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 \( \alpha_4 \) 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-ribityl-amino-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 \( \alpha \) subunits (riboflavin synthase) and 60 \( \beta \) subunits (lumazine synthase) (7–9). The genes ribB and ribH of the riboflavin operon of B. subtilis code for the \( \alpha \) and \( \beta \) subunits of the enzyme complex (10–12). The \( \beta \) subunits form an icosahedral capsid enclosing the \( \alpha \) subunit trimer (8). The structure of the \( \beta_60 \) 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. 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-ribitylaminol-2,4(1H,3H)-pyrimidinedione and 6,7-dimethyl-8-ribityllumazine were synthesized by published procedures (20, 21). Recombinant 3,4-dihydroxy-2-butane 4-phosphate synthase of E. coli (22) was used for preparation of 3,4-dihydroxy-2-butane 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 (23), 3,4-dihydroxy-2-butane 4-phosphate synthase (3), GTP cyclohydrolase II (23), deaminase, and reductase \(^2\) 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) \(^3\) 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 p69222) and the primer ORF3-2. The amplification product was cleaved with BamHI and EcoRI, and the fragment was ligated into vector p602/22, which had been treated with BamHI and EcoRI. 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 EcoRI and NsiI, and the fragment was ligated into the vector p60222-CAT, which had been prepared with EcoRI and PstI. 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 p60222. The plasmid p60222 was digested with the restriction enzymes PvuII and ScaI, and the resulting product was recovered and ligated into vector p60222-CAT, which served as the template for subcloning the ribE gene.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
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 mg 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).

Table I

<table>
<thead>
<tr>
<th>Strain/plasmids</th>
<th>Relevant characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [P’, proAB, lacI ΔM15, Tn10(tet’)]</td>
<td>Ref. 24</td>
</tr>
<tr>
<td>DSM 613</td>
<td>Wild type</td>
<td>Deutsche Sammlung von Mikroorganismen</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p602/22</td>
<td>E. coli/B. subtilis shuttle vector</td>
<td>A. van Loon</td>
</tr>
<tr>
<td>p602/22-CAT</td>
<td>E. coli/B. subtilis shuttle vector without CAT gene</td>
<td>This study</td>
</tr>
<tr>
<td>p602ribE</td>
<td>p602 with the ribE gene of E. coli with CAT gene</td>
<td>This study</td>
</tr>
<tr>
<td>p602rib4-CAT</td>
<td>p602 with the RIB4 gene of S. cerevisiae without CAT gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table II

| Oligonucleotides used for the construction of plasmids |
|-----------------|-----------------|-----------------|
| P1’ | 5’-ACACCGAATTCATTAAGAGGAGAAATTATACTATG-3’ |
| ORF3–1 | 5’-GGGAAATTTACTTAGACATTATAGGACC-3’ |
| ORF3–2 | 5’-GTTTACGAGATCCCCCCTTACTAATTC-3’ |
| rib4–1 | 5’-GGGAAATTTACCATGGCAGTTAAAGGATTAGGC-3’ |
| rib4–2 | 5’-CGCAAAATGGAAGATGCATTAGGAAGCACC-3’ |
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 leiognathi (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**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>Cell extract</td>
<td>279</td>
<td>928,000</td>
<td>3,330</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>72</td>
<td>594,000</td>
<td>8,300</td>
</tr>
<tr>
<td>Resource Q</td>
<td>40</td>
<td>475,000</td>
<td>11,800</td>
</tr>
</tbody>
</table>

**Purification of Recombinant Lumazine Synthase of E. coli**

Wet cell mass of E. coli strain XL-1 carrying the plasmid p602 rib4-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 1–0 M ammonium sulfate in 50 mM potassium phosphate buffer, pH 7.5. The column had a volume of 2 ml and was placed on a column of hydroxyapatite (Beckman, 70 Ti rotor, 45,000 rpm, 16 h).

**Analytical Ultracentrifugation—** Boundary sedimentation experiments and sedimentation equilibrium experiments were performed with a Beckman 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. The 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 negatively stained protein were obtained as described (16). For determination of protein, 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-2-butanone 4-phosphate with a specific activity of 11,800 nmol mg⁻¹ h⁻¹. 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 is secreted as the single, symmetrical boundary (data not shown). The sedimentation velocity at 20 °C in 50 mM potas-
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### TABLE IV

<table>
<thead>
<tr>
<th>Property</th>
<th>B. subtilis*</th>
<th>E. coli</th>
<th>S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ for 5-amino-6-ribitylaminopyrimidinedione (μM)</td>
<td>4.6</td>
<td>4.2</td>
<td>4.0</td>
</tr>
<tr>
<td>$K_m$ for 3,4-dihydroxy-2-butanoate 4-phosphate (μM)</td>
<td>130 62 90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}$ (nmol mg$^{-1}$ h$^{-1}$)</td>
<td>12,000 11,800 15,400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedimentation coefficient (S)</td>
<td>26.5 26.8 5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subunit mass (kDa)</td>
<td>α = 23.5, β = 16.2</td>
<td>16.2</td>
<td>18.6</td>
</tr>
<tr>
<td>No. of subunits</td>
<td>3 α, 60 β</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>Molecular mass (kDa)$^b$</td>
<td>1,000</td>
<td>977</td>
<td>90</td>
</tr>
<tr>
<td>Predicted molecular mass (kDa)$^c$</td>
<td>1,042</td>
<td>969</td>
<td>93</td>
</tr>
</tbody>
</table>

* 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.

The lumazine synthase 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 negative 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 α$_{5β}$ 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 decrations 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.
of α subunits (riboflavin synthase). Whereas β subunits occur exclusively as the α3β60 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 substrate 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 outer 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 sterical 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.
yeast strains showed a 90-fold increase in lumazine synthase
the control of the strong

The gene was cloned in the multicopy plasmid YEP352 under the control of the shuttle plasmid p602/22 under the control of the RIB4

helix. In the B. subtilis protein, this insertion is the dominant factor inhibiting 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

yeast the yeast protein as a consequence of the 532 symmetry. The interface

distincted in the icosahedral capsid of the B. subtilis protein

pentamer contacts are similar or identical to amino acid residues in 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 flavoenzymes 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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Recently, the gene RIB4 of S. cerevisiae has been sequenced. The gene was cloned in the plasmid cosm id 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 (15). 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 α4 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 α2 helix and the β3 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 flavoenzymes 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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