

Biosynthesis of Riboflavin

LUMAZINE SYNTHASE OF *ESCHERICHIA COLI**

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A gene located at 443 kilobases on the *Escherichia coli* chromosome (subsequently designated *ribE*) was expressed in a recombinant *E. coli* strain and was shown to code for the enzyme 6,7-dimethyl-8-ribityllumazine synthase. The recombinant enzyme was purified to homogeneity. The protein is an icosahedral capsid of 60 subunits with a mass of about 1 MDa as shown by hydrodynamic studies and by electron microscopy. In contrast to the icosahedral lumazine synthase-riboflavin synthase complex of *Bacillus subtilis*, the lumazine synthase of *E. coli* is not physically associated with another enzyme of the riboflavin pathway, and the core of the icosahedral capsid is empty. The *RIB4* gene of *Saccharomyces cerevisiae* was also expressed to a high level (about 40% of cellular protein) in *E. coli*. The recombinant protein is a pentamer of 90 kDa. An insertion of 4 amino acids into helix α_4 is likely to hinder the formation of an icosahedral capsid by the yeast protein. The kinetic properties of lumazine synthase of *E. coli*, *B. subtilis*, and *S. cerevisiae* are similar.

Lumazine synthase catalyzes the formation of 6,7-dimethyl-8-ribityllumazine (**3**) by condensation of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**1**) with 3,4-dihydroxy-2-butanone (**2**) (1–3) (Fig. 1). In the biosynthetic pathway this reaction is followed by the dismutation of lumazine **3**, yielding riboflavin (**4**) and pyrimidine **1**, which is catalyzed by the enzyme riboflavin synthase (4, 5). Pyrimidine **1** can subsequently be reutilized by lumazine synthase. As a consequence of the unusual reaction pathway, every second molecule of pyrimidine **1** must be processed twice by lumazine synthase (6).

Bacillaceae express a bifunctional enzyme complex with lumazine synthase and riboflavin synthase activity previously designated “heavy riboflavin synthase.” The 1-MDa protein of *Bacillus subtilis* consists of 3 α subunits (riboflavin synthase) and 60 β subunits (lumazine synthase) (7–9). The genes *ribB* and *ribH* of the riboflavin operon of *B. subtilis* code for the α and β subunits of the enzyme complex (10–12). The β subunits form an icosahedral capsid enclosing the α subunit trimer (8). The structure of the icosahedral β_{60} capsid has been determined by x-ray crystallography (13–15). The enzyme complex has unusual kinetic properties due to substrate channeling (6).

An open reading frame with sequence similarity to the *ribH* gene of *B. subtilis* is located in close proximity to the *nusB* gene

on the chromosome of *Escherichia coli* (16). This paper reports the hyperexpression of this gene for which the designation *ribE* is proposed as well as the properties of the gene product. The *E. coli* protein is a hollow icosahedral capsid of 60 subunits.

Early work by Oltmanns and his co-workers (17) indicated that the genes *RIB3* and *RIB4* of *Saccharomyces cerevisiae* are involved in the formation of lumazine **3**. Logvinenko *et al.* (18) reported the partial purification of lumazine synthase from the yeast *Pichia guilliermondii*, which appeared to have a molecular weight well below that of the *B. subtilis* enzyme. Recently, the *RIB4* gene of *S. cerevisiae* was sequenced and expressed in yeast (19). We have found that the gene can be expressed efficiently in *E. coli*. The physical properties of the recombinant protein suggest that the protein is a pentamer with close similarity to the pentamer substructure of the icosahedral enzymes from eubacteria.

EXPERIMENTAL PROCEDURES

Materials—5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and 6,7-dimethyl-8-ribityllumazine were synthesized by published procedures (20, 21). Recombinant 3,4-dihydroxy-2-butanone 4-phosphate synthase of *E. coli* (22) was used for preparation of 3,4-dihydroxy-2-butanone 4-phosphate synthase (3). Bacterial strains and plasmids used in this study are summarized in Table I.

Enzyme Assays—The assay methods for riboflavin synthase (21), lumazine synthase (6), 3,4-dihydroxy-2-butanone 4-phosphate synthase (3), GTP cyclohydrolase II (23), deaminase, and reductase¹ were performed as described.

Construction of an Expression Vector for the *ribE* Gene of *E. coli*—The coding region of the *ribE* gene was amplified by polymerase chain reaction (PCR)² using isolated chromosomal DNA from *E. coli* RR28 as template and the oligonucleotides ORF3-1 and ORF3-2 (Table II) as forward and reverse primers. The amplification product served as template in a second PCR using primer P1' (Table II) (complementary to the ribosome binding site of the expression vector p602/22) and the primer ORF3-2. The amplification product was cleaved with *Bam*HI and *Eco*RI, and the fragment was ligated into vector p602/22, which had been treated with *Bam*HI and *Eco*RI. The ligation mixture was transformed into *E. coli* XL-1 Blue host cells (24). Transformants were selected on LB solid medium supplemented with kanamycin (20 mg/liter).

Construction of an Expression Vector for the *RIB4* Gene of *S. cerevisiae*—The *RIB4* gene of *S. cerevisiae* was amplified by PCR using chromosomal DNA as template and the oligonucleotides *rib4-1* and *rib4-2* as primers (Table II). A second PCR amplification was performed with the primers P1' and *rib4-2*. The PCR product was purified and cleaved with *Eco*RI and *Nsi*I, and the fragment was ligated into the vector p602/22-CAT, which had been prepared with *Eco*RI and *Pst*I. The ligation mixture was transformed into *E. coli* XL-1 Blue cells. Transformants were selected on LB medium supplemented with kanamycin (20 mg/liter). The plasmid p602/-CAT was constructed by elimination of the CAT gene from the plasmid p602/22. The plasmid p602/22 was digested with the restriction enzymes *Pvu*II and *Sca*I, and the resulting

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¹ G. Richter, M. Fischer, S. Eberhardt, C. Krieger, I. Gerstenschläger, and A. Bacher, submitted for publication.

² The abbreviations used are: PCR, polymerase chain reaction; ORF, open reading frame; CAT, chloramphenicol acetyltransferase.

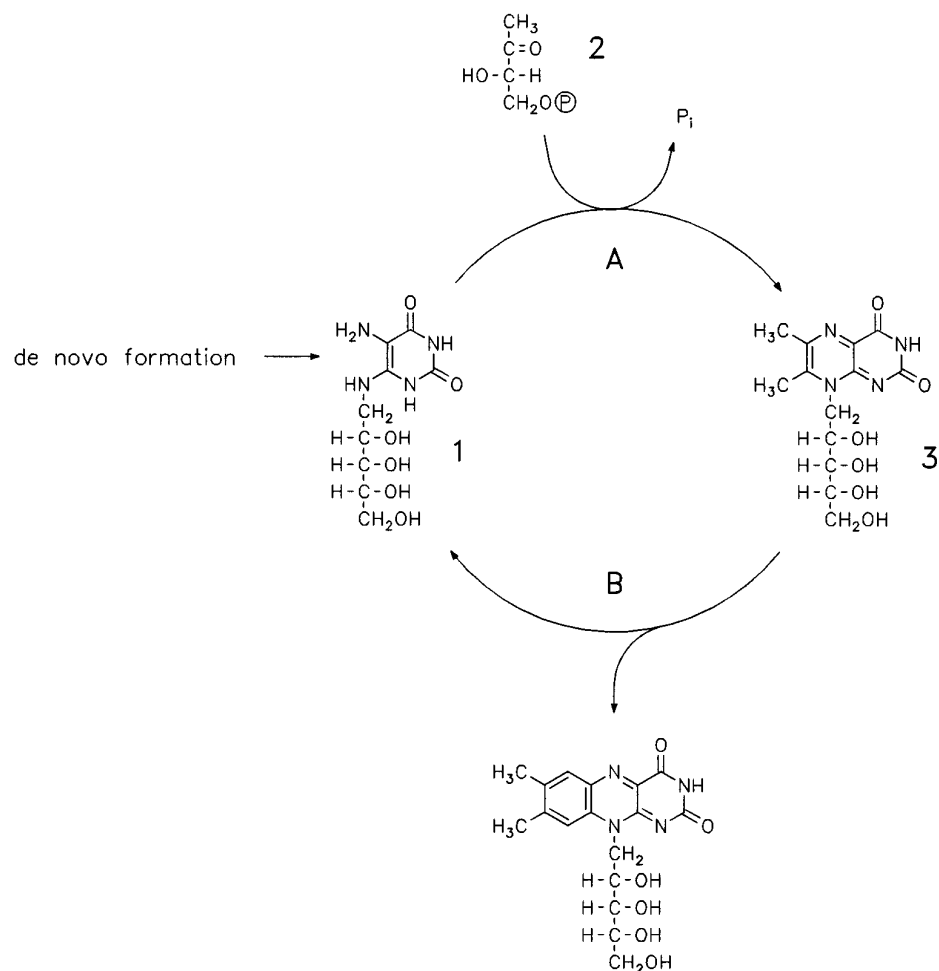


FIG. 1. Terminal reactions in the pathway of riboflavin biosynthesis. A, lumazine synthase; B, riboflavin synthase.

TABLE I
Bacterial strains and plasmids

Strain/plasmids	Relevant characteristics	Source
<i>E. coli</i> strains		
XL-1 Blue	recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [P', proAB, lacI ^q ZAM15, Tn10(tet ^r)]	Ref. 24
DSM 613	Wild type	Deutsche Sammlung von Mikroorganismen
Plasmids		
p602/22	<i>E. coli/B. subtilis</i> shuttle vector	A. van Loon
p602/22-CAT	<i>E. coli/B. subtilis</i> shuttle vector without <i>CAT</i> gene	This study
p602ribE	p602 with the <i>ribE</i> gene of <i>E. coli</i> with <i>CAT</i> gene	This study
p602rib4-CAT	p602 with the <i>RIB4</i> gene of <i>S. cerevisiae</i> without <i>CAT</i> gene	This study

TABLE II
Oligonucleotides used for the construction of plasmids

P1'	5'-ACACAGAATTCATTAAGAGGAGAAATTAACATATG-3'
ORF3-1	5'-GGAGAAATTAACATATGAACATTATTGAAGC-3'
ORF3-2	5'-GTTTCACGGATCCCCCTTACTAATTC-3'
rib4-1	5'-GGAGAAATTAACCATGGCAGTTAAAGGATTAGGC-3'
rib4-2	5'-CGCAAATGAAAGATGCATTGAAGCACCG-3'

fragments were separated by agarose gel electrophoresis. The 5,303-base pair fragment was excised from the gel, purified, and ligated with T4 ligase, yielding the plasmid p602/22-CAT.

Growth of Bacterial Cells—Recombinant *E. coli* strains were grown in LB medium containing 20 μg of kanamycin/liter. At an OD₆₀₀ of 0.7, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 2 mM. After an additional incubation of 5 h, cells were harvested and stored at -20 °C.

Purification of Recombinant Lumazine Synthase of *E. coli* Protein—Frozen cell mass of *E. coli* strain XL-1 carrying the plasmid p602ribE (4 g) was thawed in 50 ml of 50 mM phosphate buffer, pH 7, containing 8 mg of lysozyme and 0.8 mg of DNase. The mixture was incubated at

37 °C for 2 h and was then centrifuged.

The supernatant was placed on a column of DE52 cellulose (Whatman, 3 × 15 cm) which had been equilibrated with 50 mM potassium phosphate, pH 7. The column was developed with a linear gradient (50–500 mM) of potassium phosphate, pH 7 (total volume, 800 ml). Fractions were combined and dialyzed against 50 mM potassium phosphate, pH 7. Aliquots of 6 ml were placed on a Resource Q column (Pharmacia, 6 ml). The column was developed with a linear gradient of 50–500 mM potassium phosphate, pH 7 (total volume, 120 ml) at a flow rate of 5 ml/min. Fractions were combined, and the solution was concentrated by ultracentrifugation (Beckman, 70 Ti rotor, 40,000 rpm, 4 °C, 16 h).



FIG. 2. Alignments of predicted amino acid sequences. a, *ribH* of *B. subtilis* (10, 11); b, *ribE* of *E. coli* (16); c, *ORFII* of *Photobacterium leignathi* (33); d, *ribE* of *Hemophilus influenzae* (34); e, *RIB4* of yeast (19). Secondary structure elements of the *B. subtilis* lumazine synthase are shown (13). Residues that are part of the active site in *B. subtilis* are marked by stars.

TABLE III
Purification of recombinant lumazine synthase of *E. coli*

Procedure	Protein	Activity	Specific activity
	mg	units	units/mg
Cell extract	279	928,000	3,330
DEAE-cellulose	72	594,000	8,300
Resource Q	40	475,000	11,800

Purification of Recombinant Lumazine Synthase of *S. cerevisiae*—Wet cell mass of *E. coli* strain XL-1 carrying the plasmid p602rib4-CAT (4 g) was suspended in 50 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 8 mg of lysozyme and 0.8 mg of DNase. The suspension was incubated at 37 °C for 90 min and was centrifuged at 5000 rpm for 15 min. The pellet was discarded. Solid ammonium sulfate was added slowly to the crude cell extract to a final concentration of 1.5 M. The solution was adjusted to pH 7.5 and was placed on a butyl-Sepharose column (Pharmacia, 2 × 15 cm) which had been equilibrated with 1.5 M ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5. The column was developed with a linear gradient of 1.5–0 M ammonium sulfate in 50 mM potassium phosphate, pH 7.5 (total volume, 800 ml). Fractions were combined and dialyzed against 20 mM potassium phosphate, pH 6.8. The solution was placed on a column of hydroxyapatite (2 × 8 cm, DNA grade Bio-Gel HTP, Bio-Rad) equilibrated with 20 mM potassium phosphate, pH 6.8. The column was developed with a linear gradient of 20–200 mM potassium phosphate, pH 6.8 (total volume, 500 ml). Fractions were combined and concentrated by ultracentrifugation (Beckman, 70 Ti rotor, 45,000 rpm, 16 h).

Analytical Ultracentrifugation—Boundary sedimentation experiments and sedimentation equilibrium experiments were performed with an analytical ultracentrifuge XL-A Optima (Beckman) as described (8). Partial specific volumes were estimated on the basis of amino acid compositions (25, 26).

Sucrose Gradient Centrifugation—Gradients containing 5–20% sucrose in 100 mM buffer solution were prepared in 4-ml centrifuge tubes. Potassium phosphate (100 mM) was used for experiments at pH 6 and pH 7, and 100 mM Tris-HCl was used for experiments at pH 8. Crude cell extract was layered on top of each gradient. The gradients were centrifuged in a Beckman SW56 rotor for 4.5 h at 45,000 rpm and 4 °C. Fractions were collected, and activities of enzymes involved in riboflavin biosynthesis were monitored.

Electron Microscopy—Electron micrographs of negative stained protein were obtained as described (8). For decoration experiments, protein layers adsorbed on carbon-coated Formvar grids were frozen in liquid nitrogen. The specimens were then freeze-dried at –80 °C in a Balzers BAF360 freeze-etching unit and were decorated with 0.4 nm of silver at –110 °C and at 90° incidence followed by rotational shadowing with a thin layer of tantalum/tungsten at 45° (27). Micrographs showing individual decorated protein molecules were digitized with an Eikonix CCD

camera system (1,024 × 1,024-pixel arrays; step size, 15 μm) and were processed using the software packages SEMPER 6 and IMAGIC.

In the course of image processing, decorated molecule images were extracted from the micrograph. For each individual image, the cluster distribution was cross-correlated with two-dimensional projections at different orientations of a three-dimensional model of a hypothetical molecule with icosahedral symmetry (28). Specifically, the molecular model consisted of a sphere in which the icosahedral 3- and 5-fold symmetry elements were indicated by metal clusters in analogy to the decoration pattern of silver observed on the surface of the lumazine synthase-riboflavin synthase complex of *B. subtilis* (29). The cluster distributions on individual molecules correlated well with icosahedral models corresponding to different orientations of the adsorbed molecules. The molecules showing the same orientation with respect to the substrate were assigned to classes. The class members were aligned for in-plane rotation, and class averages were calculated.

RESULTS

An open reading frame located at 443 kilobases of the *E. coli* chromosome that had been sequenced by Taura and co-workers (16) showed considerable sequence similarity to the *ribH* gene of *B. subtilis* specifying the enzyme 6,7-dimethyl-8-ribityllumazine synthase (Fig. 2). Using the vector p602/22, a plasmid containing the putative *E. coli* gene under the control of the *lac* operator was constructed. This plasmid directed the synthesis of large amounts of a peptide with an apparent mass of about 16 kDa in an *E. coli* host. Cell extracts of the recombinant strain had high lumazine synthase activity, thus confirming the presumptive function of the *E. coli* gene for which the designation *ribE* is proposed.

The recombinant protein was isolated as described under "Experimental Procedures" (Table III). The protein catalyzes the formation of 6,7-dimethyl-8-ribityllumazine from 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and 3,4-dihydroxy-2-butanone 4-phosphate with a specific activity of 11,800 nmol mg⁻¹ h⁻¹ at 37 °C (Table IV and Fig. 3). This value is similar to the specific activity of lumazine synthase from *B. subtilis* (12,000 nmol mg⁻¹ h⁻¹) (6). The *E. coli* lumazine synthase requires no cofactors and shows full catalytic activity in the presence of a chelator such as EDTA. Steady-state kinetic analysis yielded values for K_m and V_{max} which are similar to the *B. subtilis* protein (Table IV).

Lumazine synthase of *E. coli* sediments in the analytical ultracentrifuge as a single, symmetrical boundary (data not shown). The sedimentation velocity at 20 °C in 50 mM potas-

TABLE IV
Properties of lumazine synthase from *B. subtilis*, *E. coli*, and *S. cerevisiae*

Property	Microorganism		
	<i>B. subtilis</i> ^a	<i>E. coli</i>	<i>S. cerevisiae</i>
K_m for 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (μM)	4.6	4.2	4.0
K_m for 3,4-dihydroxy-2-butanone 4-phosphate (μM)	130	62	90
V_{\max} ($\text{nmol mg}^{-1} \text{h}^{-1}$)	12,000	11,800	15,400
Sedimentation velocity (S)	26.5	26.8	5.5
Subunit mass (kDa)	$\alpha = 23.5; \beta = 16.2$	16.2	18.6
No. of subunits	3 α , 60 β	60	5
Molecular mass (kDa) ^b	1,000	977	90
Predicted molecular mass (kDa) ^c	1,042	969	93

^a Data refer to the lumazine synthase-riboflavin synthase complex consisting of 3 α and 60 β subunits as reported earlier (6, 7).

^b Data are from sedimentation equilibrium analysis.

^c Calculated from amino acid sequence and subunit stoichiometry.

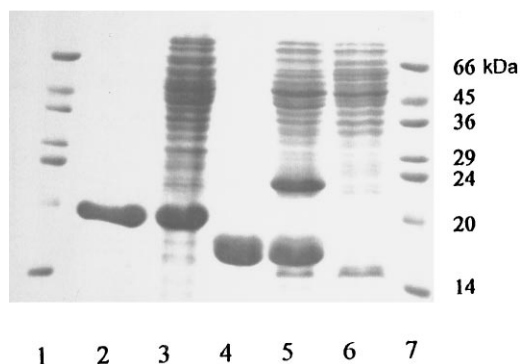


FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes 1 and 7, molecular mass markers; lane 2, recombinant lumazine synthase of *S. cerevisiae*; lane 3, cell extract of *E. coli* XL-1/p602-CAT; lane 4, recombinant lumazine synthase of *E. coli*; lane 5, cell extract of *E. coli* XL-1/p602ribE; lane 6, cell extract of *E. coli* XL-1.

sium phosphate buffer, pH 7.0, was 26.8 S. For comparison, the lumazine synthase/riboflavin synthase complex of *B. subtilis* displays a sedimentation coefficient of $s_{20,w} = 26.5$ S (7, 8).

Sedimentation equilibrium experiments performed with lumazine synthase of *E. coli* under a variety of experimental conditions indicated a molecular mass of 977 kDa (Fig. 4). The line in Fig. 4 was calculated for an ideal monodisperse system. The residuals show close agreement between the model and the experimental data. The subunit mass of 16,156 Da predicts a mass of 969 kDa for a 60-mer in close agreement with the experimental data.

An electron micrograph of *E. coli* lumazine synthase negatively stained with uranyl acetate is shown in Fig. 5. The protein molecules appear as almost spherical particles with an apparent diameter of about 17 nm.

The shape of the molecule and its composition of 60 subunits suggest that the *E. coli* enzyme has icosahedral symmetry in close analogy with the $\alpha_3\beta_{60}$ complex of *B. subtilis*. As shown in previous studies, the icosahedral symmetry of the lumazine synthase-riboflavin synthase of *B. subtilis* can be portrayed by metal decoration, *i.e.* vacuum evaporation of about one or a few monolayers of a suitable metal at normal incidence onto the frozen-hydrated protein surface (27, 29). On the surface of the *B. subtilis* enzyme, silver decorates the sites of 3- and 5-fold symmetry axes, and the decoration pattern reflects the symmetry properties of the enzyme complex. In line with these studies, the molecular symmetry of the *E. coli* enzyme was also examined by silver decoration of adsorbed and freeze-dried protein molecules. For this purpose, the distribution of the silver clusters on the surface of decorated individual molecules was compared with the decoration pattern of silver on the lumazine synthase-riboflavin synthase of *B. subtilis*. The silver decoration pattern on the surface of the *E. coli* enzyme is shown

in Fig. 6a. This pattern can be best described by an icosahedral model, where the sites of 3- and 5-fold symmetry are occupied by silver clusters, as demonstrated by the *overlay* in Fig. 6b. Thus, the silver decoration pattern on the surface of *E. coli* enzyme is indistinguishable from the pattern on the surface of the *B. subtilis* enzyme.

These data show conclusively that lumazine synthase of *E. coli* is characterized by icosahedral symmetry in analogy with the lumazine synthase-riboflavin synthase complex of *B. subtilis*. However, it should be noted that the native *B. subtilis* enzyme contains a riboflavin synthase module (trimer of α subunits) in the core space of the icosahedral capsid formed by the 60 lumazine synthase subunits. It was therefore in order to analyze the *E. coli* protein for the presence of riboflavin synthase or other enzyme activity of the riboflavin biosynthetic pathway. For this purpose, cell extract of *E. coli* wild type cells was subjected to sucrose gradient centrifugation, and fractions were analyzed for all enzymes of the riboflavin pathway (Fig. 7). To minimize the hazard that a large enzyme complex might dissociate under inappropriate pH conditions, sucrose gradient sedimentation analysis was performed at pH values from 6 to 8. In all experiments, lumazine synthase sedimented at a substantially higher rate than all other enzyme activities studied, thus indicating the absence of a physical association between lumazine synthase and another enzyme of the riboflavin pathway. We conclude from these data that lumazine synthase of *E. coli* is a hollow icosahedral capsid of 60 subunits.

The *RIB4* gene of *S. cerevisiae* has been sequenced by Revuelta and co-workers (19). The predicted amino acid sequence is similar to that of the bacterial lumazine synthases. We have cloned the *RIB4* gene into an expression vector under the control of a *lac* operator. A recombinant *E. coli* strain harboring this plasmid expressed high levels of the recombinant yeast protein, which was purified to homogeneity as described under "Experimental Procedures" (Fig. 3 and Table V). Kinetic properties of the recombinant yeast protein are similar to the proteins from *E. coli* and *B. subtilis* (Table IV).

The recombinant lumazine synthase of *S. cerevisiae* showed a sedimentation coefficient of $s_{20,w} = 5.5$ S at 20 °C in 50 mM potassium phosphate buffer, pH 7.0. Sedimentation equilibrium experiments indicated a molecular mass of 90 kDa. The predicted subunit molecular weight is 18,598. It follows that the recombinant protein is a pentamer of identical subunits. A pentameric structure has also been proposed by Garcia-Ramirez *et al.* (19) on basis of gel filtration experiments.

DISCUSSION

The structure of the lumazine synthase-riboflavin synthase complex of *B. subtilis* has been studied in considerable detail (7–9, 13–15) and is characterized by 60 β subunits (lumazine synthase) forming an icosahedral capsid that encloses a trimer

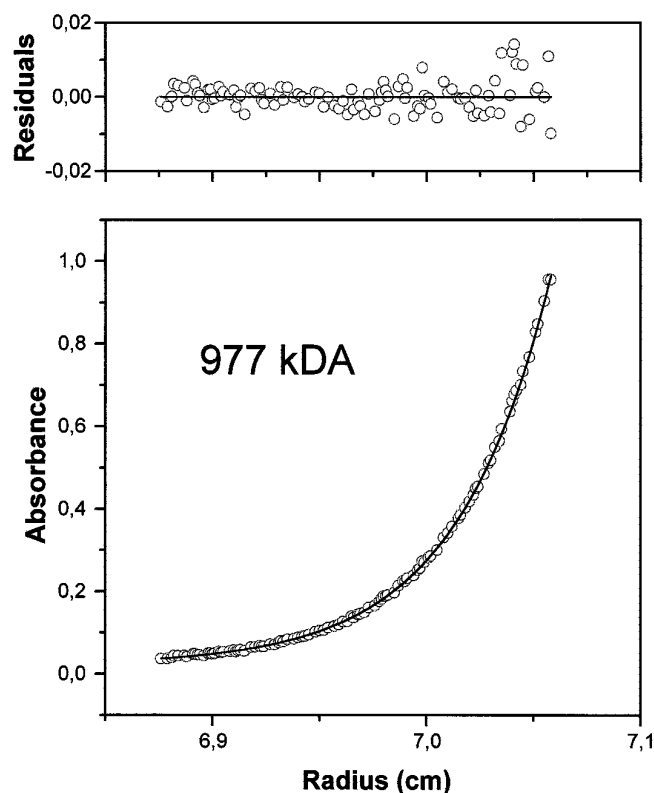


FIG. 4. Sedimentation equilibrium centrifugation of recombinant lumazine synthase of *E. coli*. A solution containing 0.3 mg of protein/ml of 50 mM potassium phosphate, pH 7.0, was centrifuged at 5,000 rpm and 4 °C for 72 h. The line was calculated for an ideal monodisperse system and a partial specific volume of $v = 0.741 \text{ ml g}^{-1}$. Residuals are shown in the top section.

of α subunits (riboflavin synthase). Whereas β subunits occur exclusively as the $\alpha_3\beta_{60}$ enzyme complex, free α subunit trimers are present in the cytoplasm of *B. subtilis* where they account for the major fraction of the total riboflavin synthase activity. Hollow β subunit capsids can be formed by reaggregation *in vitro* (8, 30) and have full lumazine synthase activity (6).

Lumazine synthase of *E. coli* is specified by the gene *ribE* located at 443 kilobases on the *E. coli* chromosome, which has been reported earlier by Taura *et al.* (16) as an unidentified open reading frame. The gene is directly adjacent to the *ribD* gene coding for a bifunctional pyrimidine deaminase-pyrimidine reductase which is involved in the formation of riboflavin precursor 1 (31). It appears likely that the *ribD* and *ribE* genes are part of an operon that may also contain the *nusB* gene and an open reading frame of unknown function (16).

The lumazine synthase of *E. coli* specified by the *ribE* gene forms a spherical oligomer of 60 subunits. In contrast to the *B. subtilis* enzyme, the lumazine synthase of *E. coli* is not physically associated with riboflavin synthase. A physical association between lumazine synthase of *E. coli* and any other enzyme of the riboflavin pathway has also been ruled out. Since the stability of the lumazine synthase-riboflavin synthase complex of *B. subtilis* is pH-dependent, sucrose gradient sedimentation analysis of cell extracts from *E. coli* wild type was performed at different pH values. The presence of complexes, even of moderate stability, between lumazine synthase and any other riboflavin biosynthetic enzyme is clearly ruled out by these experiments.

Kinetic evidence has been obtained for substrate channeling in the lumazine synthase-riboflavin synthase complex of *B. subtilis* (6). Whereas channeling is incomplete at high sub-

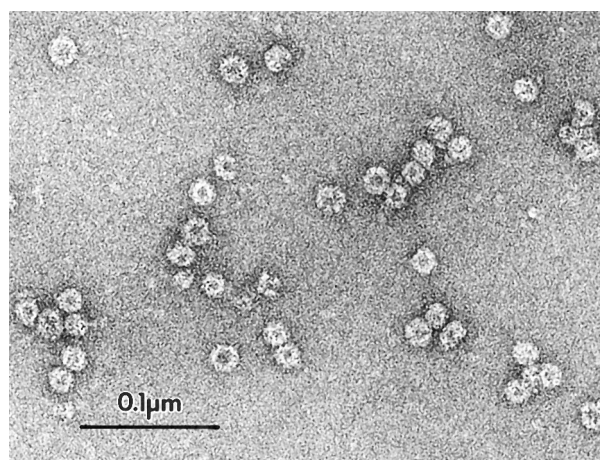


FIG. 5. Electron micrograph of lumazine synthase of *E. coli* negative-stained with 2% uranyl acetate. Note the accumulation of the stain in the central part of the molecule indicating a hollow capsid.

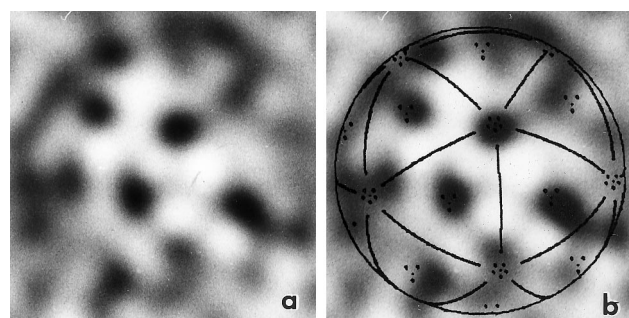


FIG. 6. Decoration pattern of silver on the surface of lumazine synthase of *E. coli*. Panel a, average of a class containing molecules with the same orientation with respect to the substrate. Panel b, same as panel a with an overlay of a model with icosahedral symmetry elements indicated.

strate concentrations, it is very efficient at low substrate concentrations. Since the channeling mechanism depends on the physical association between lumazine synthase and riboflavin synthase, it cannot be operative in *E. coli*.

The 60 active sites of the lumazine synthase of *B. subtilis* are located close to the inner surface of the icosahedral capsid. Import of substrates and export of products require penetration of the rather densely packed capsid structure. Substrates may be able to pass through the capsid wall via narrow channels along the 5-fold symmetry axes (13, 15). However, the dimensions of these channels are clearly not sufficient to explain the passage of lumazine 3, and major dynamic motions must be assumed to allow the access of substrate and products. These steric restrictions may be the structural basis for substrate channeling in the *B. subtilis* enzyme complex, and the advantage of substrate channeling may overcompensate any kinetic disadvantage caused by the sterically difficult access to the active site. In contrast, no selective advantage appears to result from the quaternary structure of the icosahedral *E. coli* protein.

Besides the lumazine synthase of *B. subtilis* and *E. coli*, the only other enzyme known to obey icosahedral symmetry is pyruvate dehydrogenase of *S. cerevisiae* (32). The three-dimensional structure of the icosahedral pyruvate dehydrogenase has not yet been determined at atomic resolution. The icosahedral module forms part of a multienzyme complex. The *E. coli* enzyme reported in this study appears to be the only known example of a hollow icosahedral capsid.

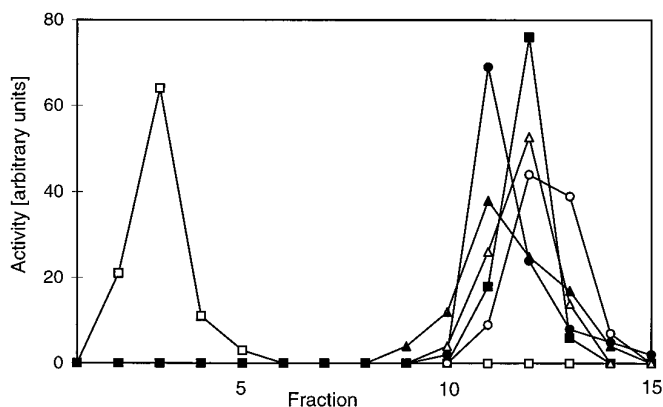


FIG. 7. **Sucrose gradient centrifugation.** Cell extract of the *E. coli* wild type strain DSM 613 (200 μ l, 12.0 mg of protein/ml) was loaded on top of linear gradients containing 5–20% sucrose in 100 mM phosphate buffer, pH 7. The gradients were centrifuged in an SW56 rotor (Beckman) at 45,000 rpm and 4 $^{\circ}$ C for 4.5 h. Fractions were collected and analyzed for enzyme activity. \square , lumazine synthase; \blacksquare , 3,4-dihydroxy-2-butanone 4-phosphate synthase; \blacktriangle , GTP cyclohydrolase II; \circ , reductase; \triangle , pyrimidine deaminase; \bullet , riboflavin synthase.

TABLE V
Purification of recombinant lumazine synthase of *S. cerevisiae*

Procedure	Protein	Activity	Specific activity
	mg	units	units/mg
Cell extract	295	1,711,000	5,800
Butyl-Sepharose	96	1,368,800	14,400
Hydroxyapatite	58	889,700	15,400

Recently, the gene *RIB4* of *S. cerevisiae* has been sequenced. The gene was cloned in the multicopy plasmid YEP352 under the control of the strong *TEF1* promoter. The recombinant yeast strains showed a 90-fold increase in lumazine synthase activity (19). We cloned the *RIB4* gene into the *E. coli/B. subtilis* shuttle plasmid p602/22 under the control of the *lac* promoter and expressed the gene in *E. coli*. Recombinant *E. coli* cell extracts showed a 500-fold increase in lumazine synthase activity. Analytical ultracentrifugation indicates that the enzyme is a pentamer that does not associate to form an icosahedral capsid even at protein concentrations above 1 mg/ml. Revuelta and co-workers (19) also obtained evidence for a relatively low molecular weight by gel filtration experiments.

Dimer, trimer, and pentamer assemblies can be formally distinguished in the icosahedral capsid of the *B. subtilis* enzyme as a consequence of the 532 symmetry. The interface contacts of the pentameric assemblies are large compared with the trimers and dimers (13). The icosahedral *B. subtilis* protein is therefore best described as a dodecamer of pentamers.

Amino acids in *B. subtilis* lumazine synthase involved in pentamer contacts are similar or identical to amino acid residues in *S. cerevisiae*. Specifically, the residues Glu-5, Arg-21, Asp-24, and Glu-145 of the *Bacillus* protein are conserved in the yeast protein. On the other hand, structure elements involved in dimer and trimer contacts in *B. subtilis* are not conserved in the yeast enzyme.

By comparison with the *Bacillus* enzyme, the yeast protein shows two insertions of 4 and 5 amino acid residues, respectively. One of these insertions is located inside the putative α_4 helix. In the *Bacillus* protein, this insertion would compromise the formation of the trimer contacts. We therefore assume that this insertion is the dominant factor inhibiting the formation of an icosahedral capsid by yeast protein. The other insertion in

the yeast protein is located between the putative α_2 helix and the β_4 sheet and would not interfere with the folding topology or the subunit assembly.

The kinetic properties of the lumazine synthase from bacteria and yeast are similar (Table IV). Whereas the access of substrates and products to the active sites of the icosahedral lumazine synthases is hard to explain in light of the densely packed icosahedral capsid, it is likely that the active sites of the pentameric yeast enzyme are more easily accessible. However, the closely similar kinetic properties of the icosahedral, bacterial enzymes and the pentameric yeast enzyme indicate that penetration of product through the icosahedral capsid is not a rate-limiting factor for catalysis.

Enterobacteria such as *E. coli* and *Salmonella* are devoid of an uptake system for riboflavin. They are therefore absolutely dependent on the intracellular synthesis of flavo-coenzymes and should be vulnerable to inhibitors of riboflavin biosynthesis. Thus, a study of the riboflavin biosynthetic enzymes in enterobacterial species could serve as the basis for the rational design of enzyme inhibitors with chemotherapeutic potential.

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