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 x-ray crystallography (13–15). The enzyme complex is located in close proximity to the genes *ribE*, which is catalyzed by the enzyme riboflavin synthase (4, 5). Pyrimidine synthase and riboflavin synthase activity previously described 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 RIB4 Gene of *S. cerevisiae***—The coding region of the *ribE* gene was amplified by polymerase chain reaction (PCR) using isolated chromosomal DNA from *E. coli* strain 212 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 p60222) and the primer ORF3-2. The amplification product was cloned into *Bam HI* and EcoRI, and the fragment was ligated into vector p60222, which had been treated with *Bam HI* and EcoRI. The ligation mixture was transformed into *E. coli* XL-1 Blue host cells (24). Transformants were selected on LB medium supplemented with kanamycin (20 mg/liter). The ligation mixture was transformed into *E. coli* XL-1 Blue host cells (24). Transformants were selected on LB medium supplemented with kanamycin (20 mg/liter). The plasmid p60222-CAT was constructed by elimination of the *CAT* gene from the plasmid p60222. The plasmid p60222 was digested with the restriction enzymes *Pst I* and *Sac 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.
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-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
Bacterial strains and plasmids

| Strain/plasmids | Relevant characteristics | Source |
|-----------------|--------------------------|--------|
| E. coli strains |                          |        |
| XL-1 Blue       | recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [P', proAB, lacI'ZΔM15, Tn10(tet')] | Ref. 24 |
| DSM 613         | Wild type                |        |
| Plasmids        |                          |        |
| p602/22         | E. coli/B subtilis shuttle vector | A. van Loon |
| p602/22-CAT     | E. coli/B. subtilis shuttle vector without CAT gene | This study |
| p602/ribE       | p602 with the ribE gene of E. coli with CAT gene | This study |
| p602/rib4-CAT   | p602 with the RIB4 gene of S. cerevisiae without CAT gene | This study |

TABLE II
Oligonucleotides used for the construction of plasmids

| Oligonucleotides | Sequence |
|------------------|----------|
| P1'              | 5'ACACGAATTCCATTAAAGGGAGAAATTTACTATG-3' |
| ORF3–1           | 5'GGAGAAATTAACCTACATGACATAATTGAGCC-3' |
| ORF3–2           | 5'GTTCACCGGTCCTTTGACTTCTTCC-3' |
| rib4–1           | 5'GGAGAAATTAACCTACATGACATAATTGAGCC-3' |
| rib4–2           | 5'CGCAGAATGAAATATGCATTGAGCACC-3' |
FIG. 2. Alignments of predicted amino acid sequences. a, ribH of \textit{B. subtilis} (10, 11); b, ribE of \textit{E. coli} (16); c, \textit{ORFII} of \textit{Photobacterium leiognathi} (34); d, ribE of \textit{Hemophilus influenzae} (34); e, ribH of \textit{yeast} (19).

Secondary structure elements of the \textit{B. subtilis} lumazine synthase is shown (13). Residues that are part of the active site in \textit{B. subtilis} are marked by stars.

| TABLE III |
|---|
| **Purification of recombinant lumazine synthase of \textit{E. coli}** |
| Procedure | Protein | Activity | Specific activity |
| --- | --- | --- | --- |
| 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 \textit{S. cerevisiae}**—Wet cell mass of \textit{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 was incubated at 37 °C for 90 min and was centrifuged at 5000 rpm for 15 min. 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 buffer, pH 7.5. Fractions were combined and dialyzed against 20 mM potassium phosphate buffer, pH 7.5. The solution was placed on a column of hydroxyapatite (3 × 8 cm, DNA grade Bio-Gel HTP, Bio-Rad) equilibrated with 20 mM potassium phosphate buffer, pH 6.8. The column was developed with a linear gradient of 20–200 mM potassium phosphate, pH 6.8 (total volume, 800 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. 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 (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 \textit{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 \textit{E. coli} chromosome that had been sequenced by Taura and co-workers (16) showed considerable sequence similarity to the ribH gene of \textit{B. subtilis} specifying the enzyme 6,7-dimethyl-8-ribityllumazine synthase (Fig. 2). Using the vector p602/22, a plasmid containing the putative \textit{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 \textit{E. coli} host. Cell extracts of the recombinant strain had high lumazine synthase activity, thus confirming the presumptive function of the \textit{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-ribitylaminolucurate, 6,7-dimethyl-8-ribityllumazine synthase (Fig. 2). Using the vector p602/22, a plasmid containing the putative \textit{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 \textit{E. coli} host. Cell extracts of the recombinant strain had high lumazine synthase activity, thus confirming the presumptive function of the \textit{E. coli} gene for which the designation ribE is proposed.
The recombinant lumazine synthase of \( \textit{B. subtilis} \) 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 \( \textit{E. coli} \) 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 \( \textit{E. coli} \) and \( \textit{B. subtilis} \) (Table IV).

The recombinant lumazine synthase of \( \textit{S. cerevisiae} \) 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 \( \textit{B. subtilis} \) has been studied in considerable detail (7–9, 13–15) and is characterized by 60 \( \beta \) subunits (lumazine synthase) forming an icosahedral capsid that encloses a trimer.

### Table IV

| Property | Microorganism |
|----------|---------------|
| \( K_m \) for 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (\( \mu \)M) | B. subtilis | E. coli | S. cerevisiae |
| 130 | 62 | 90 |
| \( V_{max} \), (nmol mg\(^{-1}\) 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 |
| \( \gamma \) | 60 | 5 | 3 |
| 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 \( \alpha \) and 60 \( \beta \) subunits as reported earlier (6, 7).

\( ^b \) Data are from sedimentation equilibrium analysis.

\( ^c \) Calculated from amino acid sequence and subunit stoichiometry.
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 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 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.
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 pRO2/22 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. Purification of recombinant lumazine synthase of *S. cerevisiae* strain and plasmids.

**Table V**

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

**REFERENCES**

1. Neuberger, G., and Bacher, A. (1986) Biochem. Biophys. Res. Commun. 139, 1111–1116
2. Volk, R., and Bacher, A. (1988) J. Am. Chem. Soc. 110, 3651–3653
3. Volk, R., and Bacher, A. (1989) J. Biol. Chem. 264, 19479–19485
4. Plaut, G. W. E., Smith, C. M., and Alworth, W. L. (1974) Annu. Rev. Biochem. 43, 899–922
5. Wacker, H., Harvey, R. A., Winestock, C. H., and Plaut, G. W. E. (1964) J. Biol. Chem. 239, 3493–3497
6. Wis, K., Volk, R., and Bacher, A. (1995) Biochemistry 34, 2883–2892
7. Bacher, A., Baur, R., Eggers, U., Harders, H.-D., Otto, M. K., and Schnepple, H. (1986) J. Biol. Chem. 261, 632–637
8. Bacher, A., Ludwig, H. C., Schnepple, H., and Bacher, A. (1986) J. Mol. Biol. 187, 57–86
9. Bacher, A., and Ladenstein, R. (1991) in Chemistry and Biochemistry of Flavodoxins (Muller, F., ed) Vol. 1, pp. 293–316, Chemical Rubber Co., Boca Raton, FL
10. Minrean, V. M., Kneve, A. S., Chernov, B. K., Ulyanov, A. V., Golova, Y. B., Popovskaya, G. E., Simonova, M. L., Gordeev, V. K., Stepovanskaya, A. I., and Skryabin, K. G. (1989) Dokl. Akad. Nauk SSSR 305, 482–487; Dokl. Biochem. New York 305, 79–83
11. Perkins, J. B., Pero, J. J., and Slama, A. (1991) Eur. Pat. Appl. EP 45370, A191020
12. Perkins, J. B., and Pero, J. G. (1996) in Bacillus subtilis and Other Gram-Positive Bacteria (Soneshine, A. L., Hoch, J. A., and Losick, R., eds) pp. 319–334, American Society for Microbiology, Washington, D.C.
13. Ladenstein, R., Schneider, M., Huber, R., Bartunik, H.-D., Wilson, K., Schott, K., and Bacher, A. (1988) J. Mol. Biol. 203, 1045–1070
14. Ladenstein, R., Ritsert, K., Huber, R., Richter, G., and Bacher, A. (1994) Eur. J. Biochem. 223, 1007–1017
15. Ritsert, K., Huber, R., Turk, D., Ladenstein, R., Schmidt-Baase, K., and Bacher, A. (1995) J. Mol. Biol. 253, 151–167
16. Taura, T., Ueguchi, C., Shiba, K., and Ito, K. (1992) Mol. Gen. Genet. 234, 429–432
17. Oltmanns, O., Bacher, A., Lingens, F., and Zimmermann, F. K. (1967) Mol. Gen. Genet. 105, 306
18. Logvinenko, E. M., Shavlovsky, G. M., and Tsarenko, N. Y. (1985) Biochemistry 50, 744; Biochemistry 50, 625
19. Garcia-Ramirez, J. J., Santos, M. A., and Revuelta, J. L. (1995) J. Mol. Biol. 250, 289–290
20. Sedlimaier, H., Muller, F., Keller, P. J., and Bacher, A. (1987) Z. Naturforsch. 42a, 425–429
21. Bacher, A. (1986) Methods Enzymol. 132, 192–199
22. Richter, G., Volk, R., Krieger, C., Lahm, H., Rottischberger, U., and Bacher, A. (1992) J. Bacteriol. 174, 4050–4056
23. Ritzi, H., Richter, G., Kazemimeyer, G., Volk, R., Koehne, A., Lassplech, F., Allendorf, D., and Bacher, A. (1993) J. Bacteriol. 175, 4045–4051
24. Bulloch, W. O., Fernandez, J. M., and Short, J. M. (1987) BioTechniques 3, 757–778
25. Edsall, J. T., and Cohn, E. J. (1943) Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions, p. 57, Rheinhold, New York
26. Lane, T. M., Eash, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E., Rowe, A. J., and Horton, J. C., eds) pp. 90–125, Royal Society of Chemistry, Cambridge, U.K.
27. Bachmann, L., Weinkauf, S., Baumeister, W., Wildhaber, I., and Bacher, A.
Biosynthesis of Riboflavin

(1989) J. Mol. Biol. 207, 575–584
28. Braun, N., Tack, J., Bachmann, L., and Weinkauf, S. (1996) Thin Solid Films, in press
29. Weinkauf, S., Bacher, A., Baumeister, W., Ladenstein, R., Huber, R., and Bachmann, L. (1991) J. Mol. Biol. 221, 637–645
30. Schott, K., Ladenstein, R., König, A., and Bacher, A. (1990) Proc. Natl. Acad. Sci. USA 87, 8656–8660
31. Fischer, M., Eberhardt, S., Richter, G., Krieger, C., Gerstenschläger, I., and Bacher, A. (1996) Biochem. Soc. Trans. 24: (suppl.) 35
32. Stoops, J. K., Baker, T. S., Schroeter, J. P., Kolodziej, S. J., Nui, X.-D., and Reed, L. J. (1992) J. Biol. Chem. 267, 24769–24775
33. Lee, L. Y., and Meighen, E. A. (1992) Biochem. Biophys. Res. Commun. 186, 690–697
34. Fleischmann, R. D., et al. (1995) Science 269, 496–512