-dimethyl-8-ribityllumazine (**9**) (lumazine protein),[203,204] 6-methyl-7-oxo-8-ribityllumazine (**51**) (blue fluorescent protein)[205–207] or riboflavin 5′-phosphate (**11**) (yellow fluorescent protein)[208,209] with high affinity and are devoid of enzymatic activity (Fig. 26). They show fluorescence emission with high quantum yields.

The fluorescent proteins are believed to act as optical transponders which acquire energy from bacterial luciferase by radiationless transitions from the excited state of bacterial luciferase.[210] The emission maximum can be shifted by comparison with direct emission from luciferase. Moreover, the luminescent proteins have been reported to enhance the overall quantum yield of bacterial luminescence.[203] The fluorophore–protein

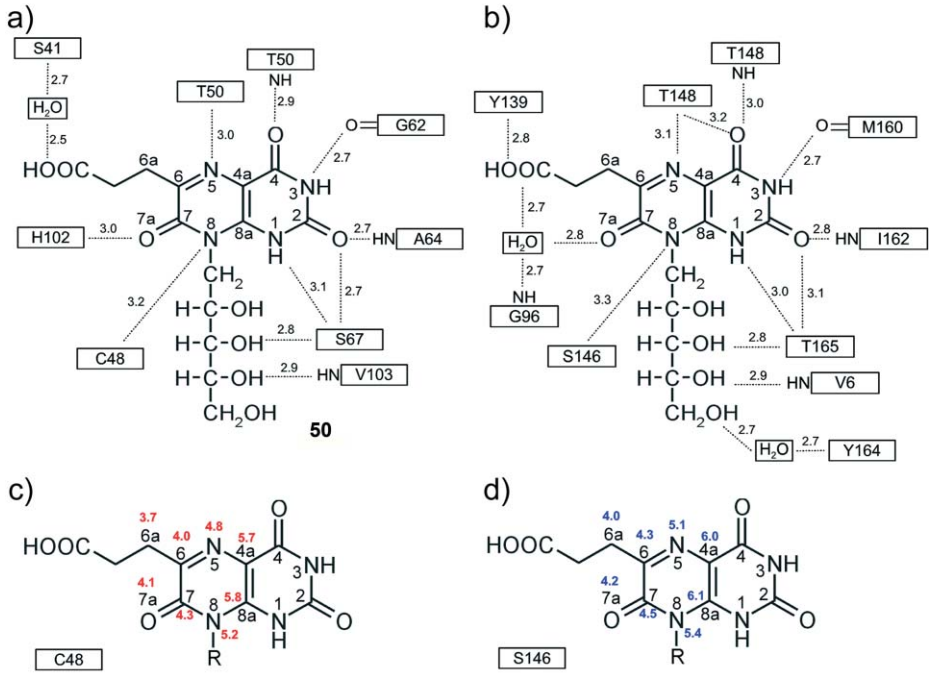

Fig. 23 Hydrogen-bonding topology of 6-carboxyethyl-7-oxo-8-ribityllumazine (**50**) bound to *S. pombe* riboflavin synthase. a) N-terminal domain; b) C-terminal domain; c) distances from the thiolate group of cysteine 48; d) distances from the side chain oxygen of serine 146; R, ribityl chain.[190] Reprinted from *Structure*, **10**, 2002, Gerhardt *et al.*, Studies on the Reaction Mechanism of Riboflavin Synthase: X-Ray Crystal Structure of a Complex with 6-Carboxyethyl-7-Oxo-8-Ribityllumazine, 1371–1381, Copyright (2002), with permission from Elsevier.

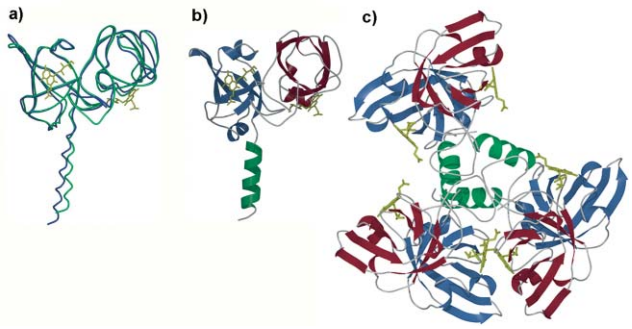

Fig. 24 X-Ray structures of riboflavin synthases from *E. coli* and *S. pombe*. a) Diagram of the superposition of one subunit of riboflavin synthase from *E. coli* (blue) and *S. pombe* (green); b) *S. pombe* riboflavin synthase monomer with bound 6-carboxyethyl-7-oxo-8-ribityllumazine (**50**, yellow); c) trimeric model of *S. pombe* riboflavin synthase with bound 6-carboxyethyl-7-oxo-8-ribityllumazine (yellow).[190] Reprinted from *Structure*, **10**, 2002, Gerhardt *et al.*, Studies on the Reaction Mechanism of Riboflavin Synthase: X-Ray Crystal Structure of a Complex with 6-Carboxyethyl-7-Oxo-8-Ribityllumazine, 1371–1381, Copyright (2002), with permission from Elsevier.

interaction was studied by time-resolved fluorescence anisotropy measurements.[211,212] The ligand in lumazine protein exhibits little or no independent mobility, and its rotational correlation time corresponds to that of the protein.[213] Complex formation between bacterial luciferase and lumazine protein has been proposed on basis of dynamic fluorescence polarisation measurements.[212,214]

^{19}F NMR spectra derived from perturbation experiments using fluorinated lumazine derivatives of 6,7-dimethyl-8-ribityllumazine in complex with lumazine proteins were well in line with the hypothesis that lumazine protein binds ligands with a 1 : 1 stoichiometry. The protein showed surprisingly strict stereoselectivity for epimer A (Fig. 17, compound **40**).[167,215]

NMR studies of lumazine protein in complex with ^{13}C- resp. ^{15}N-labeled 6,7-dimethyl-8-ribityllumazine derivatives showed that the ligand is embedded in a polar environment and that the ring system is strongly polarised.[216]

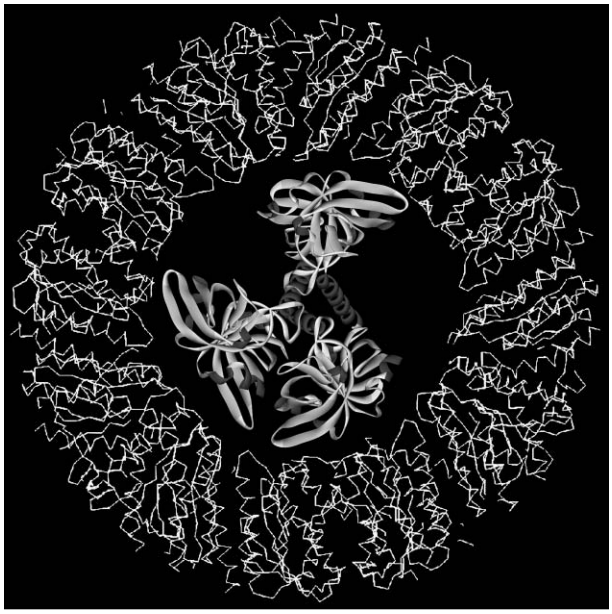

Fig. 25 Computer generated model of a heavy riboflavin synthase complex. The capsid was generated using the coordinates from the lumazine synthase 60-mer of *B. subtilis* and from the riboflavin synthase trimer of *E. coli*.

Sub-picosecond fluorescence spectroscopy has been used to characterise complexes of 6,7-dimethyl-8-ribityllumazine (**9**) and riboflavin (**10**) non-covalently bound to lumazine protein (from *Photobacterium leiognathi*) and blue fluorescent protein (from *Vibrio fischeri* Y1), respectively.[217]

Recent work with *Vibrio fischeri* Y1 suggested that the activity of the yellow fluorescent protein (YFP) is altered by redox interactions with respiratory components. Under O_2-limited conditions, the cellular YFP molecules seem to lose their florescence, possibly due to reduction *via* some respiratory components.[218]

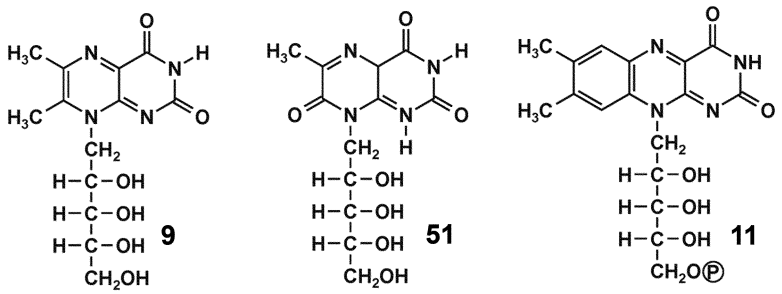

Fig. 26 Fluorophores of various luminescence proteins. **9**, 6,7-dimethyl-8-ribityllumazine; **51**, 6-methyl-7-oxo-8-ribityllumazine; **11**, riboflavin 5′-phosphate (FMN).

9 Biosynthesis of riboflavin in plants

A bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase (Fig. 1, steps **I** + **VII**) has been cloned and expressed from *A. thaliana*. The enzyme is remarkably similar to the bifunctional orthologs from eubacteria.[87]

The similarity between the riboflavin pathway in eubacteria and plants is further emphasised by the presence of a plant deaminase which converts the product **2** of GTP cyclohydrolase II (Fig. 1, step **I**) into **3**, as in eubacteria, whereas fungi first convert **2** into **4** by a reductase (Fig. 1, step **IV**). Sequence comparison showed relatively close similarity between eubacterial and plant deaminases (Fig. 27).[46]

Pyrimidine deaminases of the riboflavin pathway show sequence similarity with yeast cytosine deaminase (Fig. 28). The enzyme has been found in procaryotes and fungi, where it is a member of the pyrimidine salvage pathway. The X-ray structure of yeast cytosine deaminase has been determined in the presence of an inhibitor at 1.14 Å resolution.[219] Each active site contains a single catalytic zinc ion which is coordinated by a histidine, two cysteines, and a single bound water molecule which was found to act as a nucleophile in the deamination reaction. The coordination site for the zinc ion, which is known from the structure of the yeast cytosine deaminase, is strictly conserved over 54 bacterial and 9 plant pyrimidine deaminases of the

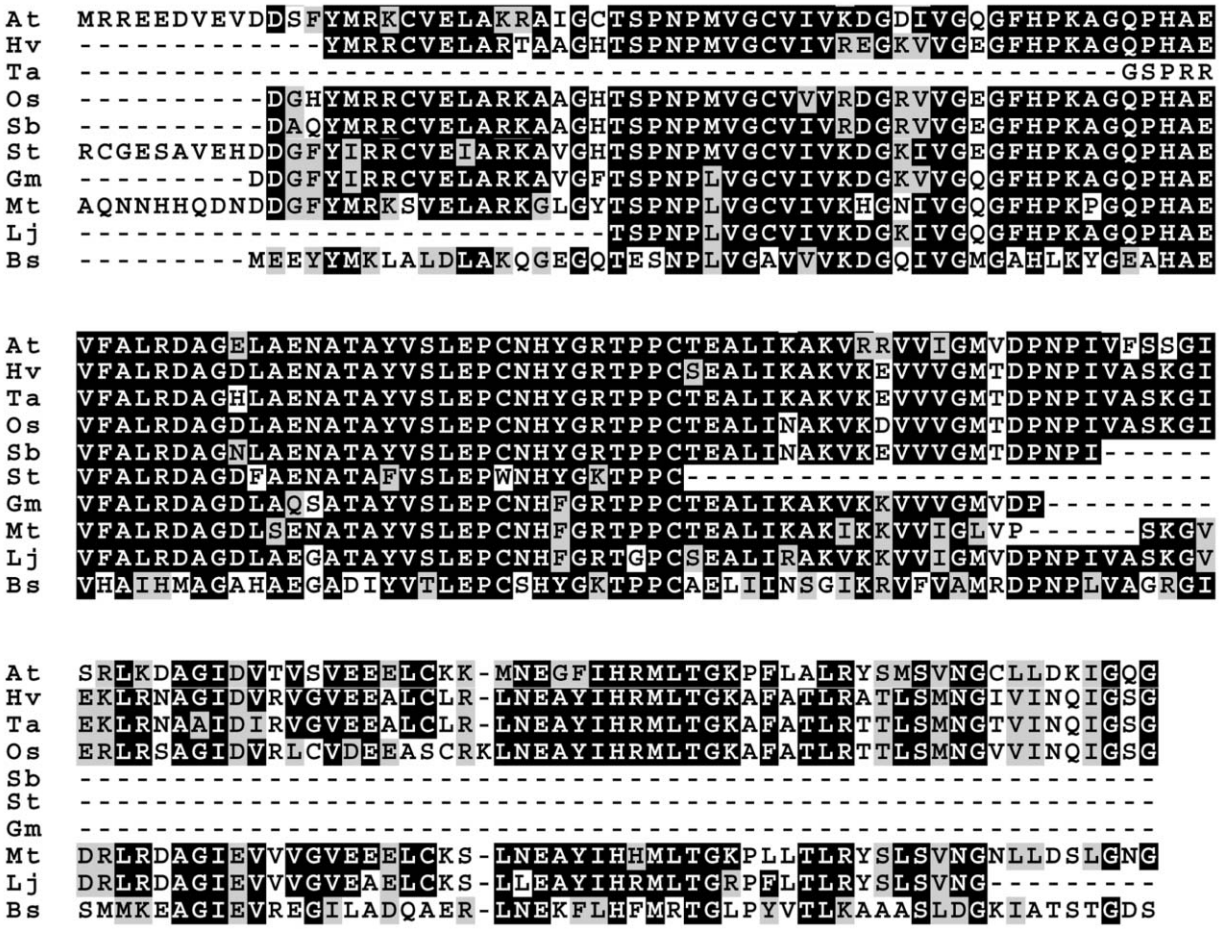

Fig. 27 Primary structures of deduced plant 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate deaminase domains and the deaminase domain of the *rib*G protein of *B. subtilis* (Bs). *A. thaliana* (At, NCBI accession CAB79096.1), *B. subtilis rib*G (Bs, NP_390209). The following open reading frames were deduced from nucleotide sequences available at The TIGR Gene Indices Database: barley, *Hordeum vulgare* (Hv, TC35422); *Medicago truncatula* (Mt, TC50740), lotus, *Lotus japonicus* (Lj, AV426745); wheat, *Triticum aestivum* (Ta, TC62433); soybean, *Glycine max* (Gm, TC129327); sorghum, *Sorghum bicolour* (Sb, BE600990); rice, *Oryza sativa* (**Os**, accession no. AK070281); potato, *Solanum tuberosum* (St, TC42758). The length of the alignment has been adapted to the deaminase domain of *A. thaliana* starting from position 64 to position 250 which can fold independently, yielding enzymatically active protein.[46] Reprinted from *J. Biol. Chem.*, **279**(35), 2004, Fischer *et al.*, Evolution of Vitamin B2 Biosynthesis. Structural and Functional Similarity between Pyrimidine Deaminases of Eubacterial and Plant Origin, 36299–36308, Copyright (2004), with permission from the Journal of Biological Chemistry.

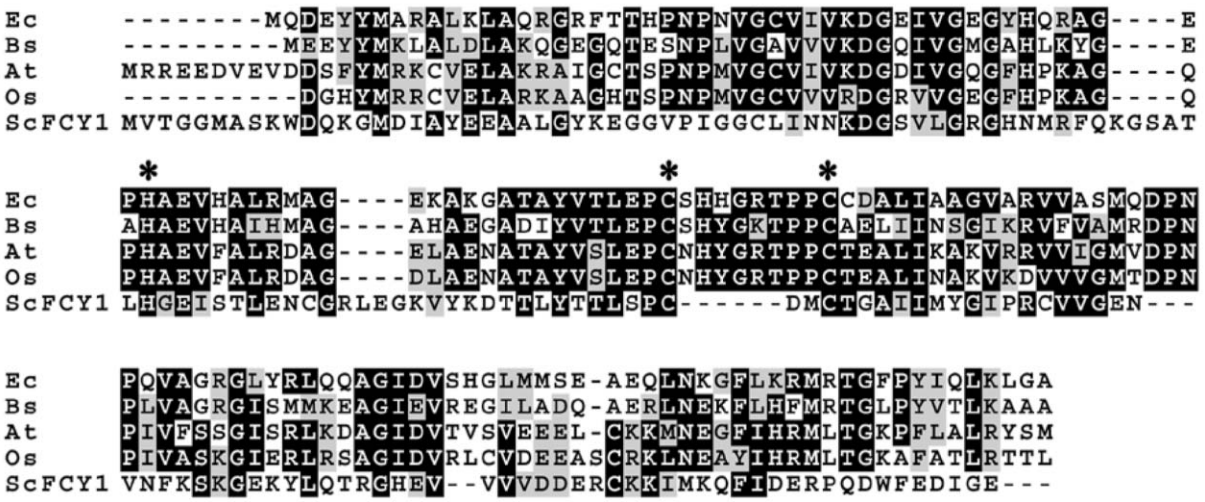

Fig. 28 Sequence alignment of 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate deaminases from eubacteria and plants and cytosine deaminase from *S. cerevisiae. A. thaliana* (At, accession At4g20960); *B. subtilis rib*G (Bs, accession no. P17618); *E. coli* (Ec, accession no. Q8FKC3); *Oryza sativa* (Os, accession no. AK070281) and cytosine deaminase of *S. cerevisiae* (ScFCY1, accession no. Q12178). The highly conserved zinc coordination site of yeast cytosine deaminase is indicated by asterisks.[46] Reprinted from *J. Biol. Chem.*, **279**(35), 2004, Fischer *et al.*, Evolution of Vitamin B2 Biosynthesis. Structural and Functional Similarity between Pyrimidine Deaminases of Eubacterial and Plant Origin, 36299–36308, Copyright (2004), with permission from the Journal of Biological Chemistry.

riboflavin pathway (Fig. 28). Sequence similarity is also observed to bacterial cytidine deaminase, *e.g.* of *E. coli*. Functional residues involved in zinc coordination or in the fixation of the substrate are present in all three paralog enzymes, the pyrimidine, the cytosine, and the cytidine deaminases.[46]

Lumazine synthase of spinach forms an icosahedral capsid of 60 subunits.[148] Again, that quaternary structure mimics the icosahedral lumazine synthase found in most eubacteria (with the exception, at the present state of information, of *B. abortus*[149,150]), whereas yeasts and fungi form C_5 symmetric, pentameric lumazine synthases.

Riboflavin synthase from *A. thaliana* has been obtained by heterologous expression in *E. coli* cells. Sequence arguments show that the enzyme has the typical two-domain architecture of riboflavin synthases from bacteria and fungi.[170,220]

All riboflavin pathway enzymes from plants comprise N-terminal segments which have been interpreted as plastid targeting peptides (*e.g.* Fig. 29).[221] It is therefore assumed that the biosynthesis of riboflavin proceeds in the plastid compartment. In this context, it should be noted that some of the enzymes for the biosynthesis of the structurally related vitamin, folic acid, are believed to be located in the cytosol of plant cells.[222]

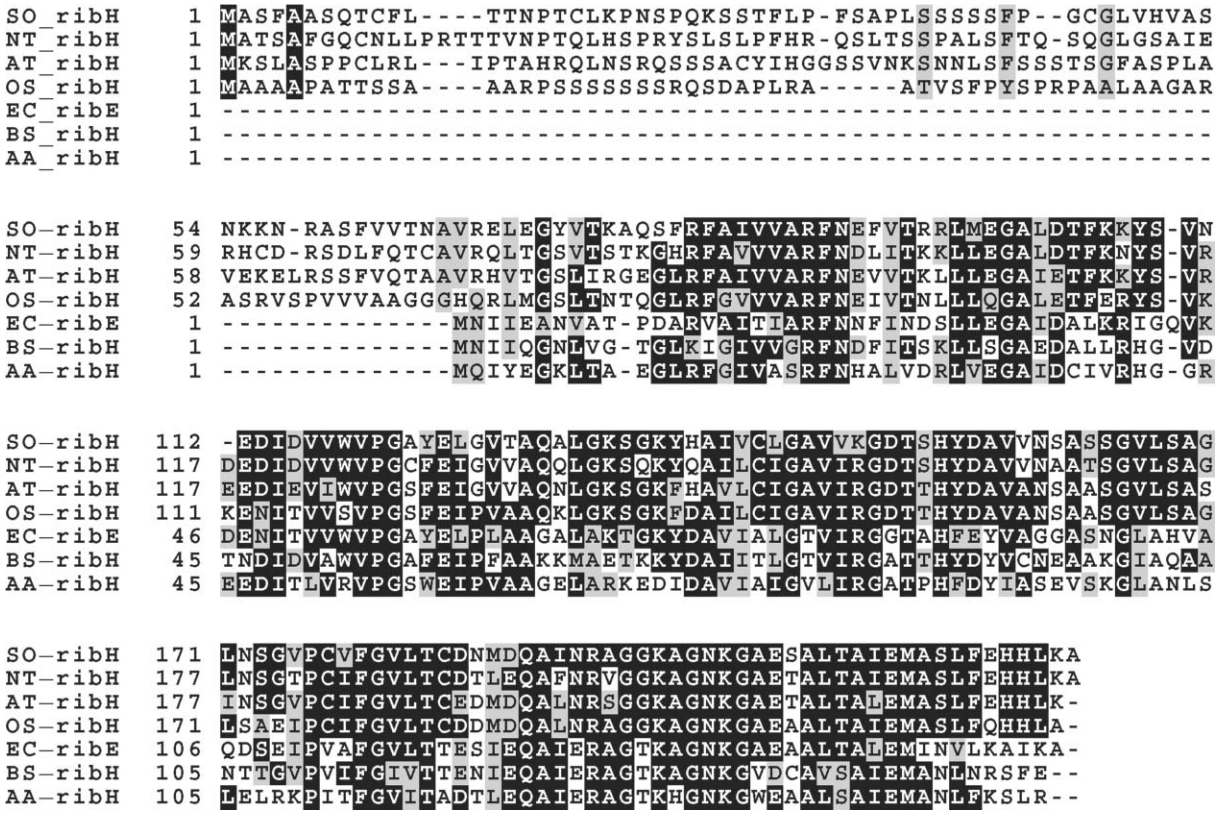

Fig. 29 Sequence alignment of lumazine synthases from eubacteria and plants. *Spinacia oleracea* (SO-ribH, accession no. AF147203); *Nicotiana tabacum* (NT-ribH, accession no. AF422802); *A. thaliana* (AT-ribH, accession no. O80575); *O. sativa* (OS-ribH, accession no. Q7XUK6); *E. coli* (EC-ribE, accession no. P61717); *B. subtilis* (BS-ribH, accession no. P11998); *A. aeolicus* (AA-ribH, accession no. O66529).

10 Biosynthesis of riboflavin in Archaea

The basic pathway of riboflavin biosynthesis in Archaea has been shown to be similar to that of eubacteria and fungi by *in vivo* studies with ^{13}C-labeled precursors.[223,224] Specifically, the xylene ring was shown to be assembled from two identical 4-carbon fragments which are derived from the pentulose phosphate pool.

Archaea have no detectable homologs of GTP cyclohydrolases I and II. Instead, they use a new type of GTP cyclohydrolase (GTP cyclohydrolase III) that produces 2-amino-5-formylamino-6-ribofuranosylamino-4(3*H*)-pyrimidone monophosphate (Fig. 30, Compound **52**). The enzyme has a steady-state turnover of 21 min^{-1}, which is much faster than those of canonical GTP cyclohydrolase enzymes.[225]

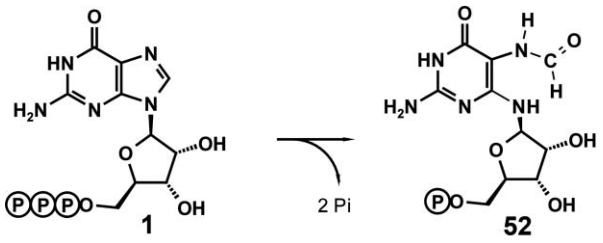

Fig. 30 Reaction catalysed by GTP cyclohydrolase III. **52**, 2-amino-5-formylamino-6-ribofuranosylamino-4(3*H*)-pyrimidinone monophosphate.[225]

The open reading frame MJ0671 of *M. jannaschii* has been reported recently to specify an enzyme catalysing the reduction of **2** affording **4** (Fig. 1, step **IV**). Thus, the pathway follows the pattern found in yeasts as opposed to that in eubacteria and plants.[47]

Heterologous expression of the putative open reading frame MJ0303 of *M. jannaschii* provided a recombinant protein catalysing the formation of the riboflavin precursor 6,7-dimethyl-8-rityllumazine.[151] The lumazine synthase of thermophilic Archaea show only relatively low similarity with those of eubacteria. In negatively stained electron micrographs, the enzyme from *M. jannaschii*, *E. coli* and *A. aeolicus* appear as essentially spherical particles with diameters around 15 nm. However, sedimentation equilibrium analysis of the *M. jannaschii* enzyme was hampered by nonideal solute behaviour.[151]

A riboflavin synthase without apparent sequence similarity to the enzymes from eubacteria, fungi and plants has been cloned from *Methanothermobacter thermoautotrophicus*.[165] Recently, the open reading frame MJ1184 of *M. jannaschii* with similarity to riboflavin synthase of *M. thermoautotrophicus* has been expressed and characterised in some detail. Hydrodynamic studies indicated a relative mass of 88 kDa suggesting a homopentameric structure. Divalent metal ions, preferably manganese or magnesium, are required for maximum activity. With the enzyme of *M. jannaschii*, NMR spectroscopy with ^{13}C-labeled samples of 6,7-dimethyl-8-ribityllumazine (**9**) as substrates showed that the regiochemistry of the dismutation reaction is the same as observed in eubacteria and eucaryotes.[142,171] The reaction catalysed by riboflavin synthase of *M. jannaschii* proceeds *via* a pentacyclic intermediate which has been shown to be a diastereomer of the pentacyclic intermediate of eubacterial and yeast riboflavin synthases.[226]

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 (Fig. 1, step **VII**; Fig. 31), thus suggesting that riboflavin synthases of Archaea are

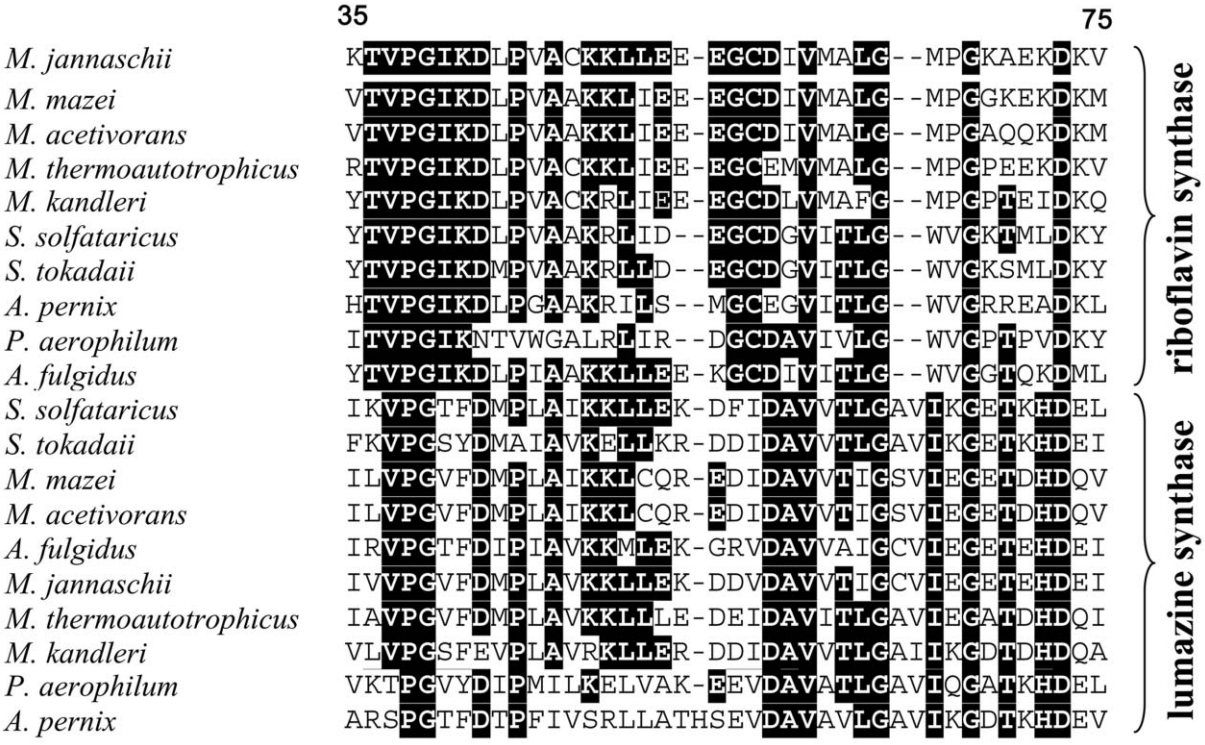

Fig. 31 Sequence comparison (highly conserved sequence from position 35 to 75 of riboflavin synthase of *M. jannaschii*; first line) of archaeal type riboflavin synthase (RS) and lumazine synthase (LS). *Methanosarcina mazei Goe I* (RS, accession no. NP632269; LS, accession no. NP632268); *M. jannaschii* (RS, accession no. Q58584; LS, accession no. Q57751); *Methanopyrus kandleri AV19* (RS, accession no. NP613646; LS, accession no. Q8TYL5); *Methanosarcina acetivorans C2A* (RS, accession no. NP616743; LS, accession no. NP616744); *Methanothermobacter thermoautotrophicus delta H* (RS, accession no. Q59587; LS, accession no. NP276506); *Pyrobaculum aerophilum* (RS, accession no. NP560637; LS, accession no. Q8ZTE3); *Aeropyrum pernix* (RS, accession no. Q9YDC5; LS, accession no. Q9YC88); *Sulfolobus solfataricus* (RS, accession no. NP341940; LS, accession no. Q980B5); *Sulfolobus tokadaii* (RS, accession no. NP376265; LS, accession no. Q975M5); *Archaeoglobus fulgidus DSM* 4304 (RS, accession no. NP070245; LS, accession no. NP070953). Identical residues are shaded in black.[171] Reprinted from *J. Mol. Biol.*, **343**(1), 2004, Fischer *et al.*, Evolution of Vitamin B2 Biosynthesis. A Novel Class of Riboflavin Synthase in Archaea, 267–278, Copyright (2004), with permission from Elsevier.

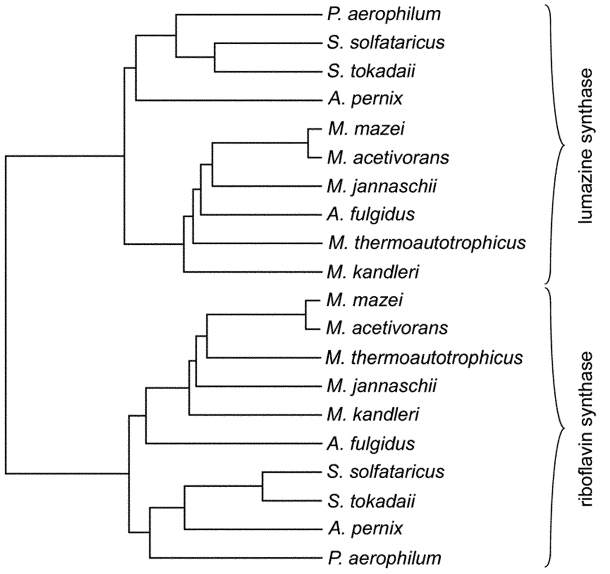

Fig. 32 Phylogenetic tree of riboflavin synthases compared with lumazine synthases from archaeal bacteria. For details see legend to Fig. 31.[171] Reprinted from *J. Mol. Biol.*, **343**(1), 2004, Fischer *et al.*, Evolution of Vitamin B2 Biosynthesis. A Novel Class of Riboflavin Synthase in Archaea, 267–278, Copyright (2004), with permission from Elsevier.

paralogs of lumazine synthase. 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 (Fig. 32).

Two archaebacterial species (*Pyrococcus furious* and *Halobacterium* NRC-1) carry putative genes with close similarity to the 6,7-dimethyl-8-ribityllumazine synthase 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. 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.

11 Biosynthesis of deazaflavin

Coenzyme F_{420} (**55**, Fig. 33), a derivative of 5-deaza-7,8-demethyl-8-hydroxy-riboflavin (factor F_0, **54**, Fig. 33), serves a central role in the reductive transformation of carbon dioxide into methane by methanogenic bacteria. Factor F_{420} has also been found in certain *Streptomycetes* where it was shown to serve as a cofactor in the biosynthesis of tetracycline and lincomycin.[227–229] *Mycobacterium* and *Nocardia* spp. use coenzyme F_{420} as a cofactor for F_{420}-dependent glucose-6-phosphate dehydrogenase.[230–232] Photolyases from the green alga *Scenedesmus* spp. and the cyanobacterium *Synechocystis* spp. contain bound F_{420}.[233,234]

The structure of coenzyme F_{420} in *Mycobacterium smegmatis* was shown to be composed of a chromophore identical to that of F_{420} from *Methanobacterium thermoautotrophicum*, with a side chain of a ribityl residue, a lactyl residue and five or six glutamate groups (F_{420}-5 and F_{420}-6). HPLC analysis indicated that *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, and *Mycobacterium fortuitum* have F_{420}-5 and F_{420}-6 as the predominant structures, whereas *Mycobacterium avium* contains F_{420}-5, F_{420}-6 and F_{420}-7 in significant amounts.[235]

Analyses of the F_{420} molecules present in *M. jannaschii* have shown that these cells contain a series of γ-glutamyl-linked F_{420} capped with a single, terminal α-linked L-glutamate. The predominant form of F_{420} was designated as α-F_{420}-3 and represented 86% of the F_{420} in these cells. Analyses of *Methanosarcina thermophila*, *Methanosarcina barkeri*, *M. thermoautotrophicus*,

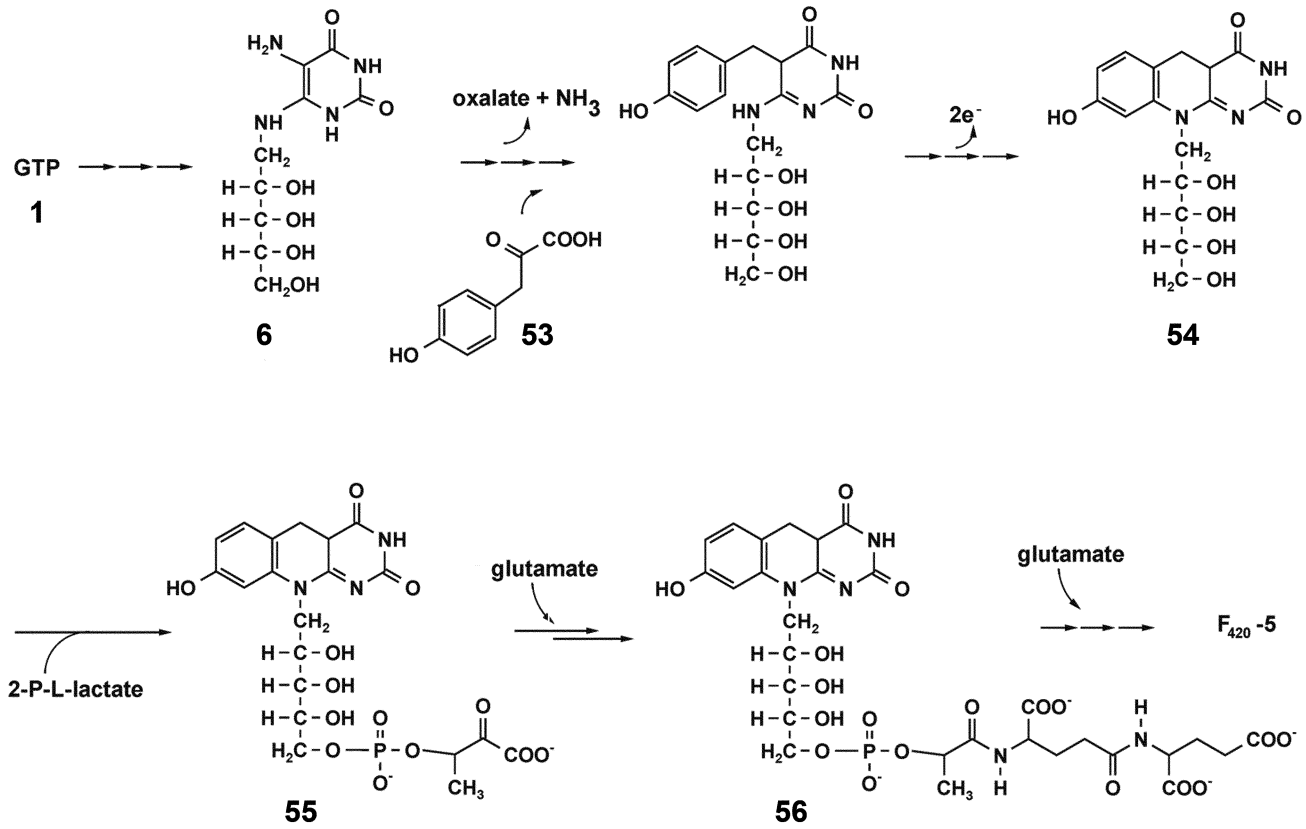

Fig. 33 Starting substrates for the biosynthesis of 5-deaza-7,8-demethyl-8-hydroxy-riboflavin (F_0, **54**). **1**, GTP; **6**, 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; **53**, 4-hydroxyphenylpyruvate; **55**, coenzyme F_{420}-0, **56**, coenzyme F_{420}-2.

Table 3 Specific activities of enzymes involved in the biosynthesis of riboflavin

Enzyme	Organism	Activity/nmol mg^{-1} min^{-1}; 37 °C	Reference
GTP cyclohydrolase II	*E. coli*	182	65
	A. thaliana (*MBP fusion protein*)	2.75	87
Pyrimidine deaminase	*E. coli*	0.36 (μmol mg^{-1} min^{-1})	48
	B. subtilis	0.17 (μmol mg^{-1} min^{-1})	48
	A. thaliana	0.9 (μmol mg^{-1} min^{-1})	46
Pyrimidine reductases	*E. coli*	0.12 (NADPH), 0.13 (NADH) (μmol mg^{-1} min^{-1})	48
	B. subtilis	0.01 (NADPH), 0.03 (NADH) (μmol mg^{-1} min^{-1})	48
	M. jannaschii	0.05 (NADPH), 0.03 (NADH) (nmol mg^{-1} min^{-1})	47
	Methanosarcina thermophila	0.04 (NADPH), 0.04 NADH) (nmol mg^{-1} min^{-1})	47
3,4-Dihydroxy-2-butanone 4-phosphate synthase	*E. coli*	283	126
	M. jannschii	174	113
	C. albicans	332	128
	Magnaporte grisea	112	133
	A. thaliana (*MBP fusion protein*)	6.7	87
Lumazine synthase	*A. aeolicus*	31	157
	B. subtilis	242	51
	E. coli	197	147
	M. jannaschii	11	151
	S. cerevisiae	257	147
	S. oleracea	275	221
	S. pombe	217	156
Riboflavin synthase	*A. thaliana*	197	167
	B. subtilis	33	283
	E. coli	21 (20 °C)	192
	M. jannaschii	24 (40 °C)	171
	M. thermoautotrophicus	32 (65 °C, recombinant); 45 (65 °C, isolated from wt)	165
	S. cerevisiae	232	59
	S. pombe	158	167

Archaeoglobus fulgidus, and *M. smegmatis* showed that they contained only γ-glutamyl-linked F_{420}.[236]

In vivo studies with *M. thermoautotrophicum* using ^{13}C-labeled precursors showed that the ribityl side chain and the pyrimidine moiety of factor F_0 are biosynthetically derived from GTP or a closely related nucleotide.[224] The carbocyclic ring, together with carbon atom 5, was shown to be derived from tyrosine. The riboflavin pathway intermediate, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**6**) was incorporated into factor F_0 with surprising efficacy. The incorporation was accompanied by the loss of the position 5 amino group. A hypothetical mechanism for the formation of factor F_0 from **6** and tyrosine is shown in Fig. 33.

Transposon mutagenesis using *M. bovis* strain BCG afforded mutants that could synthesise the biosynthesis intermediate F_0 but not F_{420}-5,6. These mutants contained transposons inserted in 2 adjacent homologs of *M. tuberculosis* genes, which have been named *fbiA* and *fbiB* (for *F*420 *bi*osynthesis). Homologs of *fbiA* were found in all microorganisms that have been fully sequenced and annotated at this time and that are known to make F_{420}. *FbiB* homologs were found in most of these organisms. Complementation data demonstrated that both genes are essential for normal F_{420}-5,6 production and suggest that the *fbiA* mutation has a partial polar effect on *fbiB*. The specific reactions catalysed by FbiA and FbiB are unknown, but both function between F_0 and F_{420}-5,6, since F_0 is made by both mutants.[237]

In *M. bovis* strain BCG, *fbiC*, a homolog of *M. tuberculosis* gene Rv1173, was found to be (i) essential for F_{420} biosynthesis and (ii) to participate in a step of the F_{420} biosynthetic pathway between pyrimidinedione (**6**) and F_0 (**54**). Homologs of *fbiC* were found in all microorganisms that have been fully sequenced at this time (2002) and that are known to make F_{420}. Four of these homologs (all from members of the aerobic actinomycetes) coded for proteins homologous over the entire length of the *M. bovis* FbiC, but in seven microorganisms two separate genes were found to code for proteins homologous with either the N-terminal or C-terminal portions of the *M. bovis* FbiC.[238]

The protein product of the *M. jannaschii* MJ1256 gene has been shown to be involved in coenzyme F_{420} biosynthesis. The protein catalyses the transfer of the 2-phospholactate moiety from lactyl (2) diphospho-(5′)guanosine (LPPG) to 7,8-didemethyl-8-hydroxy-5-deazariboflavin F_0 (**54**) with the formation of the L-lactyl phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (F_{420}-0, **55**) and GMP. On the basis of the reaction catalysed, the enzyme was named LPPG:F_0 2-phospho-L-lactate transferase. Since the reaction is the fourth step in the biosynthesis of coenzyme F_{420}, the enzyme has been designated as CofD, the product of the *cofD* gene (for *co*enzyme F_{420}).[239]

Recently, it was shown the product of the *M. jannaschii* MJ0768 catalyses the GTP-dependent addition of two L-glutamates to the L-lactyl phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (F_0) to form F_{420}-glutamyl-glutamate (F_{420}-2, **56**). Since the reaction is the fifth step in the biosynthesis of coenzyme F_{420}, the enzyme has been designated as CofE, the product of the *cofE* gene. The enzyme has no sequence similarity to any previously characterised proteins.[240]

12 Reaction rates of riboflavin biosynthetic enzymes

The reaction rates of several enzymes involved in riboflavin biosynthesis are surprisingly low, with only a few catalytic cycles per minute and per subunit (Table 3). More specifically, type II GTP cyclohydrolases from different kingdoms have specific activities in the range of 2.8–182 nmol mg^{-1} min^{-1}. The specific activity of a recombinant plant enzyme was shown to be substantially lower as compared to the homologous bacterial enzymes.[126,241] In order to increase the expression level of the plant enzyme in *E. coli*, it was fused with maltose binding protein. It has been proposed that this fusion may be in part responsible for the low activity.[87]

Lumazine synthases of eubacteria, Archaea, yeasts and plants have specific activities in the range of 11–275 nmol mg^{-1} min^{-1} equivalent to a maximum of about 0.2 to 4, resp. catalytic cycles per minute and per subunit. Not surprisingly, the activity of lumazine synthase from the hyperthermophilic eubacterium, *A. aeolicus*, is even lower when assayed at 37 °C. At higher temperatures in the range of the optimum temperature for

growth of the organism, the specific activity of the *A. aeolicus* enzyme is similar to the enzymes from mesophilic organisms at their respective temperature optimum for growth.[157] Also not surprisingly, the catalytic activity of enzyme from the thermophilic Archaeon *M. jannaschii* at 37 °C is low by comparison with mesophilic organisms. At a temperature of 70 °C, the catalytic rate of the enzyme is 90 nmol mg^{-1} min^{-1} equivalent to a turnover rate of 1.4 min^{-1}.[151]

The maximum specific activities of riboflavin synthase from plants and fungi are in the range of 158–232 nmol mg^{-1} min^{-1}, equivalent to an average turnover rate of 4.5 catalytic cycles per min and per subunit. The rate of riboflavin synthases from eubacteria and Archaea are even lower, in the range of 21–45 nmol mg^{-1} min^{-1}.

In the entire pathway, only pyrimidine reductases of eubacteria and yeast operate significantly more rapidly than the enzymes discussed above. Thus, reductases from those organisms have specific activities in the range of 5 μmol mg^{-1} min^{-1}.[48]

Although the reaction mechanisms of some of these enzymes are really quite complex, there is little reason to believe that the low catalytic rates are inherently due to that mechanistic complexity. Thus, the formation of riboflavin from 6,7-dimethyl-8-ribityllumazine, despite its mechanistic complexity, can proceed in the complete absence of any catalyst. More than that, the reaction catalysed by lumazine synthase proceeds with remarkable velocity without any catalyst, even at neutral pH and at room temperature.[136] Thus, the catalytic acceleration by lumazine synthase is really unexpectedly modest.[155] And, last but not least, the rate limiting step in the reaction catalysed by GTP cyclohydrolase II is the release of pyrophosphate from the triphosphate, at the beginning of the reaction trajectory, and not the hydrolytic opening of the imidazole ring which one would intuitively expect to involve a much higher free energy barrier that for the cleavage of a phosphoanhydride motif.[66]

Apparently, the pathway enzymes have been selected, over long periods of evolution, to provide a modest rate of catalytic acceleration, one of the reasons being that flavins are only required in small amounts to serve as cofactors for catalysis.

13 Biotechnology

The world production of riboflavin is assumed to exceed 3000 metric tones per year for use as nutritional supplement, animal feed additive and food colorant. Until recently, the vitamin was predominantly manufactured by chemical synthesis using glucose as a chiral precursor.

Certain microorganisms had been known for a long period to produce excess amounts of riboflavin. Specifically, the flavinogenic Ascomycetes *Ashbya gossypii* and *Eremothecium ashbyii* as well as some flavinogenic yeasts such as *Candida guilliermondii* and *Candida famata* have been studied in considerable detail. The early work in the area has been reviewed elsewhere.[32]

Fermentation processes based on *B. subtilis*, *A. gossypii* and *C. famata* have now essentially replaced the chemical synthesis of the vitamin.[22] Briefly, the production of riboflavin with *B. subtilis* could be increased by the deregulation of purine synthesis and a mutation in the gene coding for the riboflavin kinase. The *B. subtilis* process is further based on the recombinant, homologous overexpression of multiple chromosomal copies of the riboflavin operon under the control of a strong synthetic promoter. The introduction of an additional copy of the *ribA* gene has also been shown to increase the productivity of the strain.[88,242–244]

Industrially utilisable strains have been obtained by multiple rounds of mutagenetic treatment and selection of improved colonies. Inhibitor molecules like itaconate, which inhibits the isocitrate lyase in *A. gossypii*[245] and tubercidin (7-deazaadenosine),[246] which inhibits purine biosynthesis in *C. famata* have been applied successfully for mutant selections. In *B. subtilis* three purine analogs (8-azaguanine, decoyinine, and methionine sulfoxide plus roseoflavin (**57**)) have been used to obtain resistant mutant strains.[247] Roseoflavin resistant *B. subtilis* mutants were shown to contain mutations in regulatory genes and/or in the 5′-region of the rib-operon.

14 The riboflavin biosynthetic pathway as a potential drug target

Most pathogenic bacteria and fungi, with the possible exception of *Enterococcus faecalis*, *Listeria sp.*, *Mesoplasma sp.*, *Rickettsia sp.*, *Borrelia sp.*, *and Treponema sp.*, obtain riboflavin by endogenous biosynthesis. The endogenous production is essential at least in Gram-negative bacterial pathogens which are virtually unable to acquire riboflavin from the environment due to the lack of an uptake system. Hence, inhibitors of riboflavin biosynthetic enzymes might be used as antibiotic agents directed against Gram-negative bacteria and possibly also against pathogenic yeasts and Mycobacteria.

Whereas inhibitors of metabolic pathways have generally played only a limited role in the chemotherapy of infectious disease, it should be noted that sulfonamides, the first antibacterial agents acting against a broad spectrum of bacterial pathogens, operate by inhibition of dihydropteroate synthase in the folic acid biosynthetic pathway.[248,249] Pathogenic microorganisms have apparent difficulties to acquire folate from the environment due to the lack of an efficient uptake system and are therefore dependent on endogenous biosynthesis. Similar to the situation with folate, many human pathogens are unable to absorb riboflavin from the environment in sufficient amounts and are therefore absolutely dependent on endogenous synthesis of the vitamin.

Only a relatively small number of microbial target proteins have been addressed up to now by commercially available anti-infective drugs. In light of the rapid progression of resistance against virtually all current antibiotics, the exploration of new anti-infective targets and the development of novel antiinfective drugs appear to be urgent medical problems. Several enzymes of the riboflavin pathway catalyse mechanistically complex reactions which offer ample opportunities for the design of transition state type inhibitors. It should also be noted that the enzymes of the riboflavin pathway are potential herbicide targets.

A large number of substrate analogs of riboflavin synthase and lumazine synthase have been reported.[160,198,250–262] Certain compounds can inhibit both riboflavin synthase and lumazine synthase.

Several high resolution structures of inhibitor molecules in complex with riboflavin synthase or lumazine synthase are available.[137–139,142–145,148,190]

Recently, a high throughput screening method was developed that is based on the competitive binding of a lumazine synthase inhibitor and riboflavin to the active site of *S. pombe* lumazine synthase.[263]

15 Biosynthesis of FMN and FAD

Organisms of all taxonomic kingdoms are faced with the necessity to convert endogenous or exogenous riboflavin into FMN (**11**) and FAD (**12**), the molecular species serving as cofactors for flavoproteins. Although the early steps of riboflavin biosynthesis proceed *via* 5′ phosphoric acid esters, the phosphate residue must be removed prior to their conversion into riboflavin by the catalytic activity of lumazine synthase and riboflavin synthase which are both unable to use phosphoric acid esters as substrate.[49,50]

Riboflavin kinase (Fig. 1, step **X**) and FAD synthetase (Fig. 1, step **XI**) have been reported to occur in a wide variety of species during a period of more than 5 decades. Many of the earlier studies were focussed on aspects of substrate specificity. This earlier work has been reviewed earlier[60] and will not be covered in the present article.

A significant step in the research on riboflavin kinase and FAD synthetase was the discovery of a bifunctional flavokinase–FAD synthetase in *Brevibacterium ammoniagenes* and *Corynebacterium ammoniagenes*.[264,265] The rapid deployment of whole genome sequences during the past decade has shown that the bifunctional enzyme type is widespread in eubacteria, whereas a monofunctional FAD synthetase[266] and a monofunctional flavokinase[267] have been identified in the yeast *S. cerevisiae*.

Relatively few in depth studies on riboflavin kinases and FAD synthetases have been published over the past decade. However, the structures of the riboflavin kinases of *S. pombe* and *Homo sapiens*, and the structure of FAD synthetase of *Thermotoga maritima* have been determined by X-ray diffraction.[268–271]

16 Regulation

It has been known for a while that FMN is required for down-regulation of the *rib* operon of *B. subtilis* and that the regulation of riboflavin biosynthesis in *B. subtilis* is affected by the activity of the flavokinase–FAD synthetase encoded by *ribC*.[244,272] It has been reported that the ribR gene coding for a monofunctional riboflavin kinase is involved in regulation of the *B. subtilis* riboflavin operon.[242,273,274]

A highly conserved RNA domain, termed the RFN element, has been identified in mRNAs of prokaryotic genes required for the biosynthesis of riboflavin and FMN (**11**).[275,276] Mutations within the RFN element inactivated FMN-based regulation of the rib operon.[277,278] It was reported that the RFN domain serves as the receptor for a metabolite-dependent riboswitch that directly binds FMN in the absence of proteins, controlling gene expression by causing premature transcription termination of the rib operon. Beside this, it has also been shown that artificial RNA sequences (aptamers) can bind FMN with very high affinity.[279] Riboswitches are genetic regulatory elements found in the 5′ untranslated region of messenger RNA that act in the absence of protein cofactors. They are broadly distributed across bacteria and account for the regulation of more than 2% of all genes in *B. subtilis*, underscoring their importance in the control of cellular metabolism. The 5′ untranslated region of many mRNAs of genes involved in purine metabolism and transport contain a guanine-responsive riboswitch that directly binds guanine, hypoxanthine or xanthine to terminate transcription. Riboswitches were also found in the regulation of other metabolic pathways including the biosynthesis of vitamins (*e.g.* thiamin and cobalamin) and the metabolism of methionine and lysine (for review see ref. 280).

Recently it has been shown that the exposure of *Lactobacillus lactis* strain NZ9000 to roseoflavin (Fig. 34, Compound **57**) resulted in a spontaneous mutation, which reversed the strain from a riboflavin consumer into a riboflavin producer. This mutant contained a single base change in the regulatory region, the RFN element, upstream of the riboflavin biosynthetic genes.[281]

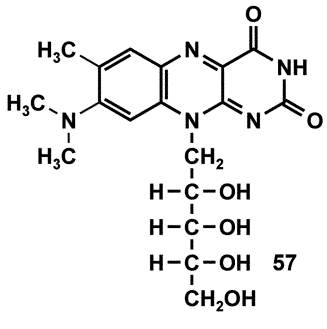

Fig. 34 **57**, Roseoflavin (8-dimethylaminoriboflavin).

17 Conclusion and perspectives

Flavocoenzymes are essential cofactors that have to be obtained by the conversion of riboflavin in all cellular organisms. Riboflavin (vitamin B_2) can be obtained by *de novo* biosynthesis or from nutritional sources. Most of the microorganisms can produce their own riboflavin and some members of this group can absorb riboflavin from the environment, facilitated by an efficient uptake system. It is an essential component of human and animal nutrition (due to the absence of an engenous biosynthesis) and it is manufactured in bulk quantity. Knowledge on the genetics, on the biochemistry, and on the regulation of the enzymes involved in the pathway may improve biotechnological processes for the production of the vitamin.

Some bacteria, like Enterobacteriaceae are unable to obtain riboflavin from the surrounding medium and those are absolutely dependent on endogenous biosynthesis. Members of this subgroup should be susceptible to inhibitors of biosynthetical enzymes involved in this pathway. The inactivation of these enzymes appearing as potential targets should cause antibacterial growth inhibition; advantageously these enzymes are usually not present in the human or animal host. It should be noted, that compounds interfering with the biosynthesis of folic acid, a vitamin characterised by many structural and biosynthetic similarities with riboflavin, have a long and highly successful history as chemotherapeutic agents. A detailed understanding of the mechanism of the riboflavin pathway could serve as a basis for the development of antiinfective agents of this novel type. It should be noted that most of the enzymes involved in this pathway catalyse extremely complex reactions, thus providing numerous opportunities for the rational design of transition state analogous inhibitors.

18 References

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