THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 31, Issue of August 5, pp. 28541–28546, 2005

Printed in U.S.A.

Biosynthesis of Vitamin B_2

DIASTEREOMERIC REACTION INTERMEDIATES OF ARCHAEAL AND NON-ARCHAEAL RIBOFLAVIN SYNTHASES*[S]

Received for publication, March 21, 2005, and in revised form, May 20, 2005
Published, JBC Papers in Press, June 8, 2005, DOI 10.1074/jbc.M503093200

Boris Illarionov‡, Wolfgang Eisenreich§, Nicholas Schramek, Adelbert Bacher, and Markus Fischer¶

From the Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Germany

The dismutation of 6,7-dimethyl-8-ribityllumazine catalyzed by riboflavin synthase affords riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. A pentacyclic adduct of two 6,7-dimethyl-8-ribityllumazines has been identified earlier as a catalytically competent reaction intermediate of the *Escherichia coli* enzyme. Acid quenching of reaction mixtures of riboflavin synthase of *Methanococcus jannaschii*, a paralog of 6,7-dimethyl-8-ribityllumazine synthase devoid of similarity with riboflavin synthases of eubacteria and eukaryotes, afforded a compound whose optical absorption and NMR spectra resemble that of the pentacyclic *E. coli* riboflavin synthase intermediate, whereas the circular dichroism spectra of the two compounds have similar envelopes but opposite signs. Each of the compounds could serve as a catalytically competent intermediate for the enzyme by which it was produced, but not *vice versa*. All available data indicate that the respective pentacyclic intermediates of the *M. jannaschii* and *E. coli* enzymes are diastereomers.

Riboflavin synthase, the last enzyme in the vitamin B_2 biosynthetic pathway, catalyzes the transfer of a 4-carbon fragment between two molecules of 6,7-dimethyl-8-ribityllumazine, affording one molecule each of riboflavin (vitamin B_2) and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (Fig. 1) (Refs. 1 and 2; for review, see also Refs. 3 and 4). The reaction can also proceed without catalysis under relatively mild conditions (boiling in neutral or acidic aqueous solution) (5–7). The regiochemical features of the enzyme-catalyzed (8) as well as the uncatalyzed (9) reaction require the two substrate molecules to be arranged with antiparallel orientation in the activated complex (10).

Initial work on the reaction mechanism of riboflavin synthase by Plaut and co-workers (2, 11–13) was focused on the enzyme from the yeast *Saccharomyces cerevisiae*. More recently, riboflavin synthases from a variety of prokaryotic and eukaryotic organisms including *Escherichia coli*, *Bacillus subtilis*, and *Schizosaccharomyces pombe* have been described in some detail (14–16). The subunits of these homotrimeric enzymes are all characterized by intramolecular sequence similarity (10) and fold into two domains with closely similar folding topology (16–19). Each of the folding domains can bind one substrate molecule in a shallow cavity (16–17), and catalysis is believed to proceed at the interface between one N-terminal domain and the C-terminal domain of an adjacent subunit (10, 16). Surprisingly, the homotrimeric enzyme from *E. coli* is devoid of trigonal symmetry (17). In line with spectroscopic data, the x-ray structure data show that only one pair of domains can be catalytically active at any given time (16, 20–25).

Reaction mechanisms for riboflavin synthase were initially proposed by Wood, Plaut, and their respective co-workers (8, 13, 26–28). Because the position 7 methyl group of 6,7-dimethyl-8-ribityllumazine is unusually acidic with a p*K* around 9 (9, 27, 29), the carbanion resulting from deprotonation was proposed to perform a nucleophilic attack on a second substrate molecule (8–9, 28). More recently, a pentacyclic adduct of two substrate molecules was identified by rapid quench experiments with an S41A mutant of riboflavin synthase of *E. coli* and shown to fulfill the criteria for a kinetically competent intermediate (30). The absolute stereochemistry of the adduct was derived from a structural comparison between the riboflavin synthases of *E. coli* and *S. pombe* (16). However, it should be noted that a different configuration has been proposed on basis of modeling studies (31).

An unusual riboflavin synthase was recently cloned from *Methanothermobacter thermoautotrophicus* by marker rescue (32). More recently, efficient expression of a similar enzyme from *Methanococcus jannaschii* was achieved using a synthetic gene that was optimized for the codon usage of *E. coli* (33). That enzyme catalyzes the formation of riboflavin with the same regiochemistry as the riboflavin synthases of eubacteria and eukaryotes described above (*i.e.* an antiparallel arrangement of the substrate molecules in the activated complex).

The riboflavin synthases of Archaea show sequence similarity with the penultimate enzyme of the riboflavin pathway, 6,7-dimethyl-8-ribityllumazine synthase, which catalyzes the condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione with 3,4-dihydroxy-2-butanone 4-phosphate under release of inorganic phosphate (34–37). Archaeal riboflavin synthases are believed to obey c_5 symmetry, in analogy with 6,7-dimethyl-8-ribityllumazine synthase (33, 38–41); structural features that would allow for the formation of a pseudo-c_2-symmetric active site in analogy to the eubacterial and eukaryotic riboflavin synthases are not present.

* This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Hans-Fischer-Gesellschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data.

‡ On leave of absence from Insitute of Biophysics, Krasnoyarsk, Russia.

§ To whom correspondence may be addressed. Tel.: 49-89-289-13336; Fax: 49-89-289-13363; E-mail: wolfgang.eisenreich@ch.tum.de.

¶ To whom correspondence may be addressed. Tel.: 49-89-289-13336; Fax: 49-89-289-13363; E-mail: markus.fischer@ch.tum.de.

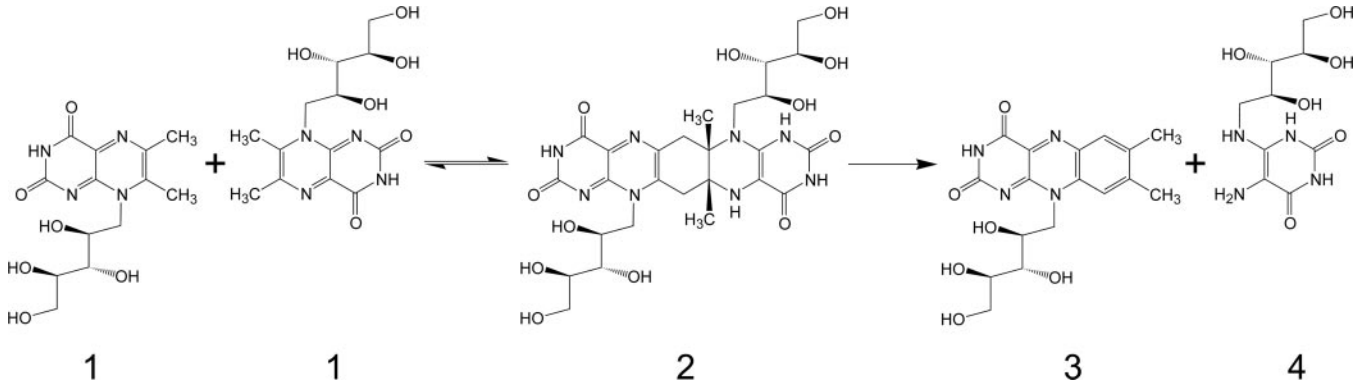

FIG. 1. **A shortened version of the reaction mechanism of *E. coli* riboflavin synthase.** *1*, 6,7-dimethyl-8-ribityllumazine; *2*, pentacyclic intermediate = Compound Q; *3*, riboflavin; *4*, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidine-dione.

This study shows that the reaction catalyzed by riboflavin synthase of *M. jannaschii* involves a pentacyclic intermediate, which is a diastereomer of the pentacyclic intermediate of riboflavin synthase of *E. coli*.

EXPERIMENTAL PROCEDURES

Materials—6,7-Dimethyl-8-ribityllumazine was prepared by published procedures (20, 42). [U-$^{13}C_{13}$]6,7-dimethyl-8-ribityllumazine and isotopolog mixtures of 6,7-dimethyl-8-ribityllumazine were prepared as described previously (43). Oligonucleotides were custom-synthesized by MWG Biotech (Ebersberg, Germany).

Proteins—Riboflavin synthases of *E. coli* and *M. jannaschii* were prepared as described elsewhere (24, 33).

Isolation of the Pentacyclic Intermediate of E. coli Riboflavin Synthase (Compound Q)—The isolation procedure for the pentacyclic intermediate of *E. coli* riboflavin synthase has been described previously (30).

Isolation of the Pentacyclic Intermediate of M. jannaschii Riboflavin Synthase (Compound Q′), Steady-state Kinetic Measurements, Analytical Reverse Phase High Pressure Liquid Chromatography, CD Spectroscopy, NMR Spectroscopy, and Computer Modeling—The detailed procedures are described in the on-line supplemental data.

RESULTS

Riboflavin synthases are inherently slow catalysts. The enzymes from *E. coli* and *M. jannaschii* catalyze the formation of product at rates of 5.3 and 0.16 molecules per minute and per subunit, respectively, at 25 °C (24, 33). That slow rate makes it possible to perform acid quenched single turnover experiments with relatively large reaction mixture volumes. In that way, the pentacyclic intermediate of riboflavin synthase of *E. coli* was obtained earlier by quenching a reaction mixture containing the S41A mutant of riboflavin synthase and 6,7-dimethyl-8-ribityllumazine (at a ratio of 2 substrate equivalents/subunit) with trifluoroacetic acid (30).

At room temperature, the *M. jannaschii* enzyme operates at an even slower rate than the *E. coli* enzyme, and it is therefore easily accessible to the same experimental approach. Specifically, a mixture containing 2.8 mM 6,7-dimethyl-8-ribityllumazine and 0.3 mM (per enzyme pentamer) *M. jannaschii* wild type riboflavin synthase was incubated for 45 s. Trichloroacetic acid was then added rapidly, with stirring to a final concentration of 0.15 M. Precipitated protein was removed by centrifugation, and the mixture was subjected to chromatographic separation affording 6,7-dimethyl-8-ribityllumazine, riboflavin, and a compound subsequently designated Q′, whose optical spectrum closely resembled that of the pentacyclic intermediate of *E. coli* riboflavin synthase, Compound Q (Fig. 2).

The isolated Compound Q′ was converted to riboflavin at a rate of 21 nmol mg^{-1} min^{-1} by riboflavin synthase of *M. jannaschii* under steady-state conditions, whereas 6,7-dimethyl-8-ribityllumazine was converted to riboflavin at a rate of 11 nmol mg^{-1} min^{-1}. Hence, Q′ fulfills the criteria for a kinetically competent intermediate. The partitioning factor for enzyme-catalyzed formation of riboflavin and 6,7-dimethyl-8-ribityllumazine from Compound Q′ was determined to be more than 2000:1, *i.e.* practically all Q′ used in this experiment was converted to riboflavin. This contrasts with earlier observation of the *E. coli* riboflavin synthase in which the transformation of Compound Q into riboflavin and 6,7-dimethyl-8-ribityllumazine proceeds with a partitioning factor of 5:1 (30).

When Compound Q (*i.e.* the putative intermediate of the *E. coli* enzyme) was tested as a substrate for *M. jannaschii* riboflavin synthase, the rates of formation for riboflavin and 6,7-dimethyl-8-ribityllumazine were below the limit of detection (<10 pmol mg^{-1} min^{-1}). Similarly, when Compound Q′ was tested as substrate for riboflavin synthase of *E. coli*, the rates of formation for riboflavin and 6,7-dimethyl-8-ribityllumazine were <10 pmol mg^{-1} min^{-1}.

Retention volumes for Q and Q′ on an RP18 column were identical. Absorption spectra of Compound Q generated by the *E. coli* enzyme and of the novel Compound Q′ generated by the *M. jannaschii* enzyme are similar under acidic as well as alkaline conditions (Fig. 2, *A* and *B*). On the other hand, circular dichroism revealed similar envelopes but opposite signs (Fig. 3).

Fig. 4*B* shows ^{13}C NMR signals of a sample of Compound Q′ prepared from [U-$^{13}C_{13}$]6,7-dimethyl-8-ribityllumazine. The multiplicity of ^{13}C signals suggests a dimer formed from 6,7-dimethyl-8-ribityllumazine, in parallel to our earlier studies with Compound Q, the intermediate of *E. coli* riboflavin synthase. With the exception of two signals (C-2 and C-2*), which appear as singlets, all ^{13}C signals appear as multiplets due to $^{13}C^{13}C$ coupling. Information on carbon/carbon connectivity was obtained from the $^{13}C^{13}C$ coupling pattern and from a two-dimensional $^{13}C^{13}C$ homocorrelation experiment (INADEQUATE), which identified 10 directly linked ^{13}C pairs (Table I). Two spin systems each comprising five ^{13}C atoms in the range of 45–75 ppm represent two topologically non-equivalent D-ribityl side chains. Their signals all appear as multiplets due to $^{13}C^{13}C$ coupling. The line widths are relatively narrow, in the range of 20–40 Hz.

The line widths of the ^{13}C signals of the heterocyclic moiety vary over a much wider range of 10–160 Hz. Unusual line broadening of certain signals had also been observed previously with Compound Q, the reaction intermediate of *E. coli* riboflavin synthase (30).

Connectivities for about one-half of the carbon atoms of Compound Q′ could be established directly by INADEQUATE spectroscopy of the universally ^{13}C-labeled samples (see above). However, certain carbon atoms escaped detection in the INADEQUATE spectrum due to their increased line widths.

To unequivocally assign all ^{13}C NMR signals, we prepared Compound Q′ from a mixture of 6,7-dimethyl-8-ribityllumazine

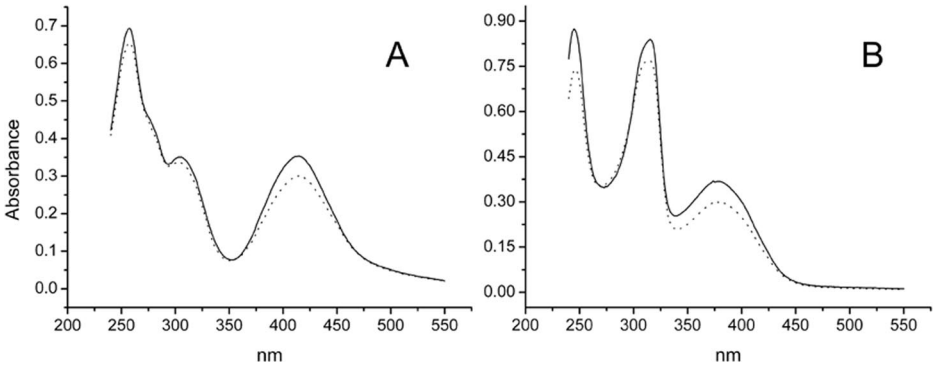

FIG. 2. **Absorbance spectra of Compound Q (29 μM, *dotted line*) and Compound Q′ (31 μM, *solid line*) in universal buffer (0.1** M acetic acid, 0.1 M phosphoric acid, and 0.1 M boric acid) pH 3.3 (*A*) and pH 10.2 (*B*).

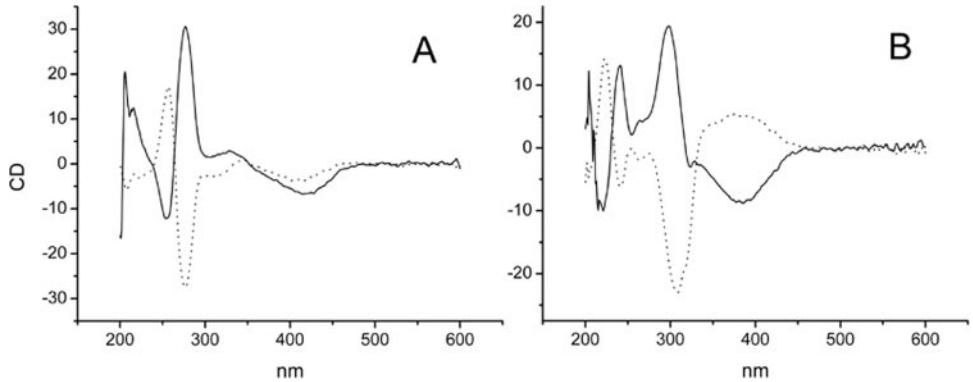

FIG. 3. **Circular dichroism spectra of Compound Q (147 μM, *dotted line*) and Compound Q′ (181 μM, *solid line*).** *A*, at pH 1.0 (0.1 M HCl), the CD peak values are −3.7 (418 nm), −27.4 (276 nm), and 17.2 (257 nm) for Compound Q and −6.6 (421 nm), 30.6 (277 nm), and −13.1 (255 nm) for Compound Q′. *B*, at pH 11.0 (0.1 M sodium phosphate and 0.1 M boric acid), the CD peak values are 5.5 (376 nm), −23.0 (309 nm), −6.1 (240 nm), and 14.2 (223 nm) for Compound Q and −8.8 (386 nm), 19.3 (298 nm), 13.2 (241 nm), and −10.0 (221 nm) for Compound Q′.

isotopologs obtained by fermentation with [3-^{13}C]glucose as carbon source (43). That mixture predominantly contained the [2′-^{13}C]-, [3′-^{13}C]-, [6-^{13}C]-, [8a-^{13}C]-, [7-^{13}C]-, and [4-^{13}C]-labeled isotopologs of 6,7-dimethyl-8-ribityllumazine; ^{13}C enrichments of these species were shown previously (43) to vary between 20% and 70% ^{13}C. Naturally, the amplitudes of the Compound Q′ sample derived from that isotopolog mixture must reflect the different ^{13}C abundance in different carbon positions (Table II). This amplitude variation can be used to trace individual carbon atoms in Compound Q′ to the atoms in 6,7-dimethyl-8-ribityllumazine from which they were derived. Two sections of the ^{13}C NMR spectrum of Q′ obtained from this library of selectively ^{13}C enriched 6,7-dimethyl-8-ribityllumazines are shown in Fig. 4*A*. The signal integrals are given below the respective signals. The relative ^{13}C enrichments of the sample whose spectrum is shown in Fig. 4*A* were calculated from the ratios of the integrals in Fig. 4, *A* and *B*. Taking the absolute ^{13}C enrichments at C-6 of 6,7-dimetyl-8-ribityllumazine as a reference value (73%), the relative enrichments of Q′ at different carbon atoms were normalized (observed enrichments in Table II). The observed values are in almost perfect agreement with the predicted ones and establish the basis for the signal assignments shown in Figs. 4 and 5 and Table I.

Based on these data, we conclude that Compounds Q and Q′ have the same constitution but diastereomeric configurations. This implies that the two lumazine substrates at the respective active sites of the *E. coli* and the *M. jannaschii* enzyme must be arranged with different topologies relative to each other.

DISCUSSION

The terminal enzyme reaction in the biosynthesis of riboflavin has a surprisingly large number of unique features. (i) The substrate, 6,7-dimethyl-8-ribityllumazine, has an extremely acidic methyl group with a p*K* around 9, and the facile formation of the cognate lumazine anion is believed to represent an early step in

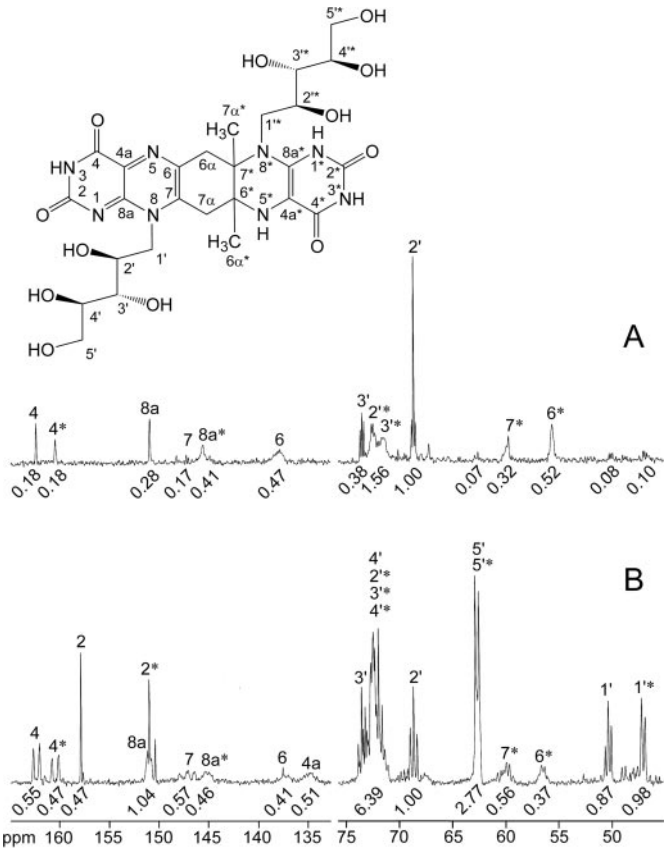

FIG. 4. **^{13}C NMR spectra of Compound Q′ obtained from a mixture of ^{13}C-labeled [2′-^{13}C]-, [3′- ^{13}C]-, [6-^{13}C]-, [8a-^{13}C]-, [7-^{13}C]-, and [4-^{13}C]6,7-dimethyl-8-ribityllumazine isotopologs (*A*) or from [U-^{13}C$_6$]6,7-dimethyl-8-ribityllumazine (*B*).** The integral values of ^{13}C NMR signals are given *below* the respective signals.

TABLE I
NMR data of [U-$^{13}C_{26}$]Compound Q′

d, doublet signal; t, triplet signal.

Position	Chemical shift ($\delta^{13}C$)	Line width ($V_{½}$)	Coupling constant (J_{cc})	Correlation pattern (INADEQUATE)
7α*	18.4	160		
6α*	20.5	150		6*
7α	34.5	150		
6α	36.5	140		
1′*	47.0	23	41 (d)	2′*
1′	50.2	17	37 (d)	2′
6*	56.5	90		6α*
7*	59.8	90		
5′, 5′*	62.7	40	40 (d)	4′*, 4′
2′	68.7	20	40 (t)	
2′*, 3′*, 4′*, 4′	72.9 to 70.9			1′*, 1′, 5′, 5′*
3′	73.6	20	42 (t)	
4a*	87.4	70		
4a	134.6	150		8a
6	137.1	150		
7	144.9	120		
8a*	146.6	40	82 (d)	
8a	150.8	85		4a
2*	150.8	10		
2	157.7	10		
4*	160.3	25	85 (d)	
4	162.2	20	76 (d)	

TABLE II
^{13}C pattern of Compound Q′ synthesized from an isotopolog mixture of ^{13}C-labeled 6,7-dimethyl-8-ribityllumazine

Values larger than 15% are shown in bold.

Position	^{13}C Enrichment	
	Predicted	Observed
	%	%
7α*	1	<5
6α*	5	<5
7α	1	<5
6α	5	<5
1′*	6	6
1′	6	5
6*	**73**	**73**[a]
7*	**32**	**32**
5′, 5′*	1	1
2′	**62**	**57**
2′*, 3′*, 4′*, 4′, 3′	**23**	**17**
4a*	4	<5
4a	4	<5
6	**73**	**73**[a]
7	**32**	ND[b]
8a*	**43**	ND
8a	**43**	ND
2*	1	<5
2	1	<5
4*	**31**	**22**
4	**31**	**18**

[a] Used as the reference value
[b] ND, not determined.

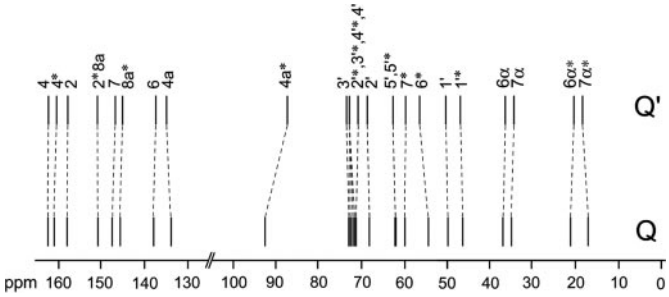

FIG. 5. **Correlation diagram of ^{13}C NMR chemical shifts of Compounds Q and Q′.**

the biosynthesis of vitamin B_2 (28). (ii) In a formal sense, the conversion of the lumazine derivative **1** into riboflavin under formation of a benzenoid ring is a dismutation. However, the reaction is set apart from typical dismutations by the sheer size of the exchanged module, a C_4H_6 fragment, whereas typical dismutations involve the exchange of hydride ions. (iii) The trajectory of the reaction under study must proceed via a complex series of transition states and reaction intermediates, but despite that mechanistic complexity, the reaction can proceed without catalysis even under mild conditions (neutral aqueous solution). (iv) Two different riboflavin synthase types have evolved independently, and these enzyme classes are not only entirely different with respect to their amino acid sequences but also differ with respect to the inherent symmetry properties of their respective active sites (for details, see below).

Earlier work with *E. coli* riboflavin synthase identified a pentacyclic intermediate that can be formally described as an adduct of two identical substrate molecules and affords riboflavin by molecular fragmentation.

In the present study, we isolated a putative intermediate of the reaction catalyzed by an archaeal riboflavin synthase that has the same constitution as the pentacyclic eubacterial intermediate but different stereochemical features. Each of the two different pentacyclic intermediates of eubacterial and archaeal origin is metabolized exclusively by the respective enzyme by which it can be formed.

To discuss in more depth the stereochemical properties of the pentacyclic intermediates, it must be noted that each of the two 6,7-dimethyl-8-ribityllumazine molecules that serve as substrate for the formation of riboflavin has three chiral centers, *i.e.* the 2′, 3′, and 4′ carbon atoms of the ribityl side chain. Hence, the pentacyclic intermediates have a total of six chiral centers located to their ribityl side chains. Moreover, the adduct formation involves the formation of two additional chiral carbons (carbons 6* and 7*) that bridge the two lumazine moieties.

The stereochemical features of the pentacyclic intermediate of *E. coli* have been derived previously by a comparison between the x-ray structures of the riboflavin synthases of *E. coli* and *S. pombe* (16). The structures of both domains of the yeast enzyme in complex with a bound intermediate analog were determined by x-ray crystallography (16). However, because the conditions used for crystal growth were conducive to the dissociation of the trimeric protein into monomers, no active site capable of adduct formation was present in the crystals. On

the other hand, the *E. coli* protein had been crystallized as a homotrimer, but without a bound ligand (17). By homology modeling of the ligands bound to the yeast enzyme into the *E. coli* structure, it became apparent that a single active site is formed in the *E. coli* crystal structure by the close apposition of an N-terminal domain of one subunit with the C-terminal domain of a second subunit. In that complex, the two substrate molecules are in close contact and form a quasi-c_2-symmetric pair that is located in a pseudo-c_2-symmetric environment formed by the apposition of the two domains described. Based on this unique topology, we proposed a cis linkage between the donor and acceptor pteridine systems in the pentacyclic intermediate of *E. coli*. Notably, a trans linkage was later proposed by other authors on the basis of computer modeling studies (31). However, that hypothesis is at odds with the experimental data reported in our earlier study.

Based on the comparison of the CD and NMR data of Compounds Q and Q′, we propose that both molecular species share (i) the same constitution, (ii) the same stereochemistry of their ribityl side chains (which is of course not affected in the course of the riboflavin synthase-catalyzed reaction), and (iii) the cis linkage of the two pteridine systems, but that the pentacyclic ring systems of the two compounds are related by mirror symmetry. Both compounds have eight chiral centers (the ring carbon atoms 6* and 7* and the chiral centers of each respective ribityl side chain). Because the chiral centers of both ribityl side chains have the same configuration, Compounds Q and Q′ differ by their configurations at the ring carbon atoms 6* and 7* and must be designated as diastereomers (Fig. 6). This is very well in line with the CD spectra. The chromophores of both compounds under study are inherently asymmetric due to the impact of the chiral ring atoms 6* and 7*, and the CD spectra should predominantly reflect the influence of these chiral centers. However, due to the additional influence of chiral centers, the CD spectra can be expected to differ in the details of the spectral envelopes, not just with respect to the signs, and this expectation is perfectly in line with the experimental findings. Based on previous mechanistic hypotheses (7, 9, 16, 28), the stereochemical features of hypothetical reaction intermediates in the trajectories of the two enzyme types are summarized in Fig. 6.

Amino acid sequence arguments indicate that riboflavin synthases have developed independently in Archaea on one side and in eubacteria and eukaryotes (plants and fungi) on the other side (33). Specifically, completely sequenced archaeal genomes comprise sets of two similar genes, which specify paralogous riboflavin synthases and 6,7-dimethyl-8-ribityllumazine synthases. On the other hand, in eubacteria, plants, and fungi, single genes of the 6,7-dimethyl-8-ribityllumazine synthases type are accompanied by genes specifying riboflavin synthases of the eubacterial/eukaryotic type characterized by internal sequence similarity and without similarity with 6,7-dimethyl-8-ribityllumazine synthase. Sequence arguments also showed that the divergence between the two domains of eubacteria/eukaryotic riboflavin synthase occurred very early in evolution; similarly, the divergence between 6,7-dimethyl-8-ribityllumazine synthase and archaebacterial riboflavin synthase occurred early in evolution (33).

The structure and mechanism of 6,7-dimethyl-8-ribityllumazine synthase has been studied in considerable detail. For example, the enzymes from *S. cerevisiae* (41), *S. pombe* (44), *Brucella abortus* (45), and *Magnaporthe grisea* (40) are homopentamers; the enzymes of *B. subtilis* (46), *M. jannaschii* (47), and *E. coli* (48) form icosahedral capsids, which are best described as dodecamers of pentamers. Each of the topologically equivalent active sites is located at the interface between two adjacent subunits in a pentamer.

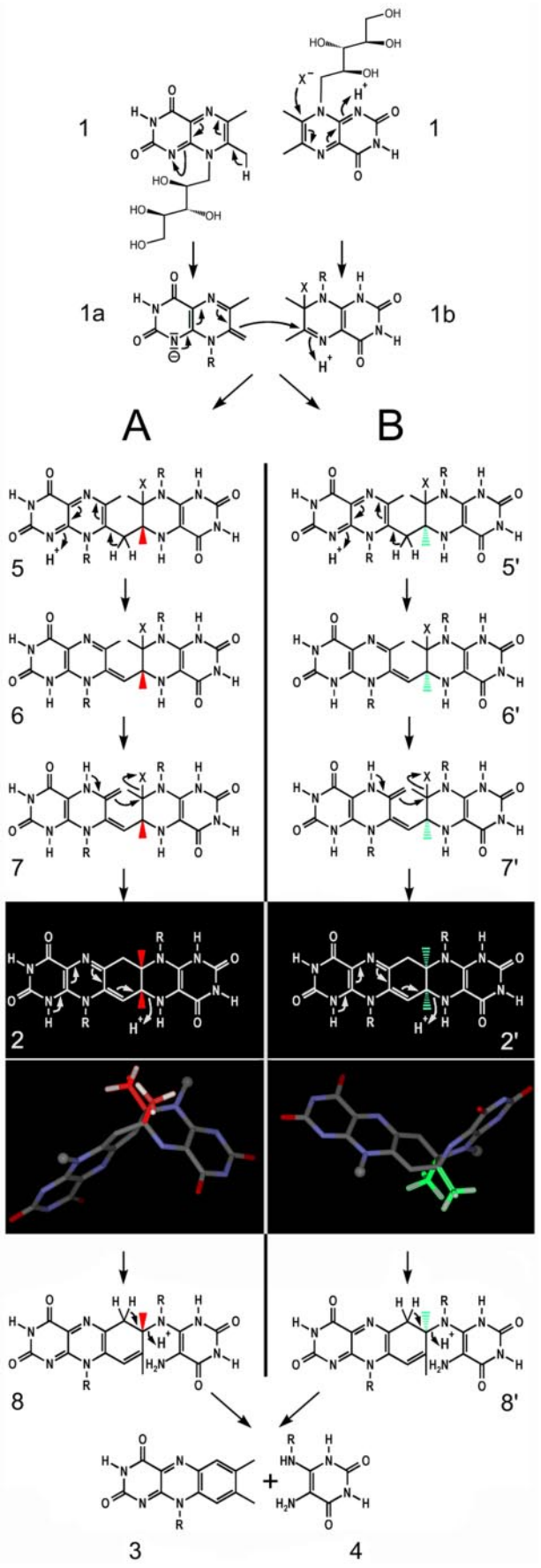

FIG. 6. **Stereochemistry of 6,7-dimethyl-8-ribityllumazine conversion into riboflavin catalyzed by eubacterial (*A*) and archaeal (*B*) riboflavin synthase.** *2*, Compound Q; *2′*, Compound Q′. *R*, ribityl.

Hydrodynamic measurements show that riboflavin synthase of *M. jannaschii* is a homopentamer (33). It is therefore virtually certain that the enzyme also shares the 5-fold rotational symmetry that is a common feature of all known lumazine synthases (because even the icosahedral lumazine synthases are best described as dodecamers of pentamers).

The active sites of lumazine synthases are invariably located at the interfaces between adjacent subunits in the pentameric modules, and it is likely that the active sites of archaebacterial riboflavin synthases have the same approximate location. The orientation of the product, 6,7-dimethyl-8-ribityllumazine in the enzyme-product complex of 6,7-dimethyl-8-ribityllumazine synthase is well documented by x-ray structures (44, 49). The heterocyclic ring system of the product has been shown to form a π complex with an aromatic amino acid residue (phenylalanine or tryptophan). It is highly plausible that one of the substrate molecules in the paralogous riboflavin synthase could occupy the same place as the product lumazine molecule in 6,7-dimethyl-8-ribityllumazine synthase. It is also likely that a second molecule of 6,7-dimethyl-8-ribityllumazine must be embedded in the spacious active site cavity with the ribityl side chains of the two substrate molecules pointing into opposite directions, *i.e.* in a quasi-c_2-symmetrical arrangement. However, whereas the pseudo-c_2-symmetry of the respective N-terminal and C-terminal domains that together form the active site of the *E. coli* enzyme is by necessity conducive to the required quasi-c_2-topology of the substrate molecules, the archaeal enzyme is devoid of any symmetry properties that would inherently enforce the required symmetry properties of the substrate topology. We are not aware of any precedent in which a given reaction occurs via stereochemically different intermediates under the catalytic influence of structurally unrelated enzymes. More importantly, however, the data are relevant in terms of the evolution of the flavin biosynthetic pathway, which affords one of the central catalysts of all organisms. The evolution of a riboflavin synthase would be expected to depend to a significant degree on the availability of the substrate, 6,7-dimethyl-8-ribityllumazine. Hence, it appears plausible that the evolution of lumazine synthase, in the case of Archaea, should not have been preceded by the evolution of the paralog enzyme, riboflavin synthase. The pathway intermediate, 6,7-dimethyl-8-ribityllumazine, differs structurally from the flavocoenzymes by the absence of the benzenoid ring. Despite that, the redox properties of 8-substituted pteridines and 10-substituted isoalloxazine-type flavocoenzymes are similar (50). It is therefore conceivable that lumazine-type coenzymes could have preceded present day flavocoenzymes. In that context, it is notable that 8-substituted lumazines function as cofactors of certain luminescent proteins such as lumazine protein and blue fluorescent protein in certain luminescent bacteria that are believed to serve as optical transponders (51, 52). Notably, however, these proteins appear not to be involved in any redox biochemistry and may in fact be entirely devoid of catalytic activity.

REFERENCES

1. Plaut, G. W. E. (1963) *J. Biol. Chem.* **238,** 2225–2243
2. Harvey, R. A., and Plaut, G. W. (1966) *J. Biol. Chem.* **241,** 2120–2136
3. Plaut, G. W. E. (1971) in *Comprehensive Biochemistry* (Florkin, M., and Stotz, E. H., eds) pp. 11–45, Elsevier, Amsterdam
4. Bacher, A. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed.) Vol. 1, pp. 215–259, Chemical Rubber & Co., Boca Raton, FL
5. Rowan, T., and Wood, H. C. S. (1963) *Proc. Chem. Soc. London* 21–22
6. Rowan, T., and Wood, H. C. S. (1968) *J. Chem. Soc. C* 452–458
7. Beach, R. L., and Plaut, G. W. (1969) *Tetrahedron Lett.* **6,** 3489–3492
8. Beach, R. L., and Plaut, G. W. (1970) *J. Am. Chem. Soc.* **6,** 2913–2916
9. Paterson, T., and Wood, H. C. S. (1969) *J. Chem. Soc. Chem. Commun.* 290–291
10. Schott, K., Kellermann, J., Lottspeich, F., and Bacher, A. (1990) *J. Biol. Chem.* **265,** 4204–4209
11. Wacker, H., Harvey, R. A., Winestock, C. H., and Plaut, W. E. (1964) *J. Biol. Chem.* **239,** 3493–3497
12. Plaut, G. W., Beach, R. L., and Aogaichi, T. (1970) *Biochemistry* **17,** 771–785
13. Plaut, G. W., Smith, C. M., and Alworth, W. L. (1974) *Annu. Rev. Biochem.* **43,** 899–922
14. Eberhardt, S., Richter, G., Gimbel, W., Werner, T., and Bacher, A. (1996) *Eur. J. Biochem.* **242,** 712–719
15. Bacher, A., Baur, R., Eggers, U., Harders, H. D., Otto, M. K., and Schnepple, H. (1980) *J. Biol. Chem.* **255,** 632–637
16. Gerhardt, S., Schott, A.-K., Kairies, N., Cushman, M., Illarionov, B., Eisenreich, W., Bacher, A., Huber, R., Steinbacher, S., and Fischer, M. (2002) *Structure* (*Camb.*) **10,** 1371–1381
17. Liao, D. I., Calabrese, J. C., Wawrzak, Z., Viitanen, P. V., and Jordan, D. B. (2001) *Structure* (*Camb.*) **9,** 11–18
18. Truffault, V., Coles, M., Diercks, T., Abelmann, K., Eberhardt, S., Luttgen, H., Bacher, A., and Kessler, H. (2001) *J. Mol. Biol.* **15,** 949–960
19. Meining, W., Eberhardt, S., Bacher, A., and Ladenstein, R. (2003) *J. Mol. Biol.* **331,** 1053–1063
20. Cushman, M., Patrick, D. A., Bacher, A., and Scheuring, J. (1991) *J. Org. Chem.* **56,** 4603–4608
21. Cushman, M., Patel, H. H., Scheuring, J., and Bacher, A. (1992) *J. Org. Chem.* **57,** 5630–5643
22. Scheuring, J., Fischer, M., Cushman, M., Lee, J., Bacher, A., and Oschkinat, H. (1996) *Biochemistry* **35,** 9637–9646
23. Cushman, M., Mavandadi, F., Yang, D., Kugelbrey, K., Kis, K., and Bacher, A. (1999) *J. Org. Chem.* **64,** 4635–4642
24. Illarionov, B., Kemter, K., Eberhardt, S., Richter, G., Cushman, M., and Bacher, A. (2001) *J. Biol. Chem.* **276,** 11524–11530
25. Fischer, M. J., Schott, A. K., Kemter, K., Feicht, R., Richter, G., Illarionov, B., Eisenreich, W., Gerhardt, S., Cushman, M., Steinbacher, S., Huber, R., and Bacher, A. (2003) *BMC Biochem.* **4,** 18
26. Paterson, T., and Wood, H. C. S. (1972) *J. Chem. Soc. Perkin Trans. I* 1051–1056
27. Plaut, G. W., and Beach, R. L. (1975) in *Chemistry and Biology of Pteridines* (Pfleiderer, W., ed) pp. 101–123, de Gruyter, Berlin
28. Plaut, G. W., and Beach, R. L. (1976) in *Flavins and Flavoproteins* (Singer, T. P., ed) pp. 737–746, Elsevier, Amsterdam
29. Bown, D., Keller, P. J., Floss, H. G., Sedlmaier, H., and Bacher, A. (1986) *J. Org. Chem.* **51,** 2461–2467
30. Illarionov, B., Eisenreich, W., and Bacher, A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7224–7229
31. Zheng, Y.-J., Jordan, D. B., and Liao, D.-I. (2003) *Bioorg. Chem.* **31,** 278–287
32. Eberhardt, S., Korn, S., Lottspeich, F., and Bacher, A. (1997) *J. Bacteriol.* **179,** 2938–2943
33. Fischer, M., Schott, A.-K., Römisch, W., Ramsperger, A., Augustin, M., Fidler, A., Bacher, A., Richter, G., Huber, R., and Eisenreich, W. (2004) *J. Mol. Biol.* **343,** 267–278
34. Volk, R., and Bacher, A. (1988) *J. Am. Chem. Soc.* **110,** 3651–3653
35. Volk, R., and Bacher, A. (1990) *J. Biol. Chem.* **265,** 19479–19485
36. Kis, K., Volk, R., and Bacher, A. (1995) *Biochemistry* **34,** 2883–2892
37. Kis, K., and Bacher, A. (1995) *J. Biol. Chem.* **270,** 16788–16795
38. Ladenstein, R., Meyer, B., Huber, R., Labischinski, H., Bartels, K., Bartunik, H. D., Bachmann, L., Ludwig, H. C., and Bacher, A. (1986) *J. Mol. Biol.* **187,** 87–100
39. Ladenstein, R., Schneider, M., Huber, R., Bartunik, H. D., Wilson, K., Schott, K., and Bacher, A. (1988) *J. Mol. Biol.* **203,** 1045–1070
40. Persson, K., Schneider, G., Jordan, D. B., Viitanen, P. V., and Sandalova, T. (1999) *Protein Sci.* **8,** 2355–2365
41. Meining, W., Mortl, S., Fischer, M., Cushman, M., Bacher, A., and Ladenstein, R. (2000) *J. Mol. Biol.* **299,** 181–197
42. Bacher, A., Eberhardt, S., Fischer, M., Mörtl, S., Kis, K., Kugelbrey, K., Scheuring, J., and Schott, K. (1997) *Methods Enzymol.* **280,** 389–399
43. Illarionov, B., Fischer, M., Lee, C. Y., Bacher, A., and Eisenreich, W. (2004) *J. Org. Chem.* **69,** 5588–5594
44. Gerhardt, S., Haase, I., Steinbacher, S., Kaiser, J. T., Cushman, M., Bacher, A., Huber, R., and Fischer, M. (2002) *J. Mol. Biol.* **318,** 1317–1329
45. Braden, B. C., Velikovsky, C. A., Cauerhff, A. A., Polikarpov, I., and Goldbaum, F. A. (2000) *J. Mol. Biol.* **297,** 1031–1036
46. Schott, K., Ladenstein, R., Konig, A., and Bacher, A. (1990) *J. Biol. Chem.* **265,** 12686–12689
47. Haase, I., Mortl, S., Kohler, P., Bacher, A., and Fischer, M. (2003) *Eur. J. Biochem.* **270,** 1025–1032
48. Mörtl, S., Fischer, M., Richter, G., Tack, J., Weinkauf, S., and Bacher, A. (1996) *J. Biol. Chem.* **271,** 33201–33207
49. Zhang, X., Meining, W., Haase, I., Fischer, M., Bacher, A., and Ladenstein, R. (2003) *J. Mol. Biol.* **328,** 167–182
50. Harders, H., Forster, S., Voelter, W., and Bacher, A. (1974) *Biochemistry* **13,** 3360–3364
51. Lee, J., Matheson, I. B. C., Müller, F., O'Kane, D. J., Vervoort, J., and Visser, A. J. W. G. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed) Vol. 2, pp. 109–151, CRC Press, Boca Raton, FL
52. Petushkov, V. N., and Lee, J. (1997) *Eur. J. Biochem.* **245,** 790–796