

Recent Advances in Riboflavin Biosynthesis

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Abstract

Riboflavin is biosynthesized from GTP and ribulose 5-phosphate. Whereas the early reactions conducting to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate show significant taxonomic variation, the subsequent reaction steps are universal in all taxonomic kingdoms. With the exception of a hitherto elusive phosphatase, all enzymes of the pathway have been characterized in some detail at the structural and mechanistic level. Some of the pathway enzymes (GTP cyclohydrolase II, 3,4-dihydroxy-2-butanone 4-phosphate synthase, riboflavin synthase) have exceptionally complex reaction mechanisms. The commercial production of the vitamin is now entirely based on highly productive fermentation processes. Due to their absence in animals, the pathway enzymes are potential targets for the development of novel anti-infective drugs.

Key words Biosynthesis of flavocoenzymes, Riboflavin synthase, Lumazine synthase, GTP cyclohydrolase II, Riboflavin biosynthesis

1 Introduction

There is reason to believe that flavocoenzymes derived from riboflavin (vitamin B₂) are essential in all living cells where they are involved in a wide variety of redox processes (it has been estimated that up to 2 % of enzymes may be using flavocoenzymes as cofactors; in line with that, several percent of structures in the Protein database are flavoproteins). More recently, a variety of flavoproteins has been found to mediate functions other than redox catalysis, such as dehydration, DNA repair, blue light sensing, and circadian timekeeping. Some of these more recently discovered functions have been reviewed elsewhere [1–3].

On the practical side, riboflavin is a bulk commodity that is manufactured on a scale of about 3,000 metric tons per year, predominantly for use in animal husbandry, with a minor fraction directly diverted to human nutrition in the form of food supplements, food colorants and as components of multivitamin preparations [4].

The discovery of riboflavin and its role in redox catalysis has been recognized by several Nobel prizes to Warburg, Kuhn, Karrer, and Theorell, and the pioneering work on the structure determination of the vitamin was instrumental in the generation of the early technology affording riboflavin by chemical synthesis. The investigation of the vitamin's biosynthesis was in part driven by attempts to replace chemical synthesis by fermentation. That approach was so successful that highly advanced fermentation processes have by now completely replaced the chemical synthesis as manufacturing process.

The large amount of work on riboflavin biosynthesis in the second half of the twentieth century has been reviewed repeatedly [5–10]. Rather than reiterating those papers, this review is focused on the considerable advances that have been achieved in the first decade of the present century.

2 Biosynthesis of Riboflavin

Most aspects of riboflavin biosynthesis (Fig. 1) are now firmly established. GTP (**1**) serves as the universal precursor, and the initial reaction steps (**B** in Fig. 1) involve the hydrolytic release of its C(8) as formate and of pyrophosphate to afford 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinedione 5'-phosphate (**2**) which is subsequently converted to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**7**) by three reaction steps involving reduction of the ribosyl side chain (**E&F**), deamination of the pyrimidine moiety (**D&G**), and dephosphorylation (**H**). Thus, the ribityl side chain and the pyrimidine moiety of the vitamin are entirely derived from the nucleotide precursor, GTP.

On the other hand, all carbon atoms of the xylene moiety of the vitamin are derived from the pentose pool. Specifically, a skeletal rearrangement followed by formate release (**I** in Fig. 1) converts ribulose phosphate (**8**) into 3,4-dihydroxy-2-butanone 4-phosphate (**9**) which reacts with **7** under formation of 6,7-dimethyl-8-ribityllumazine (**10**) (**J**). Riboflavin (**11**) is then generated by a mechanistically unique dismutation (**K**).

Whereas the reactions in the second part of the biosynthetic pathway are universal, the early part of the reaction sequence is a complex maze due to the fact that the sequence of events shows variations in different taxonomic kingdoms. It had long been known that the sequence of ring deamination and side chain reduction is inverse in eubacteria (**D&E**) and fungi **F&G**. As a relatively recent and surprising finding, plants were shown to use the eubacterial pathway with initial deamination (**D**) and subsequent reduction (**E**). Archaea, on the other hand, follow the fungal pattern with regard to side chain reaction preceding deamination (**F&G**); however, two enzymes (**A&C**) are required in archaea for the

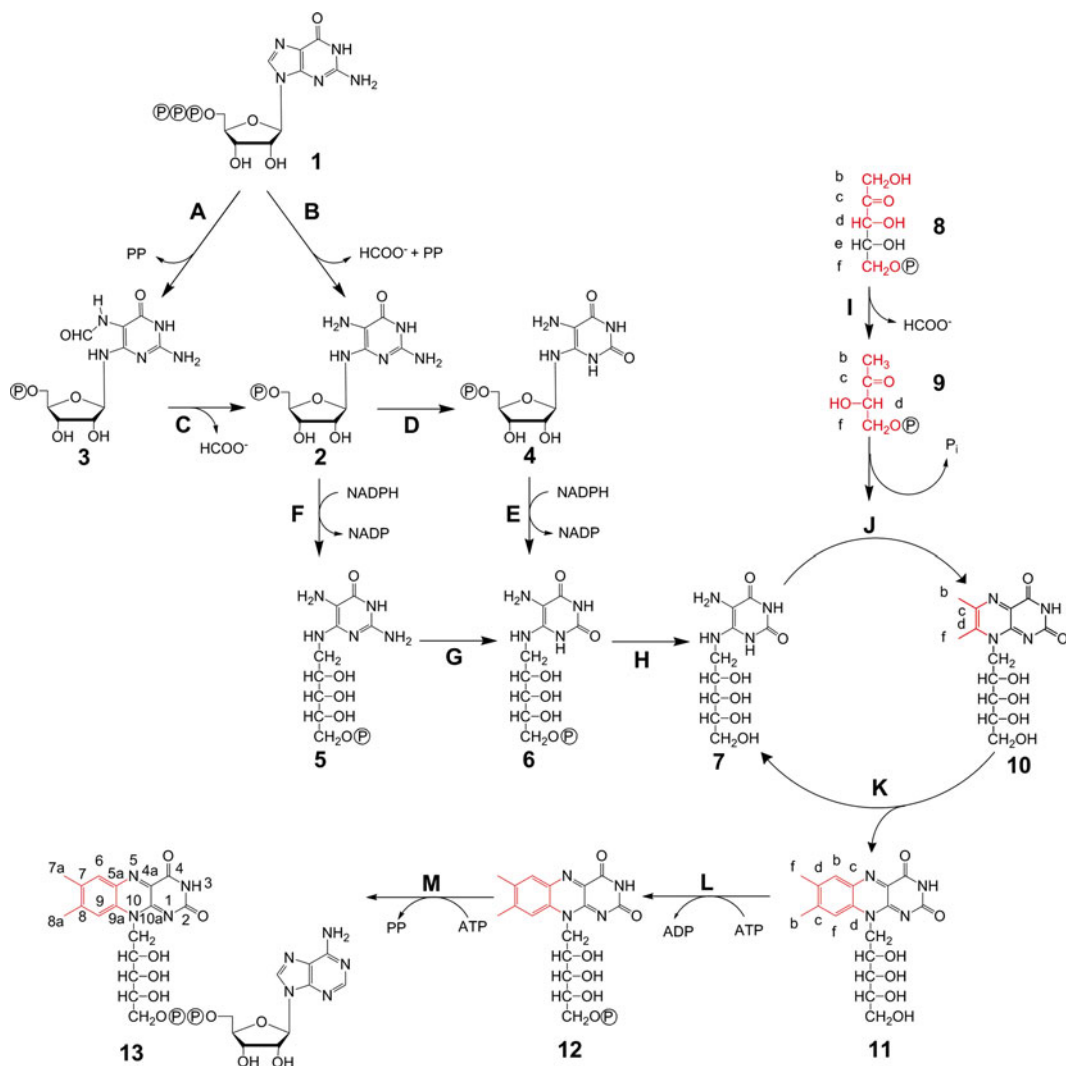


Fig. 1 Biosynthesis of flavocoenzymes. Enzymes are designated by capital letters which are used throughout the manuscript for reference to the Figure. Reprinted (adapted) with permission from (Römisch W., Eisenreich W., Richter G., Bacher A. (2002) Rapid one-pot synthesis of riboflavin isotopomers. *J Org Chem.* 67, 8890–8894). Copyright (2002) American Chemical Society

conversion of GTP into **2** which is catalyzed by a single enzyme (GTP cyclohydrolase II, **B**) in eubacteria and eukarya.

In all taxonomic kingdoms, the early reaction steps converge at the level of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate (**6**). Since that compound is unable to serve directly as substrate for lumazine synthase, which accepts only the dephosphorylated form, a dephosphorylation step **H** is mandatory. However, the details remain to be discovered. Possibly, the dephosphorylation step could be performed by some hydrolases with low substrate specificity.

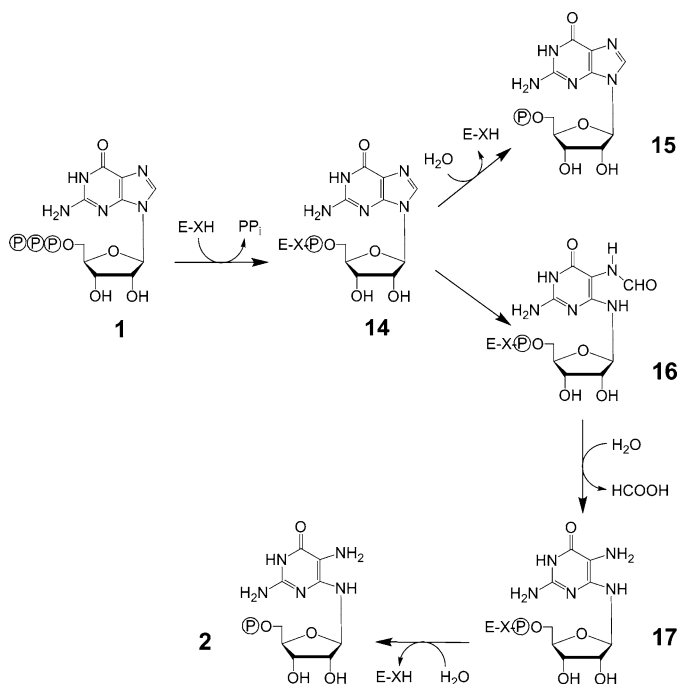


Fig. 2 GTP cyclohydrolase II reaction

Apart from the elusive phosphatase, the enzymes of riboflavin biosynthesis have been studied in considerable detail. GTP cyclohydrolase II (**B** in Fig. 2) and 3,4-dihydroxy-2-butanone synthase (**I**) catalyze multistep reaction trajectories which have been studied in some detail. However, the reaction catalyzed by riboflavin synthase (**K**) is mechanistically without parallel, and the recent developments on this topic will be a central aspect of this progress report.

2.1 GTP Cyclohydrolase II (Reaction B)

The conversion of GTP into **2** requires the hydrolytic cleavage of two carbon nitrogen bonds (affording formic acid as second product) and the hydrolysis of a phosphoanhydride bond affording inorganic pyrophosphate. In eubacteria and eukaryotes, these reaction steps are all catalyzed by GTP cyclohydrolase II (Fig. 2) [11, 12].

Besides the formation of the first committed intermediate of riboflavin synthase, GTP cyclohydrolase II also produces GMP (**15**) by release of pyrophosphate from GTP. The product ratio of GMP and **2** is about 1:10 [13]. The action of GTP cyclohydrolase II in H_2^{18}O is conducive to the incorporation of ^{18}O into the reaction product **2** as well as into **15** produced as a side product (see above). The first reaction step is therefore believed to involve the covalent linkage of a GMP moiety to an amino acid side chain, most likely Arg128 [14], under release of pyrophosphate. The hydrolytic cleavage of the phosphoamide bond affords the minor

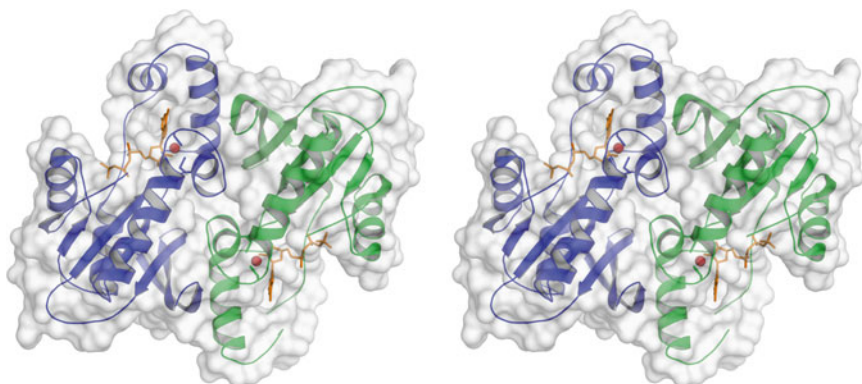


Fig. 3 Crystal structure of *E. coli* GTP cyclohydrolase II in complex with a non-hydrolyzable GTP analog (orange) and zinc (red) [14]

product, GMP. Alternatively, the imidazole ring of the covalently enzyme-bound guanyl moiety can be opened in two consecutive steps assisted by the zinc ion at the active site. More specifically, the initial cleavage of the C(8)–N(9) bond affords the formamide **16** which could be isolated using a His179 mutant of *E. coli* GTP cyclohydrolase II [15]. Cleavage of the formamide motif and subsequent hydrolysis of the phosphoamide bond affords the main product **2** which serves as the first committed intermediate of riboflavin biosynthesis. Surprisingly, the first reaction step, i.e. the formation of the covalent guanyl intermediate, appears to be rate-limiting for the overall reaction.

The structure of GTP cyclohydrolase II of *E. coli* has been determined by X-ray crystallography at a resolution of 1.5 Å (Fig. 3) [14]. The catalytic zinc ion is coordinated by Cys54, Cys65, and Cys567. It is located in close proximity to C(8) of the bound substrate. Initially, the zinc ion is believed to mediate the addition of a water molecule to C(8) under formation of a covalent hydrate, which can then be opened under formation of the formamide intermediate. The addition of a second water molecule to the formamide intermediate is also believed to be mediated by the zinc ion. A magnesium ion is coordinated to the triphosphate motif of the substrate, GTP, and appears to be essential for formation of the covalent guanylate intermediate **14**.

Under in vivo conditions, the rate of GTP cyclohydrolase II may be rate-determining for the overall formation of riboflavin. In vitro evolution of GTP cyclohydrolase II of *Bacillus subtilis* afforded a rate enhancement by a factor of about 2 which could be attributed to an increase of K_M by a factor of about 4 [16]. The engineered enzyme was conducive to increased riboflavin production in a recombinant producer strain.

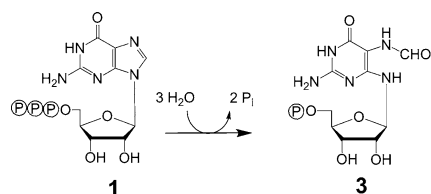


Fig. 4 GTP cyclohydrolase III reaction

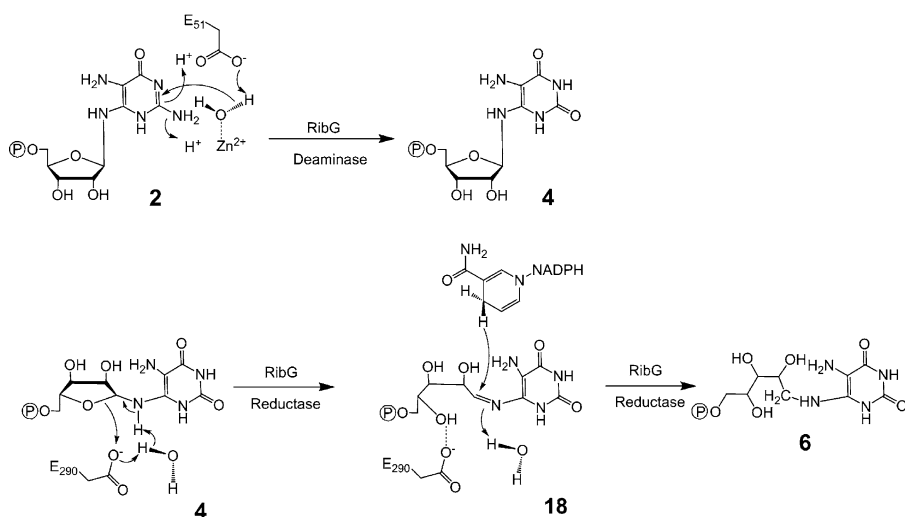


Fig. 5 Reaction mechanism of deaminase and reductase

2.2 GTP Cyclohydrolase III (Reaction A) and Formamidelyase (Reaction C)

In archaea, GTP cyclohydrolase III (**A** in Fig. 4) cleaves only one carbon nitrogen bond and thus yields the amide **3** as product (Fig. 4) [17, 18]. Formate is then released by a second hydrolase (**C**) [19]. The product **3** of GTP cyclohydrolase III can also be obtained with certain mutants of GTP cyclohydrolase II of *E. coli* [15, 20].

2.3 Pyrimidine Deaminase (Reactions D&G) and Pyrimidine Reductase (Reactions E&F)

The product of GTP cyclohydrolase II, 5-amino-6-ribosylamino-4(3H)-pyrimidinedione 5'-phosphate (**2**), is converted into 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate (**6**) by reduction of the ribosyl side chain and deamination of the pyrimidine ring (Fig. 5). The sequence of these reactions can be different, Fungi and archaea start with the reduction step, plants and eubacteria start with deamination (Fig. 1). Nevertheless, the deaminases of all taxa are homologous, and the reductases of all taxa are homologous. Many eubacteria use fusion proteins comprising an N-terminal deaminase domain and a C-terminal reductase domain.

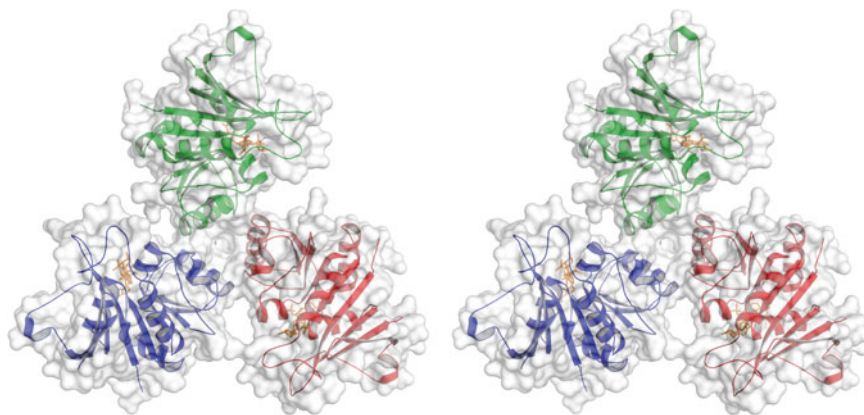


Fig. 6 Structure of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5'-phosphate reductase of *Methanocaldococcus jannaschii* in complex with NADP [21]. See also [22–24]

The pyrimidine deaminases of the riboflavin pathway are members of the pyrimidine deaminase superfamily. The zinc ion that is essential for catalysis is chelated by two cysteine residues and one histidine residue. The reductases of the riboflavin pathway are paralogs of dihydrofolate reductase. Structures of bifunctional deaminase–reductase fusion proteins have been determined by X-ray crystallography (Fig. 6) [22, 23, 25].

2.4 3,4-Dihydroxy-2-Butanone 4-Phosphate Synthase (Mutase, Reaction I)

The second substrate of lumazine synthase, 3,4-dihydroxy-2-butanone 4-phosphate (9), had escaped detection until the late 1980s, when it was shown to be formed from ribulose phosphate (8) by an enzyme requiring magnesium ions but no other cofactors (Fig. 7) [26–31].

The reaction involves the extrusion of C(4) of the ribulose phosphate substrate 8 as formate [32]. The complex reaction mechanism is believed to involve the initial formation of an endiol 19 from ribulose phosphate (8) that could then undergo the elimination of the hydroxy group at position 1 that results in the formation of the diketone 22 (Fig. 8). A sigmatropic rearrangement is then supposed to generate the branched aldose 24 that can release formate. The resulting endiol could then tautomerize under formation of the product 9. Whereas the enzyme-catalyzed tautomerization affords the product with L configuration as the naturally occurring intermediate [31], it has been shown that lumazine synthase can also use the non-natural D enantiomer [33].

The reaction mechanism of 3,4-dihydroxy-2-butanone 4-phosphate synthase has some similarity with that of the more recently discovered methylerythritol 4-phosphate synthase (IspC) catalyzing the first committed step in the non-mevalonate pathway for the biosynthesis of the universal isoprenoid precursors, IPP and DMAPP [34, 35]. The initial reaction steps convert the substrate,

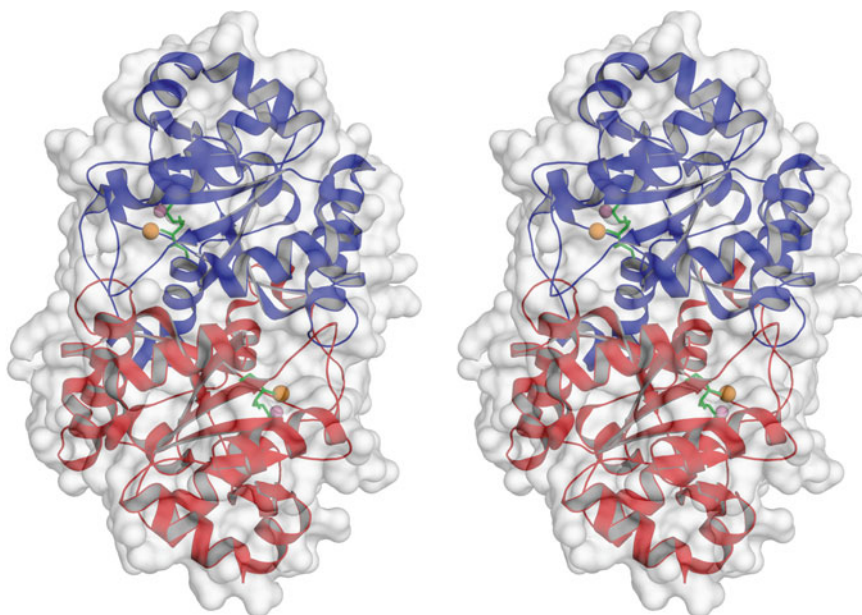


Fig. 7 Structure of 3,4-dihydroxy-2-butanone 4-phosphate synthase from *Methanocaldococcus jannaschii* in complex with calcium (orange), zinc (purple), and the substrate ribulose 5-phosphate (green) [32]. See also [36–43]

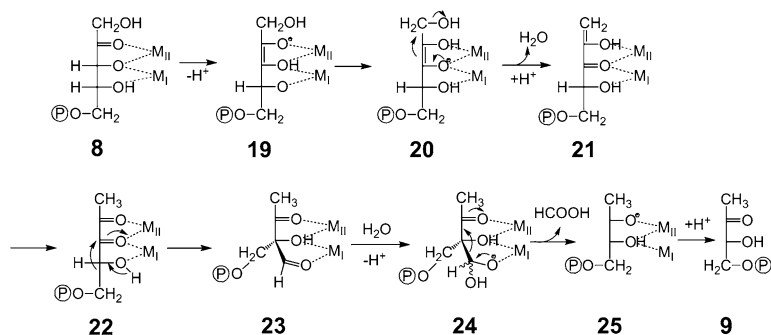


Fig. 8 Reaction mechanism of 3,4-dihydroxy-2-butanone 4-phosphate synthase

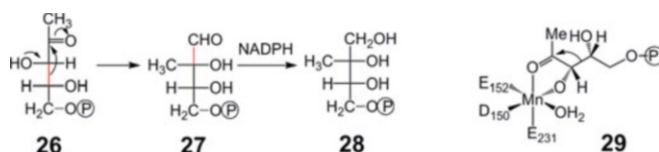


Fig. 9 Reaction mechanism of methylerythritol 4-phosphate synthase; *right*: substrate coupled to the active site

1-deoxyxululose phosphate (26), into the branched aldose (27). The aldehyde group is then reduced using NADPH as coenzyme (Fig. 9).

Significant similarity also exists with the mechanism of ribulose biphosphate carboxylase (RUBISCO), which catalyzes the first step of carbon fixation in the plant photosynthetic cycle and has

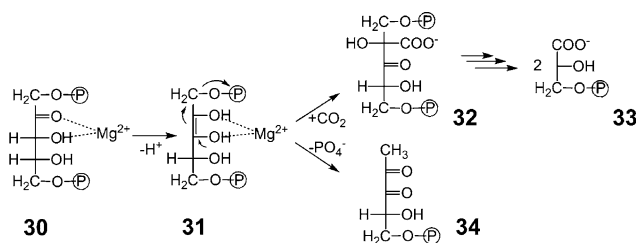


Fig. 10 Reaction mechanism of ribulose biphosphate carboxylase (RUBISCO)

been estimated to be the most abundant protein on earth. RUBISCO generates 2-carboxy-3-keto-D-arabinitol 1,5-bisphosphate (**32**) that undergoes fragmentation under formation of two equivalents of phosphoglycerate (**33**). As a side reaction, the early endiol intermediate **31** of RUBISCO can undergo phosphate elimination under formation of the diketo sugar **34** (Fig. 10) [32, 44–47].

The three enzymes have all been the subject of intense structural biology investigation. In fact, for 3,4-dihydroxy-2-butanone 4-phosphate synthase, IspC protein, and RUBISCO, at least 22, 38, and 60 respective X-ray structures have been published since 2000. Without doubt, this massive investment was in part driven by practical aspects such as drug development, crop protection (3,4-dihydroxy-2-butanone 4-phosphate synthase, IspC), or plant breeding (RUBISCO). Moreover, the structure of 3,4-dihydroxy-2-butanone 4-phosphate synthase has also been studied by NMR [42].

For catalytic activity, 3,4-dihydroxy-2-butanone 4-phosphate synthase depends on magnesium ions that cannot be replaced by zinc or calcium ions [42]. However, complexes of the protein with substrate or with glycerol that has been interpreted as a substrate analog have a tendency to include zinc and/or calcium ions from the crystallization buffer instead of the magnesium cofactor. The situation is further complicated by the apparent flexibility of a loop that is believed to serve as a lid, which occludes the active site after substrate loading. Although structures with very high resolution (better than 1 Å) have been reached, it has not been possible to directly investigate enzyme-bound intermediates or intermediate analogs.

Monofunctional 3,4-dihydroxy-2-butanone 4-phosphate synthases are c_2 -symmetric homodimers whose topologically equivalent active sites are located at the subunit interfaces (Fig. 7). Despite the caveats mentioned above, it appears safe to assume that catalysis involves two metal ions which are complexed by the oxygen substituents at carbon atoms 2, 3 and 4 of substrate or intermediates and by Glu25 of one subunit and His164 of the second subunit (residue numbers refer to the *M. jannaschii* protein). The phosphate residue of the substrate is embedded in a hydrogen bond network including Arg25, Arg161 and Thr165 (Fig. 11).

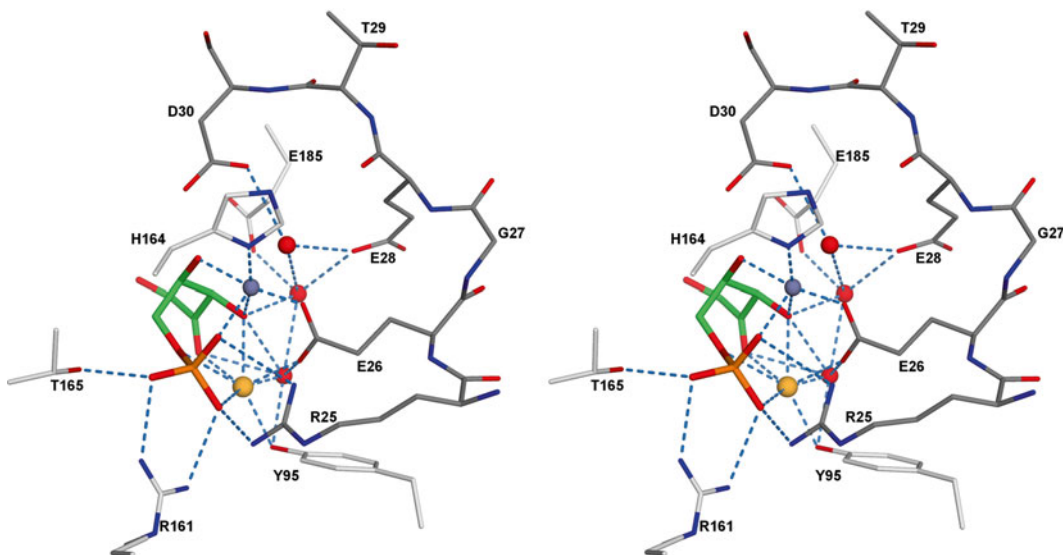


Fig. 11 Active site of 3,4-dihydroxy-2-butanone 4-phosphate synthase from *Methanocaldococcus jannaschii* comprising ribulose 5-phosphate (green), calcium (orange), zinc (purple), and several water molecules (red). Grey: Acidic active site loop. White: Further side chains of the active site [32]

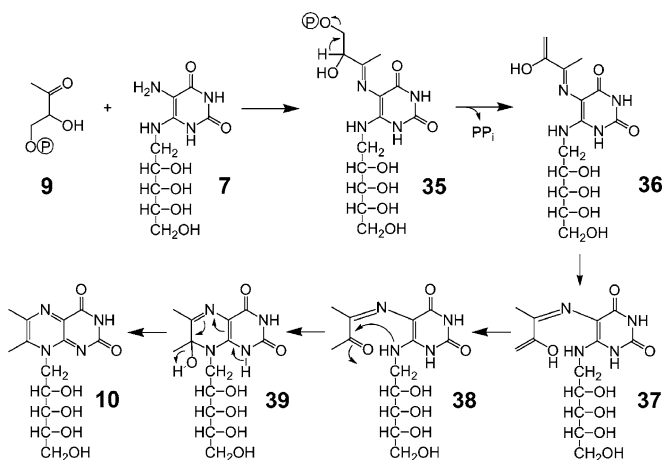


Fig. 12 Reaction mechanism of lumazine synthase

2.5 Lumazine Synthase (Reaction J)

Lumazine synthase catalyzes the penultimate step in the biosynthesis of riboflavin which involves the condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (7) with 3,4-dihydroxy-2-butanone 4-phosphate (9) under release of inorganic phosphate and two water molecules. The multistep reaction mechanism appears mechanistically straightforward. The initial formation of a Schiff base (35) is followed by elimination and ring closure (Fig. 12).

The reaction can proceed without enzyme catalysis at room temperature in dilute aqueous solution at neutral pH [33]. In fact,

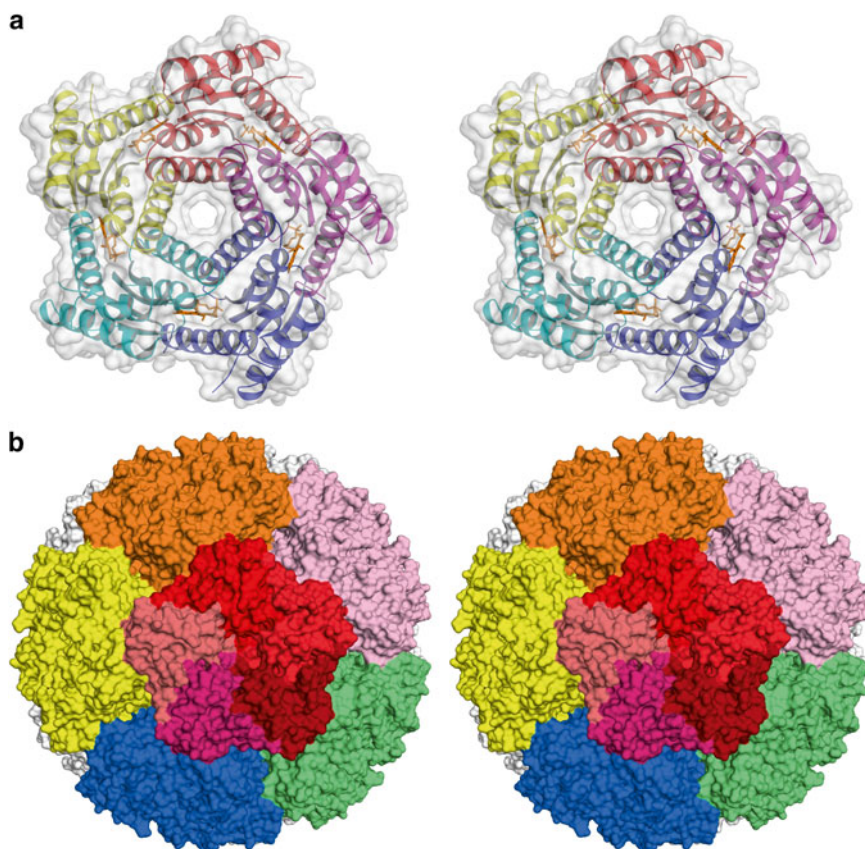


Fig. 13 Crystal structures of pentameric [56] and icosahedral lumazine synthases. See also [48–55, 57–64]

the rate acceleration by the enzyme is only modest. Lumazine synthase has been studied extensively by X-ray crystallography [48–64]. The number of over 40 structures, with most of them reported during the last decade, is larger than the number of structures of all other riboflavin biosynthesis enzymes combined. The main reason for the intense structural investigation may have been the structural complexity and structural versatility of lumazine synthases. Whereas the lumazine synthases of fungi, archaea, and certain eubacteria are c_5 -symmetric homopentamers, the enzymes from plants and many eubacteria are 532-symmetric dodecahedral/icosahedral capsids comprising 60 monomers which are best described as dodecamers of pentamers (Fig. 13). Under certain *in vitro* conditions, larger capsids comprising more than 100 subunits can also be formed [65]. Last not least, the icosahedral lumazine synthase capsids of *Bacillaceae* can enclose the homotrimeric riboflavin synthase in the central core [66, 67]. Also of note, the riboflavin synthases of archaea (but not those of eukarya and eubacteriaceae) are paralogs of lumazine synthase [68].

Analogues of the Schiff base intermediate **36** have been synthesized; they have been shown to bind to lumazine synthase in an extended conformation which most likely mimics the conformation of the early Schiff base intermediate **35** [63]. That, however, implicates that a cis-trans-isomerization of the imide **36** is necessary in a subsequent step.

Lumazine synthase subunits comprise about 150 amino acid residue. The subunit folds into an $\alpha\beta$ motif consisting of a four-stranded β sheet that is flanked on both sides by pairs of α helices. The c_5 symmetric pentamer assembly has a channel running along the fivefold axis which is formed by the α_3 helices of all respective subunits which can be viewed as a fivefold superhelix [64]. The topologically equivalent active sites are all located at interfaces of mutually adjacent monomers. The N-termini of lumazine synthases from *Bacillaceae* have short N-terminal extensions that connect with the adjacent subunit where they serve as a fifth strand of the central β sheet.

The dodecahedral/icosahedral lumazine synthases are best described as dodecamers of pentamers. The resulting, quasispherical capsid with icosahedral 532 symmetry has an outer diameter of about 150 Å and an inner diameter of about 75 Å (in case of lumazine synthases from *Bacillaceae*, the central core contains a riboflavin synthase homotrimer). Whereas icosahedral capsids are used by numerous spherical virions in order to package their genetic material, that structure principle is relatively rare in the world of enzymes. However, it should be noted that certain pyruvate dehydrogenases comprise icosahedral modules [69].

2.6 Riboflavin Synthase (Reaction K)

The final step in the biosynthesis of riboflavin is a mechanistically unique dismutation involving the transfer of a 4-carbon unit between two molecules of 6,7-dimethyl-8-ribityllumazine (**10**) affording equivalent amounts of riboflavin (**11**) and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**7**).

Notably, besides 6,7-dimethyl-8-ribityllumazine, the enzyme requires neither additional substrates nor cofactors. The reaction can be interpreted as a partial undoing of the lumazine synthase action in so far as it regenerates the lumazine synthase substrate which can be recycled by lumazine synthase. Almost incredibly, the reaction can proceed even without catalysis under relatively mild conditions (boiling of an aqueous solution of 6,7-dimethyl-8-ribityllumazine under anaerobic conditions) [70–72], for review see also [10].

Half a century of research into the mechanism of this unique reaction has yielded an impressive harvest of mechanistic hypotheses. There are basically two outstanding experimental observations that must be satisfied. (a) The transfer is regiospecific, and the two identical substrate molecules are aligned at the active site with c_2 pseudosymmetry (Fig. 12). (b) The reaction proceeds via a

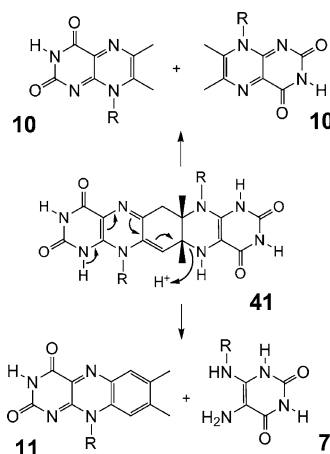


Fig. 14 Reaction of the pentacyclic intermediate **41** under regeneration of two substrate molecules (**10**) or under formation of the product riboflavin (**11**) and **7**

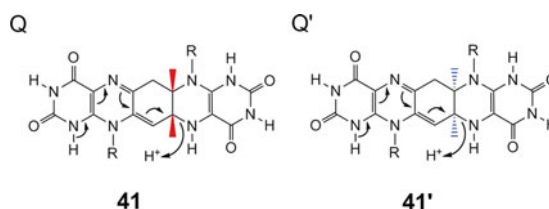


Fig. 15 Pentacyclic reaction intermediates of trimeric eubacterial **41** and pentameric archaeal **41'** riboflavin synthase

pentacyclic adduct **41** which can fragment in two possible ways, either under regeneration of the substrate, 6,7-dimethyl-8-ribityllumazine, or under formation of one molecule each of riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (Fig. 14) [73, 74].

The formation of the pentacyclic intermediate involves the generation of two novel stereocenters, but these are destroyed by fragmentation in either direction. Notably, however, the riboflavin synthases of archaea, on the one hand, and of eukarya and eubacteria, on the other hand, proceed via pentacyclic intermediates with different stereochemistry (Fig. 15) [75, 76].

The pentacyclic intermediate has been discovered at the turn of the century and was therefore not considered in the various mechanistic hypotheses that had been formulated earlier. An attempt to unite this crucial piece of evidenced with hypotheses proposed earlier by Plaut, Wood and their respective coworkers suggested the pathway shown in Fig. 16a (notably, a detailed discussion of the early mechanistic hypotheses is beyond the scope of this article, and the reader is directed to earlier reviews for an in depth discussion [72, 77–85]. Briefly, Fig. 16a implicates the initial formation of a lumazine exomethylene anion (**42**), which performs a

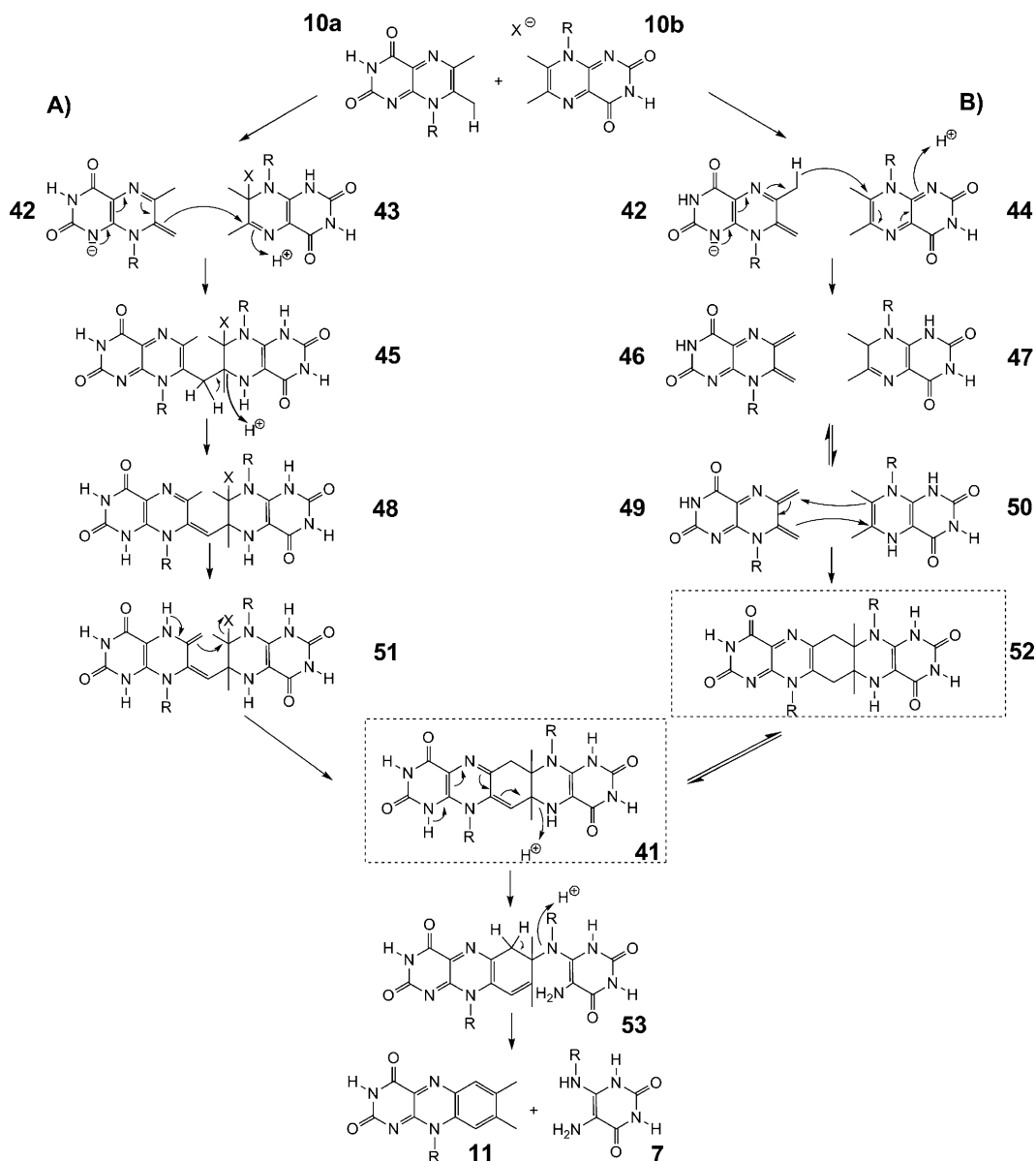


Fig. 16 Two proposed reaction mechanisms of riboflavin synthase [73, 86]

nucleophilic attack on the second substrate molecule which is then followed by ring closure under formation of the pentacyclic system **41**.

A more recent proposal shown in Fig. 16b also starts with the formation of the lumazine exomethylene anion (**42**), which subsequently donates a hydride anion to the second substrate [86]. The hydride donor is thereby converted into a quinonoid bis-exomethylene system (**46**), and the hydride acceptor is converted into a dihydrolumazine derivative (**47**); these two

moieties are then suggested to undergo a 4+2 cycloaddition affording **52**. The exomethylene structure **46** is amply documented by NMR studies and has been shown to be stabilized by complexation to the enzyme.

Whereas the formation of the pentacyclic intermediate **41** from two identical lumazine substrates is mechanistically complex, its fragmentation affording riboflavin and **7** by a sequence of two elimination reactions is mechanistically straightforward.

The riboflavin synthases of eubacteria and eukarya are homotrimers of 25 kDa subunits. The N-terminal and C-terminal half of the subunit shows a high degree of sequence similarity suggesting the formation of two similarly folded domains. That has been indeed confirmed by X-ray structure analysis which could also show that each domain can bind one respective substrate molecule in a shallow groove. Trimerization occurs by formation of a triple helix from the N-terminal domains of three subunits. The single active site is formed at the interface of the N-terminal domain of one subunit and the C-terminal domain of a second subunit; these interacting domains are related by pseudo- c_2 symmetry. Moreover, the N-terminal and C-terminal domain of each respective subunit are related by pseudo- c_2 symmetry. Surprisingly, the trimeric riboflavin synthases of eubacteria and eukarya are devoid of trimeric symmetry, and only two subunits can interact under formation of an active site. However, different subunits might be involved in the formation of a single active site by way of dynamic fluctuations.

The riboflavin synthases of archaea have no similarity with the trimeric enzymes of eubacteria and eukarya (Fig. 17). Rather, they are c_5 -symmetric homopentamers with close similarity with lumazine synthase [68]. The 5 topologically equivalent active sites are all located at the interfaces of adjacent subunits. The lumazine serving as the donor of the 4-carbon moiety has a position that is analogous to that of the pyrimidine substrate in lumazine synthase.

2.7 The Lumazine Synthase/Riboflavin Synthase Complex

Bacillaceae form a unique complex consisting of a riboflavin synthase trimer inside an icosahedral lumazine synthase. Under certain experimental conditions, the overall transformation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione into riboflavin is somewhat accelerated by intermediate channeling due to the encapsidation of the riboflavin synthase capsid; however, it is hard to imagine how this could have been a selective factor that would have driven the evolution of the enzyme complex [90]. Another unsolved riddle of the enzyme complex is the pathway for the transfer of substrates and products into and out of the capsid. Computer modeling has suggested fluctuations of the capsid structure as a way to temporarily increase the diameter of the channels running along the fivefold axes of the capsid [60]. Recent studies have resulted in the incorporation of protein modules other than

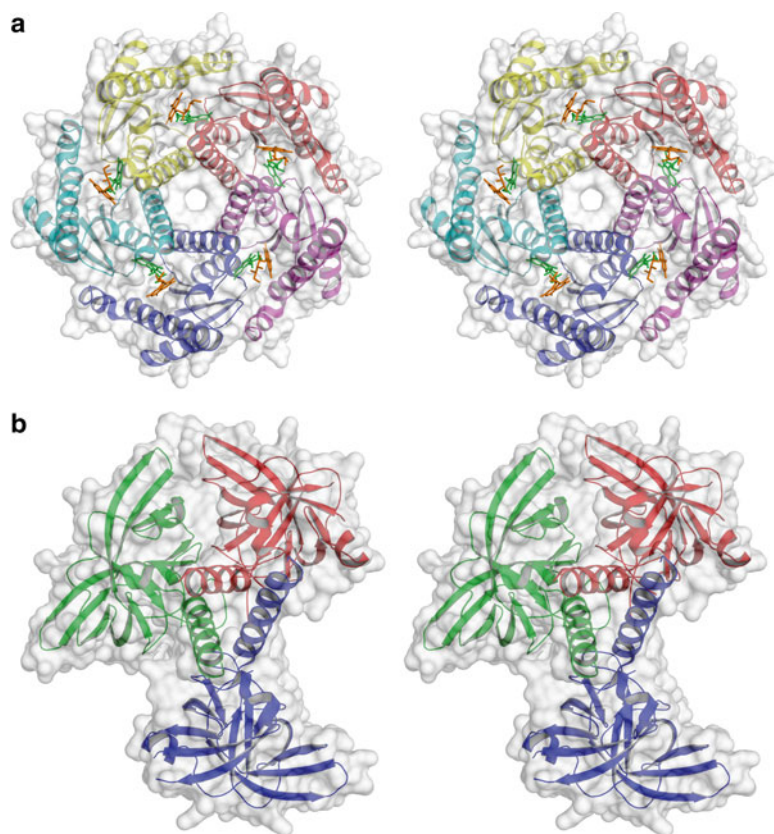


Fig. 17 Crystal structure of archaeal pentameric [75] and eubacterial trimeric [40] riboflavin synthase. The archaeal enzyme has bound ten 6,7-dioxo-5H-8-ribitylamino molecules (*orange/green*). See also [87–89]

riboflavin synthase (such as HIV protease and green fluorescent protein) into the lumazine synthase capsid of *Aquifex aeolicus*; the formation of the artificial protein complexes was mediated by mutations designed to provide charge interactions as a basis for association driven by electrostatic interaction [91, 92].

3 Deazaflavin Cofactors

The emerging research on methanogenic bacteria in the 1970s and beyond has resulted in the discovery of a fascinating bouquet of novel coenzymes that are essential for the conversion of CO₂ into methane. Thus, the structure of the deazaflavin cofactor F420 was reported in 1978 [93]. More recently, deazaflavins have also been detected in some eubacteria and in very early branch-offs from the plant evolutionary tree where they are involved in DNA photorepair [94–98].

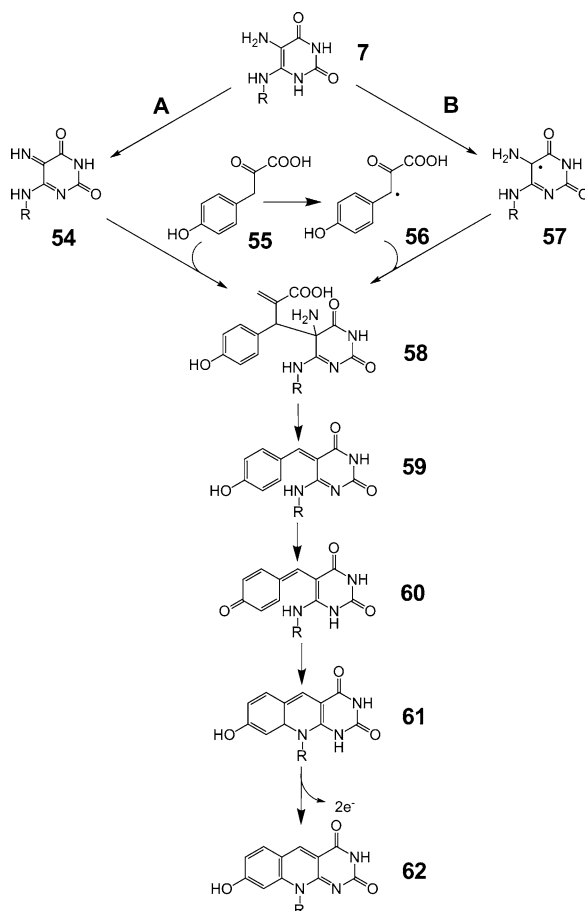


Fig. 18 Deazaflavin biosynthesis. (a) Via the quinoid pyrimidine intermediate [99, 100]; (b) by free radical recombination [101]

The deazaflavin chromophore (62) is biosynthesized from the riboflavin precursor 7 and from 4-hydroxyphenylpyruvate (55). The condensation of these building blocks is believed to proceed via free radical intermediates; two hypothetical reaction mechanisms are summarized in Fig. 18.

4 Roseoflavin

Roseoflavin (65) was isolated from *Streptomyces davawensis* as an antibacterial agent [102]. It has recently found renewed interest as an experimental tool for flavin biophysics. The producer organism has been shown to convert isotope-labeled riboflavin into roseoflavin [103]. A methyltransferase catalyzing the transfer of two methyl groups to 8-amino-riboflavin (63), specified by *rosA*, has been characterized recently as the first known enzyme of roseoflavin biosynthesis (Fig. 19) [104].

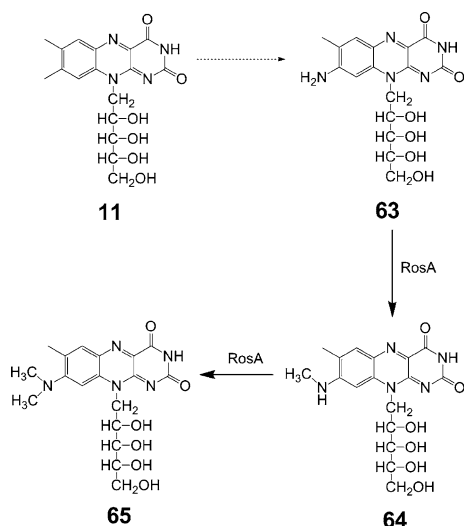


Fig. 19 Biosynthesis of roseoflavin

5 A Riboflavin Synthase Paralog as Optical Transponder

Certain marine bacteria use paralogs of riboflavin synthase designated lumazine protein, yellow fluorescent protein and blue fluorescent protein, respectively, as optical transponders for bioluminescence emission. The proteins are monomeric analogs of the trimeric riboflavin synthase which lack the C-terminal trimerization helix [105]. The proteins bind 6,7-dimethyl-8-ribityllumazine, FMN, or 6-methyl-8-ribityl-2,4,7(1*H*,3*H*,8*H*)-pteridinetrione as chromophores which can be excited by radiationless transfer from luciferase [106]. Notably, ligands are only bound by the N-terminal domain of lumazine protein [105, 107, 108].

6 Lumazine Synthase as Protein Container Model

The unique architecture of the lumazine synthase/riboflavin synthase complex has prompted successful attempts to incorporate proteins that are unrelated to riboflavin into the icosahedral shell. By implementation of glutamate residues at the inner surface, the lumazine synthase from the hyperthermophilic *Aquifex aeolicus* was enabled to incorporate the monomeric green fluorescent protein that had been tagged with 10 arginine residues in order to arrange for charge complementarity between the host and guest [91]. More recently, the incorporation of HIV protease into the thermostable lumazine synthase was improved by an *in vivo* evolution strategy [92, 109–111]. The inclusion of proteins into host protein capsids has been advocated as a tool that might be able to serve a variety of purposes.

7 Riboflavin Biosynthesis Genes as Potential Anti-infective Drug Targets

Whereas plants and many microorganisms generate riboflavin *de novo*, animals depend on dietary sources. Thus, riboflavin biosynthesis may provide an opportunity for the development of novel anti-infective drugs that should be exempt from target-related toxicity. Admittedly, biosynthesis of low molecular weight metabolites, with the exception of tetrahydrofolate, has not played a major role in anti-infective therapy. On the other hand, the biosynthesis of riboflavin and folate share certain interesting similarities; most notably, both vitamins are produced from GTP which undergoes opening of the imidazole ring as a first reaction step.

Potent inhibitors of riboflavin synthase have been discovered already in the 1960s by work in the research groups of Plaut and Wood. Most notably, 6-ribitylamino-2,4,6,7(1*H*,3*H*,5*H*,8*H*)-pteridinetetraone inhibits riboflavin synthase of *E. coli* with a K_i of 6.2 nM [112], and the compound can be viewed as an analog of the hypothetical quinoid intermediate (46) of the riboflavin biosynthesis reaction.

More recently, numerous substrate and intermediate analogs of lumazine synthase and riboflavin synthase have been synthesized and have yielded important contributions to our understanding of the reaction mechanisms (Fig. 20) [63, 112–124]. However, whereas some of the synthetic analogs are strong inhibitors of the target enzymes, they are devoid of antibacterial activity. This failure is probably due to their inability to reach their molecular targets.

Riboflavin synthase and lumazine synthase are both well suited for high throughput screening. This approach has resulted in the identification of 70 which inhibits riboflavin synthase with a K_i of 23 ± 14 μ M and has some activity against growing as well as non-growing *Mycobacterium tuberculosis* [123].

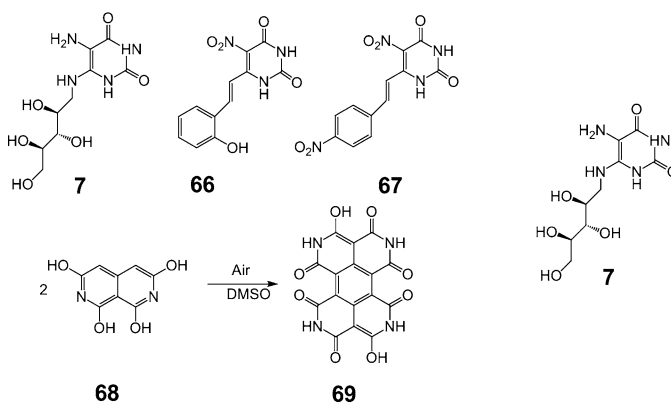


Fig. 20 Inhibitors of lumazine synthase 66, 67, 69 [122, 125], and riboflavin synthase 70 [123]

8 Riboflavin Production by Fermentation

Riboflavin is manufactured on an approximate scale of 3,000 metric tons per year, predominantly for use in animal husbandry and, on a smaller scale, as direct supplement for human nutrients and vitamin formulations [126]. The manufacture by chemical synthesis starting from ribose has been completely replaced by fermentation using *B. subtilis* or *Ashbya gossypii* [4]. In *B. subtilis*, the four genes specifying all proteins required for riboflavin biosynthesis, with the exception of the elusive phosphatase, form part of a single operon. Overexpression of that operon enables a process for the efficient in vivo biotransformation of glucose into the vitamin at high yield and with short process times. By contrast, the production of riboflavin by the Ascomycete, *A. gossypii*, is predominantly based on lipids as carbon supplement.

9 Lumazine Synthase as Vaccine

The study of *Brucella* antisera surprisingly identified lumazine synthase as the major antigen [127]. This prompted a detailed study of the *Brucella* enzyme which revealed the presence of two lumazine synthase genes, one of them coding for an icosahedral capsid and the other for a d₅-symmetric dimer of pentamers. Recently, a fusion protein with a *Brucella* membrane protein attached to the lumazine synthase moiety has been proposed as *Brucella* vaccine [128].

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