Annu. Rev. Nutr. 2000. 20:153–67

BIOSYNTHESIS OF VITAMIN B_2 (RIBOFLAVIN)

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Key Words riboflavin, lumazine, 3,4-dihydroxy-2-butanone 4-phosphate, GTP, ribulose 5-phosphate, deaminase, reductase

■ **Abstract** The biosynthesis of one riboflavin molecule requires one molecule of GTP and two molecules of ribulose 5-phosphate as substrates. The imidazole ring of GTP is hydrolytically opened, yielding a 4,5-diaminopyrimidine which is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by a sequence of deamination, side chain reduction and dephosphorylation. Condensation of 5-amino-6-ribitylamino-2,4(1H,3H)-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(1H,3H)-pyrimidinedione, which is recycled in the biosynthetic pathway. The structure of the biosynthetic enzyme, 6,7-dimethyl-8-ribityllumazine synthase, has been studied in considerable detail.

CONTENTS

INTRODUCTION

Riboflavin (vitamin B_2) is biosynthesized in plants and in many bacteria. Vegetables and milk are major sources of the vitamin in human nutrition. Ruminants

can derive vitamin B_2 from their intestinal flora. The daily recommended allowance for vitamin B_2 is 1.8 mg (59). Although the flavocoenzymes are absolutely indispensable in all cellular organisms, symptoms of riboflavin deficiency are rarely observed in humans. However, latent riboflavin deficiency may be relatively common, especially in women and adolescents (36)—especially in developing countries (1).

Apart from natural sources, vitamin B_2 is manufactured in bulk amounts for vitamin supplementation of human and animal nutrients. Fermentation processes, which are progressively replacing chemical manufacturing processes, have a long history. They were initially prompted by the natural occurrence of bacteria, yeasts, and fungi, which produce riboflavin in levels exceeding their apparent metabolic requirements (37). Early studies on the biosynthesis of vitamin B_2 were intimately linked with attempts to increase the production of riboflavin by flavinogenic microorganisms.

The discovery in 1952 by MacLaren (67) that the production of riboflavin can be increased by the addition of purine derivatives to the culture medium of *Eremothecium ashbyii* suggested a connection between purine and riboflavin. Numerous studies have subsequently shown that the pyrimidine moiety of riboflavin is biosynthetically related to guanine (12, 16, 71, 72, 87). Subsequent work identified guanosine triphosphate (GTP) as the committed precursor of riboflavin supplying the pyrimidine ring and the nitrogen atoms of the pyrazine ring, as well as the ribityl side chain of the vitamin (41, 42, 68).

The early work on the riboflavin biosynthetic pathway has been reviewed repeatedly (2, 4–7, 30–32, 37, 88, 90, 94). This article focuses on recent structural and mechanistic studies of enzymes involved in riboflavin biosynthesis. Some of the reactions are mechanistically complex and still incompletely understood.

AN OVERVIEW OF THE RIBOFLAVIN PATHWAY

The biosynthetic pathway is summarized in Figure 1. The imidazole ring of GTP (structure 1 of Figure 1) is opened hydrolytically under release of formate accompanied by release of pyrophosphate, which is catalyzed by GTP cyclohydrolase II (12, 13, 16, 26, 41, 42, 58, 83, 108, 109). The enzyme product 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate (2 in Figure 1) is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5′-phosphate (5 in Figure 1) by two reaction steps, involving the hydrolytic cleavage of the position 2 amino group of the heterocyclic ring and the reduction of the ribosyl side chain affording the ribityl side chain of the vitamin (33, 83). The sequence of these reaction steps varies in different organisms. In eubacteria, the deamination precedes the side chain reduction (33). In yeasts and fungi, the reduction precedes the deamination (10, 54, 64, 103).

5′-Phosphate of structure 5 can not serve as substrate for 6,7-dimethyl-8-ribityllumazine synthase. Hence, the compound must be dephosphorylated prior to further conversion (49, 75). Nothing is known about that reaction step.

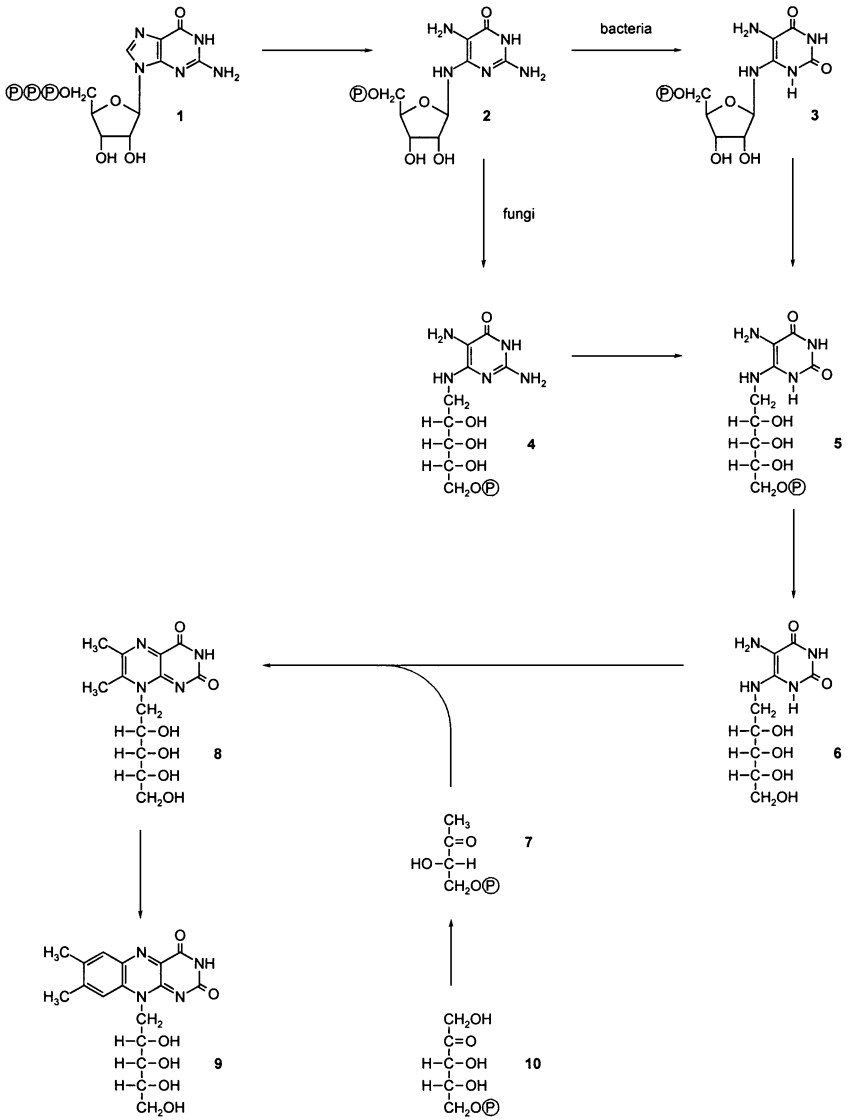

Figure 1 Biosynthesis of riboflavin.

The dephosphorylated 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (6 in Figure 1) is condensed with 3,4-dihydroxybutanone 4-phosphate (7 in Figure 1) by 6,7-dimethyl-8-ribityllumazine synthase (57, 75, 111). The carbohydrate type substrate (7 in Figure 1) of that enzyme has been discovered only relatively recently (111, 112). It is formed from ribulose 5-phosphate (10 in Figure 1) by an unusual reaction involving the loss of carbon atom 4 via an intramolecular rearrangement (113).

The final step of the biosynthetic pathway is the dismutation of 6,7-dimethyl-8-ribityllumazine (8 in Figure 1) catalyzed by riboflavin synthase (48, 89, 92, 93). The second product of the dismutation is 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (6 in Figure 1) (114). This compound is a substrate of lumazine synthase and is recycled in the biosynthetic pathway. Stoichiometrically, the formation of riboflavin requires one equivalent of GTP and two equivalents of ribulose 5-phosphate. Of the 17 carbon atoms of the riboflavin molecule, all but four are thus derived from the pentose phosphate pool.

The following section describes the reaction steps in closer detail.

GTP CYCLOHYDROLASE II

GTP cyclohydrolase II was first isolated from cell extracts of *Escherichia coli* (41). In an apparent tandem reaction, the enzyme catalyzes the release of C-8 of the imidazole ring of GTP as formate and the release of pyrophosphate from the triphosphoribosyl side chain. The enzyme requires magnesium ions for activity. The mechanism of the two hydrolytic reactions occurring at distant sites of the substrate molecule remains to be explained. Conceivably, the reaction could proceed in three steps: (*a*) covalent phosphoguanylation of the enzyme with release of pyrophosphate; (*b*) hydrolytic release of formate from the covalently bound phosphoguanosyl moiety; and (*c*) hydrolytic cleavage of the phosphodiester bond with the release of product 2 (Figure 1).

GTP cyclohydrolase II of *E. coli* is specified by the *ribA* gene (98). The 21.8-kDa protein is likely to form a homodimer.

Certain bacteria, such as *Bacillus subtilis*, form bifunctional proteins with GTP cyclohydrolase and 3,4-dihydroxy-2-butanone 4-phosphate synthase activity (53, 99).

DEAMINASE AND REDUCTASE

Bifunctional bacterial enzymes catalyzing the deamination of compound 2 (Figure 1) and the subsequent reduction of the phosphoribosyl side chain of 3 (Figure 1) have been found in *E. coli* and *B. subtilis* (95). Both enzyme activities require Mg^{2+}. The reductase requires NADPH or NADH as cofactor (33, 52). Genes specifying similar putative proteins have been found in a variety of microorganisms.

The reductase of the yeast *Saccharomyces cerevisiae* uses the product of GTP cyclohydrolase II as substrate and converts it to 2,5-diamino-6-ribitylamino-pyrimidinone 5′-phosphate (4 in Figure 1). The similarity between the reductase specified by the *RIB7* gene of *S. cerevisiae* and the bacterial deaminase/reductase is relatively low (95).

Enzymes catalyzing the deamination of ribitylaminopyrimidine (4 in Figure 1) have been partially purified from extracts of *S. cerevisiae* and *Ashbya gossypii* (52, 77).

3,4-DIHYDROXY-2-BUTANONE 4-PHOSPHATE SYNTHASE

The formation of the bicyclic riboflavin precursor 6,7-dimethyl-8-ribityllumazine by condensation of a 2,5-diamino-pyrimidine derivative with a four-carbon compound was anticipated by early investigators (for review, see 2). However, the actual four-carbon compound remained elusive despite numerous studies.

In vivo experiments using a wide variety of ^{13}C-labeled precursors indicated a biosynthetic relationship between the elusive four-carbon compound and the pentose pool via comparison of ^{13}C labeling patterns of the xylene ring with that of the ribityl side chain of riboflavin (8, 9). More specifically, in vivo studies suggested the assembly of the four-carbon precursors from carbon atoms 1, 2, 3, and 5 of a compound of the pentose/pentulose pool.

Based on these data, an enzyme was subsequently isolated from the flavinogenic yeast *Candida guilliermondii*, which catalyzes the formation of 3,4-dihydroxy-2-butanone 4-phosphate from ribulose 5-phosphate (65, 74, 111, 112). Studies with isotope-labeled ribulose 5-phosphate compounds showed that C-4 of the substrate is extruded as formate via an intramolecular rearrangement that reconnects carbon atoms 3 and 5 of the pentulose precursor (113). The hydrogen atoms at C-3 of the product are introduced from the solvent. Based on these data, a multistep mechanism featuring a series of tautomerization reactions and a sigmatropic rearrangement was proposed (Figure 2). More specifically, it has been proposed that the crucial reaction is the formation of endiol (11 in Figure 2) by tautomerization. 1-Phosphate of that hypothetical intermediate had been proposed earlier as an intermediate in the reaction catalyzed by ribulose bisphosphate carboxylase (81, 84, 85). The elimination of water from generated endiol followed by tautomerization could yield diketone (12 in Figure 2). A sigmatropic rearrangement subsequently generates a branched carbohydrate (13 in Figure 2). Elimination of formate and stereospecific reprotonation affords the enzyme product 3,4-dihydroxy-2-butanone 4-phosphate. The stereochemical course of this sigmatropic rearrangement was studied only recently using stereospecifically 5-^{2}H-labeled ribulose 5-phosphates (47). According to this study, the rearrangement proceeds with retention of the configuration at C-4 of 7 (Figure 2), which is well in line with a 1,2-sigmatropic rearrangement (76, 115).

The 3,4-dihydroxy-2-butanone 4-phosphate synthase of *E. coli* is a homodimer of 47-kDa specified by the *ribB* gene (96, 97, 99). Despite the relatively large molecular weight, structure analysis of the enzyme by nuclear magnetic resonance has been initiated successfully using multiple stable isotopic labeling and differential labeling of specific amino acid types (55, 96).

In numerous bacteria and in plants, 3,4-dihydroxy-2-butanone 4-phosphate synthase occurs as a bifunctional protein also comprising a GTP cyclohydrolase II domain (see above).

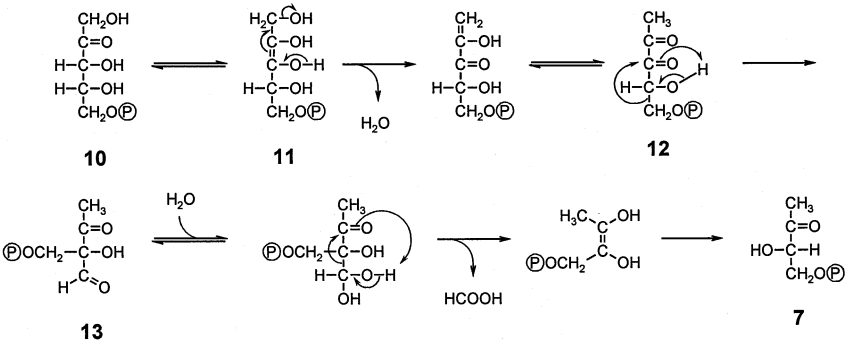

Figure 2 Proposed mechanism of 3,4-dihydroxy-2-butanone 4-phosphate synthase.

LUMAZINE SYNTHASE

The condensation of the pyrimidine (6 in Figure 3) with 3,4-dihydroxy-2-butanone 4-phosphate (7 in Figure 3) affords one equivalent of 6,7-dimethyllumazine (8 in Figure 3), two equivalents of water, and one equivalent of orthophosphate. The enzyme-catalyzed reaction is regiospecific (57, 78). This suggests that the reaction is initiated by the formation of a Schiff base via reaction of the position 5 amino group of the pyrimidine (6 in Figure 3) with the carbonyl group of 7 (Figure 3). The conjugation of the imine bond with the pyrimidine ring may facilitate the abstraction of a proton from intermediate 14 (Figure 3) followed by elimination of phosphate. Ring closure could then occur by a nucleophilic attack of the position 6 amino group on the carbohydrate side chain in conjunction with tautomerization steps (Figure 3).

The stereoselectivity of lumazine synthase with respect to the carbohydrate substrate is relatively low. The velocity of the lumazine formation with the naturally occurring L-3,4-dihydroxy-2-butanone 4- phosphate exceeds the velocity with the D-enantiomer only by a factor of 5. The K_M values for 6 and 7 (Figure 3) are 5 μM and 63 μM, respectively (57).

The lumazine synthase of plants and of many microorganisms have masses of approximately 1 MDa. These enzymes consist of 60 identical subunits that form a spherical capsid with icosahedral 532 symmetry (3, 15, 61, 62, 63, 106).

In Bacillaceae, the icosahedral 532 lumazine synthase capsids enclose a trimeric riboflavin synthase module (105, 106). This enzyme complex can catalyze the last two steps in the biosynthesis of riboflavin. The enzyme shows unusual steady state kinetics, which have been attributed to substrate channeling (56). In *B. subtilis*, this enzyme complex represents only 20% of the total riboflavin synthase activity, whereas 80% of the riboflavin synthase activity can be attributed to 75-kDa trimers, which are not associated with lumazine synthase.

Reconstituted, hollow capsids of lumazine synthase of *B. subtilis* have been studied by X-ray diffraction (62, 63, 100, 106). The virtually spherical molecules

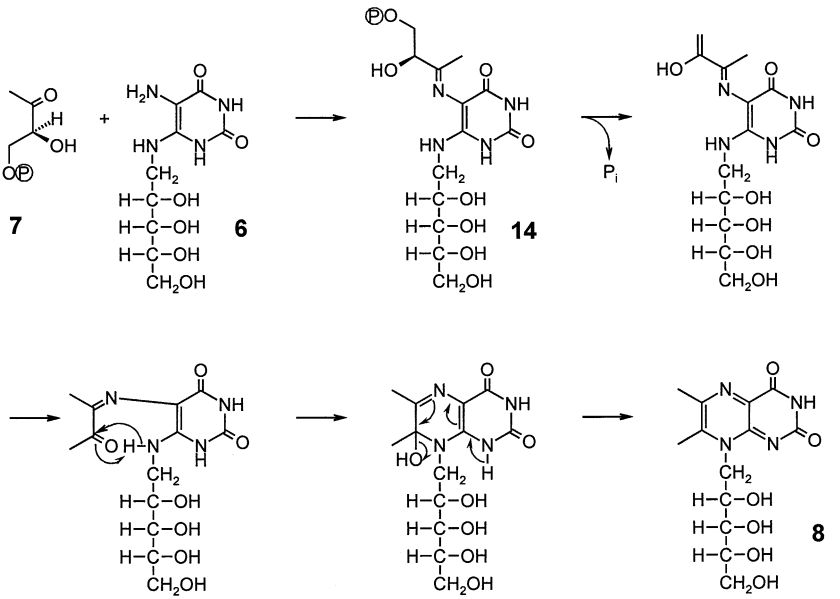

Figure 3 Mechanism of lumazine synthase.

are best described as dodecamers of pentamers. Each of the 60 equivalent active sites is located at the interface of two adjacent subunits in a pentamer module. Both adjacent subunits together form the surface of the active site cavity.

The ribityl side chain of the pyrimidine substrate can form hydrogen bonds with the peptide backbone as well as with amino acid side chains (62). The hypothetical reaction mechanism in Figure 3 suggests several proton transfer reactions expected to implicate specific amino acid residues. However, no specific amino acid residue participating in acid/base catalysis could be identified by active site mutagenesis (M Fischer, K Kugelbrey, K Kis, M Cushman, R Ladenstein, et al, manuscript in preparation). These findings suggest that the enzyme acts mainly as a positioner for the two substrates.

The condensation of the pyrimidine 6 (Figure 3) with carbohydrate 7 (Figure 3) can proceed at room temperature in neutral, dilute, aqueous solution in the absence of lumazine synthase (60). It is also remarkable that the turnover number of the enzyme from *B. subtilis* is only 0.076 s^{-1} per subunit (57).

More recently, it was found that the lumazine synthase of certain microorganisms are homopentamers (44, 73). The structure of the pentameric enzyme from *S. cerevisiae* has been determined by X-ray crystallography. Not surprisingly, the folding patterns of the pentameric and the icosahedral lumazine synthase are similar.

Lumazine synthase of *Brucella abortus* was found to dominate the human antibody response against that microorganism (38, 46, 51).

RIBOFLAVIN SYNTHASE

6,7-Dimethyl-8-ribityllumazine was detected by Masuda in 1956 (69, 70) in cultures of the flavinogenic ascomycete *Eremothecium ashbyii*. The green fluorescent material, designated G compound, could be converted to riboflavin by the enzyme riboflavin synthase (89, 107, 114). The enzyme catalyzes an unusual dismutation reaction, affording one molecule each of riboflavin and 5-amino-6-ribitylamino-2,4(^{1}H,^{3}H)-pyrimidinedione (6 in Figure 4) from two molecules of 6,7-dimethyl-8-ribityllumazine (8 in Figure 4) (89). The pyrimidine type product of riboflavin synthase is a substrate of lumazine synthase and can be recycled in the biosynthetic pathway (75).

Surprisingly, the dismutation of lumazine (8 in Figure 4) can proceed spontaneously in boiling aqueous solutions in the absence of a catalyst (17, 101, 102). The enzyme-catalyzed and the uncatalyzed reactions have the same regiochemistry (18, 79, 80).

Deprotonation of 6,7-dimethyl-8-ribityllumazine, which has a pK of about 8.5, generates a complex equilibrium mixture of anionic species (19). The equilibrium is dominated by three-cyclic species, but exomethylene form (15 in Figure 4) has also been demonstrated as a minor species (Figure 4).

It has been assumed that the reaction catalyzed by riboflavin synthase is initiated by the nucleophilic attack of a lumazine anion on a second lumazine molecule. Based on the regiospecificity of the reaction, the mechanism shown in Figure 4 was proposed (18, 79, 80, 89, 91–94); it remains to be verified in closer detail.

The sequence of riboflavin synthase from eubacteria is characterized by internal sequence similarity (105). More specifically, the N-terminal and C-terminal parts of *E. coli* riboflavin synthase have 25 identical amino acid residues and 22 conservative replacements (40). This suggests that each subunit folds into two topologically similar domains, each of which can bind one lumazine substrate. The catalytic site of the enzyme could then be located at the domain interface in the homotrimeric enzyme.

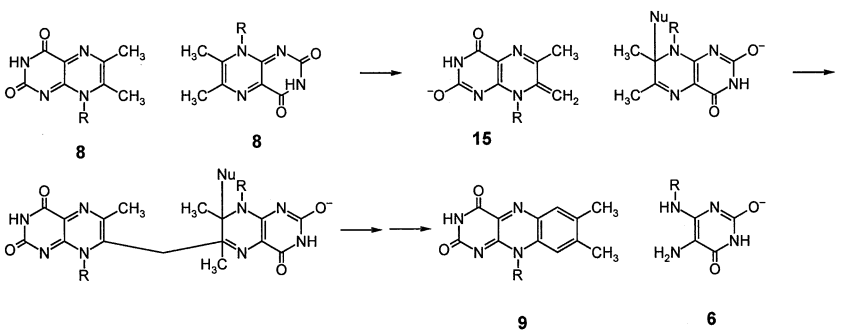

Figure 4 Mechanism of riboflavin synthase. R, D-ribityl.

Archaea have riboflavin synthases devoid of any similarity with the eubacterial enzymes (39). The archaeal riboflavin synthase subunits are considerably shorter than those of eubacterial enzymes. Moreover, they do not show internal sequence similarity. In contrast to the enzymes from eubacteria and yeast, they require Mg^{2+} for catalytic activity.

GENETICS AND REGULATION OF RIBOFLAVIN BIOSYNTHESIS

In *B. subtilis*, the enzymes involved in riboflavin biosynthesis are clustered in an operon that also comprises an additional open reading frame *ribT* of unknown function (20, 21, 23–25, 27, 45, 86). The transcriptional activity of this operon can be regulated over a wide range (14). A gene *ribC* that had been assumed to specify a repressor protein was recently shown to code for a bifunctional riboflavin kinase/FAD synthetase (20–22, 27–29, 34, 35, 66). The regulatory function of this enzyme remains to be explained.

BIOTECHNOLOGY OF RIBOFLAVIN

Riboflavin is a technical bulk product for use in human and animal nutrition. Until recently, the vitamin was produced predominantly by chemical synthesis. Currently, fermentation processes using *B. subtilis*, *Ashbya gossypii*, or Candida yeasts are progressively replacing the synthetic preparation method (50, 82). Recombinant *B. subtilis* strains for the production of vitamin B_2 have been described in considerable detail elsewhere (35, 66, 104).

Recently, a method was proposed to use riboflavin-deficient mutants of *Actinobacillus pleuropneumoniae* as vaccines for pigs (43). Enterobacteriaceae have no uptake system for riboflavin. Therefore, riboflavin-deficient mutants cannot grow in a mammalian host, although they can be cultured in vitro in the presence of large amounts of riboflavin.

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