The pathway of riboflavin biosynthesis is a potential target for antibacterial chemotherapy because enterobacteria such as Escherichia and Salmonella species are absolutely dependent on the endogenous synthesis of the vitamin because they lack an uptake system. A detailed understanding of the structure and mechanism of the enzymes involved in the biosynthetic pathway could serve as a starting point for the rational design of enzyme inhibitors with antibacterial activity.

Riboflavin is biosynthesized by a convergent pathway using one molecule of GTP and two molecules of ribulose 5-phosphate as substrates. The intermediate, 5-amino-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione (1, Figure 1) is generated from GTP by a sequence of ring opening, deamination, side chain hydrogenation and dephosphorylation (for review see [1,2]). The other key intermediate, 3,4-dihydroxy-2-butanone 4-phosphate (2), is obtained by an unusual rearrangement reaction involving the release of C-4 of ribulose 5-phosphate as formate [3,4].

5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1) reacts with 3,4-dihydroxy-2-butanone 4-phosphate (2) with formation of 6,7-dimethyl-8-ribityllumazine (3) and inorganic phosphate. This reaction is catalysed by the enzyme lumazine synthase [5]. The lumazine 3 is subsequently converted to riboflavin (4) by an unusual dismutation reaction yielding 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1) as the second product [6–8].

All carbon atoms of the xylene ring of riboflavin are ultimately derived from 3,4-dihydroxy-2-butanone 4-phosphate. The pyrimidine 1 formed in the second reaction step is also a substrate for the formation of the lumazine 3 and can be re-used in the biosynthetic process. More specifically, every second pyrimidine molecule passes twice through the enzymatic process for conversion to the final product, riboflavin.

In Bacillus subtilis, the two terminal reaction steps of the riboflavin pathway are catalysed by a 1-MDa enzyme complex consisting of 60 β-subunits and three α-subunits [9]. The β-subunits (lumazine synthase) form a capsid with icosahedral 532 symmetry [10,11]. The α-subunits (riboflavin synthase) form a trimer that is enclosed in the central core of the icosahedral β-subunit capsid. The α- and β-subunits are specified by the genes ribB and ribH of the riboflavin operon, which is located at 209° on the B. subtilis chromosome [12].

When expressed in a recombinant E. coli strain, the ribH gene of B. subtilis directs the synthesis of hollow icosahedral βho capsids which can be purified to homogeneity in the absence of substrate analogues such as 5 or 6 (Figure 1), they reaggregate with formation of a hollow icosahedral βho capsids [13].

When expressed in a recombinant E. coli strain, the ribH gene of B. subtilis directs the synthesis of hollow icosahedral βho capsids which can be purified to homogeneity in the absence of substrate analogues such as 5 for stability. The reasons for the increased stability of βho capsids assembled in vitro as compared with in vitro assembly are as yet not understood.

Crystals of the αβho enzyme complex were obtained in the presence of the substrate analogues 5 and 6 (Figure 1). X-ray structure analysis of these crystals yielded a structure for the icosahedral βho subunit capsid at 3.3 Å resolution [14]. However, no structural information was obtained for the α-subunits in the capsid core. The α-trimers are unable to follow the 532 symmetry of the icosahedral capsid and are thus probably disordered with respect to the crystal lattice.

More recently, crystals of hollow βho capsids reconstituted in vitro were obtained in a hexagonal and a monoclinic modification [13]. The monoclinic crystals diffract to a resolution of 2.4 Å. Both crystals modifications were used for X-ray analysis [15,16]. The three-dimensional capsid structure obtained with the αβho complex...
Figure 1
Biosynthesis of riboflavin (top) and structure of substrate and intermediate analogue (bottom)

(A) Luminase synthase; (B) riboflavin synthase.

and with the reconstituted \( \beta_{60} \) capsids are closely similar. Apparently, the \( \alpha \)-subunit trimer can be integrated into the capsid core without significant reorganization of the capsid structure.

The overall topology of the icosahedral capsid has been described earlier [14]. The structure is best described as a dodecamer of pentamers. The interface contacts between adjacent subunits in the pentamer are large by comparison with the other subunit interface regions.
Channels formed by bundles of five skewed helices surround the five-fold symmetry axes and have been discussed as potential gates for the transfer of substrates and products into and out of the capsids.

The icosahedral capsid has 60 symmetry-related active sites. Each site is located at a pentamer interface in close proximity with the inner capsid surface. The active site topology is summarized in Figure 2. The pyrimidine ring of the substrate forms a sandwich complex with the phenyl ring of Phe-22. The ligand is embedded in a network of hydrogen bonds.

The presence of a fixed phosphate ion in the proximity of the bound ligand was inferred from the electron density. It appears possible that this inorganic phosphate ion occupies the binding site for the phosphate moiety of the second substrate, 3,4-dihydroxy-2-butanone 4-phosphate (2).

The enzyme-catalysed condensation of the pyrimidine 1 with the 3,4-dihydroxy-2-butanone 4-phosphate (2) is strictly regiospecific [5]. A hypothetical reaction mechanism is shown in Figure 3. It is assumed that the carbonyl group of 2 forms a Schiff's base with the position 5 amino group of 1. This intermediate could then eliminate phosphate, and the resulting carbonyl group could react with the position 6 amino group with formation of the pyrazine ring.

A variety of mutant proteins were prepared by a site-directed mutagenesis to analyse the catalytic role of individual amino acid residues. The replacement of Phe-22, which forms a coplanar complex with the heterocyclic ring of the substrate, did not change the catalytic activity appreciably.

The exchange of Glu-58 was accompanied by the loss of catalytic activity. It appears likely that this residue is essential for substrate binding via the ribityl side chain. In line with this hypothesis, earlier studies had shown that the correct stereochemistry of the ribityl side chain is essential for binding of the substrate 1 [17]. The replacement of Ser-142 by glycine is also accompanied by the loss of catalytic activity. The structural data suggest that this residue is also involved in ligand binding via an interspersed water molecule.

$^{35}$F-NMR spectroscopy using trifluoromethyl-substituted intermediate analogues has been used to characterize the ligand binding sites of riboflavin synthase and lumazine protein [18-21]. This approach could also be applied to
Hypothetical mechanism for the condensation of \( \text{l-3,4-dihydroxy-2-butanone 4-phosphate (2) with 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (1)} \)

\[
\begin{align*}
\text{2} & \quad + \quad \text{1} \\
\text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} \\
\text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{H-C-OH} & \quad \text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} \\
\end{align*}
\]

The mechanism proposed in Figure 3 involves the abstraction of a proton from C-3 of the phosphorylated carbohydrate side chain. The initial hypothesis that His-88 could serve as a base involved in proton abstraction from the putative reaction intermediate 8 (Figure 3) was not confirmed by the mutagenesis experiments. Thus, it appears likely that a water molecule could serve as a base for proton abstraction. It appears possible that the enzyme serves essentially as a scaffold that facilitates the reaction pathway by exerting conformational control on substrate and transition states.

The \( \alpha_3\beta_{22} \) enzyme complex has unusual kinetic properties [23]. Based on standard kinetic arguments, it could be expected that the formation of riboflavin from 1 and 2 should show a lag phase characteristic for sequential reactions. On the contrary, it was found that the velocity of riboflavin formation from 1 and 2 was maximum time \( t = 0 \). This suggests the involvement of intermediate channelling between the active sites of the \( \alpha \)- and \( \beta \)-subunits.

The enzyme-catalysed reaction shows partitioning, suggesting a 'leaky channel' model [24].
Whereas part of the intermediate lumazine is directly converted to riboflavin, a fraction of the intermediate is transferred to the bulk solvent. This transient bulk lumazine is converted relatively slowly by the enzyme, and the rate of riboflavin formation shows a drastic deceleration after the consumption of the substrates 1 and 2 despite the presence of transient lumazine in the bulk solvent. The partitioning factor depends on the concentration of the substrates 1 and 2. At low substrate concentrations, it approaches a value of 1 (i.e. almost perfect channelling).

The kinetic data could be simulated using a heuristic numerical model based on the hypothesis that both reaction steps can be treated by a Michaelis-Menten approach [23]. The observed substrate channelling may be due to the encapsidation of the z-subunits inside the capsid. The active sites of the lumazine synthase module are located close to the inner surface of the icosahedral capsid. On the basis of the X-ray structure, it appears likely that the passage of products through the capsid structure is sterically hindered. Under these topological constraints, the intermediate lumazine 3 has a high probability of being captured by the active side of an z-subunit rather than of exiting into the bulk solution.

The kinetic properties of the enzyme complex are favourable for the production of riboflavin at high velocity even at a low substrate concentration. One would expect that these kinetic properties evolved under considerable selection pressure.

The riboflavin requirement of B. subtilis is quite low. Nevertheless, the riboflavin operon can be up-regulated to a level at which the z;βαω complex constitutes about 0.5% of cellular protein [9,23]. Under laboratory conditions, B. subtilis wild strains do not use this massive capacity to make riboflavin. The regulatory mechanism and the kinetic fine-tuning may owe their origin to the same selective pressure. The role of this potential for increased riboflavin production in the natural lifestyle of the micro-organism is still not understood.

Recently, we could show that an open reading frame at 9.5 min of the E. coli chromosome [25] codes for lumazine synthase. This gene, which was subsequently designated ribE, specifies a peptide of 16 kDa consisting of 156 amino acids. The ribE gene can be expressed to very high levels in recombinant E. coli strains. The native protein is an icosahedral capsid of 60 subunits, as shown by hydrodynamic studies indicating a molecular mass of approximately 1 MDa. The icosahedral symmetry was directly observed by electron microscopy of gold-decorated molecules. In contrast to the B. subtilis enzyme, the E. coli enzyme does not enclose a riboflavin synthase module.

A putative gene for lumazine synthase of Saccharomyces cerevisiae was recently sequenced by J. J. Garcia-Ramirez, M. A. Santos and J. L. Revuelta (unpublished work). We could express this gene to high levels in a recombinant E. coli strain. The native protein has a molecular mass of about 90 kDa as shown by sedimentation equilibrium analysis. These data suggest a pentamer structure. The protein has been crystallized.

It has been shown previously that the last step of riboflavin biosynthesis, i.e. the dismutation of the lumazine 3, can proceed in boiling aqueous buffer without enzyme catalysis [26]. More recently, Strupp and Eschenmoser [27,28] were able to produce the lumazine 3 by reaction of the pyrimidine 1 with ribulose 1,5-bisphosphate in alkaline aqueous solutions. This non-enzymic reaction mimics the biosynthesis of the intermediate 2 from ribulose phosphate as described by Volk and Bacher [3,4].

We have found recently that the formation of the lumazine intermediate 3 from 1 and 2 can also proceed as an uncatalysed reaction under extremely mild conditions. Product formation occurs at pH 7 and room temperature at substrate concentrations around 1 mM. This facile non-enzymic reaction must be contrasted with the relatively low catalytic activity of lumazine synthase, which has a turnover number of 0.07 per second and per subunit [23].

In contrast to the enzyme-catalysed formation of 6,7-dimethyl-8-ribityllumazine, the uncatalysed reaction has a low regiospecificity. The present evidence suggests partitioning between the pathway shown in Figure 2 and a second mechanism involving the formation of butanedione as a reaction intermediate conducive to non-regioselective reaction.

Ample evidence now indicates that the final steps in the biosynthesis of riboflavin could have proceeded spontaneously under the conditions of prebiotic evolution. Although this has yet to be demonstrated for the formation of the pyrimidine 1, it appears possible that riboflavin could have been present as a redox co-factor in an early RNA world. It should be noted that the modern
biosynthetic pathway requires only GTP and ribulose phosphate but no complex amino acid as precursor.

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