6,7-Dimethyl-8-ribityllumazine, the immediate biosynthetic precursor of riboflavin, is synthesized by condensation of 5-amino-6-ribitylaminono-2,4(1H,3H)-pyrimidinedione with 3,4-dihydroxy-2-butane 4-phosphate. The gene coding for 6,7-dimethyl-8-ribityllumazine synthase in Saccharomyces cerevisiae (RIB4) has been cloned by functional complementation of a mutant accumulating 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione, which can grow on riboflavin- or diacetyl-but not on 3,4-dihydroxy-2-butane-supplemented media. Gene disruption of the chromosomal copy of RIB4 led to riboflavin auxotrophy and loss of enzyme activity. Nucleotide sequencing revealed a 169-base pair open reading frame encoding a 18.6-kDa protein. Hybridization analysis indicated that RIB4 is a single copy gene located on the left arm of chromosome XV. Overexpression of the RIB4 coding sequence in yeast cells under the control of the strong TEF1 promoter allowed ready purification of 6,7-dimethyl-8-ribityllumazine synthase to apparent homogeneity by a simple procedure. Initial structural characterization of 6,7-dimethyl-8-ribityllumazine synthase by gel filtration chromatography and both nondenaturing pore limit and SDS-polyacrylamide gel electrophoresis showed that the enzyme forms a pentamer of identical 16.8-kDa subunits. The derived amino acid sequence of RIB4 shows extensive homology to the subunits of riboflavin synthase from Bacillus subtilis and other prokaryotes.

Riboflavin, vitamin B2, is the precursor of flavin mononucleotide and flavin adenine dinucleotide, which function as coenzymes for a wide variety of enzymes in intermediate metabolism. Whereas lower organisms are able to biosynthesize riboflavin, mammals have lost this capacity and, therefore, rely on its dietary ingestion to meet their metabolic needs. As a consequence, this compound is commercially important as an additive in food industries, and several flavinogenic microorganisms, including some yeast species, are used in industry to produce riboflavin by fermentation (1).

The yeast Saccharomyces cerevisiae has been thoroughly used to analyze flavinogenesis biochemically and genetically (2) and some yeast species have been developed for the biotechnological production of riboflavin (1). Six complementation groups of S. cerevisiae riboflavin auxotrophs (rib1 to rib5 and rib7) have been identified (3, 4). The immediate precursor of riboflavin, 6,7-dimethyl-8-ribityllumazine (see Fig. 1, 4), is formed by condensation of 5-amino-6-ribitylaminono-2,4(1H,3H)-pyrimidinedione (see Fig. 1, 3) with 3,4-dihydroxy-2-butane 4-phosphate (see Fig. 1, 2). Two types of mutants, rib3 and rib4, have been shown to be impaired in the synthesis of the immediate precursor of riboflavin, 6,7-dimethyl-8-ribityllumazine, but they have not been correlated with specific defects in the synthesis of riboflavin. We report here that rib4 mutants are defective in 6,7-dimethyl-8-ribityllumazine synthase.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagent chemicals were of the highest commercial grade. 6,7-Dimethyl-8-ribityllumazine and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione were synthesized as described (10). 3,4-dihydroxy-2-butane was synthesized from acetol and formaldehyde under previously described conditions (11). Restriction enzymes and DNA modification enzymes were from Pharmacia Biotech Inc., New England Biolabs, or Boehringer Mannheim. Thermus aquaticus DNA polymerase was from Perkin-Elmer. Oligonucleotides RIB4 (5′-AGTTTAAATCAAAAATGGCAGTTAAA-3′), RIB4 (5′-GGACAAAACACAGTTGAGT-3′), RIB4 (5′-CCACCGGCGATAGTACGCCGCAC-3′) (see Fig. 1), Tef1 (5′-GAATCTTGTATCATAGTCATACACAAGGA-3′), and Tef2 (5′-CATTTTGTAATTAAACTTGA-3′) were synthesized on an Applied Biosystems synthesizer.

**Strains and Media**—The following S. cerevisiae strains were used in this study. X2180–1A (MATa, and TD30 (MATa ura3–52 leu2–3 ino1–9)) were obtained from F. del Rey (Universidad de Salamanca). JC2a (MATa leu2, 3–112 his3–11 ura3–52) and SI502B (MATa leu2, 3–112 his3–11 ura3–52) were a kind gift of M. J ayaram (University of Texas). Strains YNN295 (MATa ura3–52 lys2 ade2 ade2 his7 trpl–11) and YPH149 (MATa ura3–52 lys2–801 ade2–101 his7–11 trpl–11 CFVII RAD2, pYPH149) (CFVII/RAD2, pYPH149/CFVII/RAD2/3pYPH149/CFVII/RAD2/3pYPH149) were obtained from the Yeast Genetic Stock Center (University of California). Riboflavin auxotrophs J A19 (MATa rib3–1 leu2, 3–112 ura3–52), AJ 21 (MATa rib4–1 leu2, 3–112 his3–11 ura3–52), and AJ 22 (MATa rib4–2 leu2, 3–112 ura3–52) were derived by ethyl methanesulfonate mutagenesis from the parental strains SI502B and JC2a (12). The rib4 deletion strain AJ 106 (MATa rib4–1:URA3 leu2, 3–112 ino1 ura3–52) was constructed in strain TD30 by the one-step gene disruption method described by Rothstein (13). General methods for the genetic manipulations of yeast cells were carried out as described previously (14). Yeast strains auxotrophic for riboflavin, rib3 and rib4 mutants, were cultured in media with riboflavin at a concentration of 20 mg/liter.

Plasmids and Gene Deletion—The URA3 plasmid YEp13 and the genomic library made in this vector have been described earlier (15). A rib4 deletion/substitution allele, designated rib4Δ11::URA3, was constructed by replacing the 0.5-kb PstI-SspI fragment, which contains most of the RIB4 open reading frame, with the URA3 gene. First, the 3.0-kb XbaI–PstI and 1.3-kb SspI–SalI fragments, flanking the 0.5-kb...
PstI–SspI fragment, were successively cloned into the XbaI and PstI site and the EcoRV and SalI site, respectively, of pBluescript KS+ (Stratagene) yielding pJR586. Finally, pJR586 was digested with EcoRI, treated with the Klenow fragment of DNA polymerase I to generate blunt ends, and ligated to a 1.1-kb blunt-ended fragment containing the URA3 gene of YEp24 (16) to generate pJR591. The linear 3.3-kb BglII–ClaI fragment of this plasmid was used for the transformation of haploid strain TD30. Ura− colonies were selected, and gene deletion was confirmed by Southern blot analysis.

To construct the TEF1–RIB4 gene fusion, appropriately designed oligonucleotides and polymerase chain reactions were used. Firstly, a fragment containing the 5′ region of the TEF1 gene (nucleotides 635 to 3308) was amplified using oligonucleotides TEF1 and TEF2 and genomic DNA from strain X2180–1A as template. Secondly, another fragment containing nucleotides 18 to 3 of TEF1 joined to the coding and terminator sequences of RIB4 (nucleotides 3–726) was amplified using oligonucleotides RIB41 and RIB42 and DNA of pJR633 as template. Finally, the two partially overlapping fragments generated in the two previous reactions were joined by polymerase chain reaction in a contiguous fragment (containing nucleotides 635 to 1 of TEF1 fused to nucleotides 1–726 of RIB4) using oligonucleotides TEF1 and RIB42 and the two previously amplified fragments as templates. The resulting fragment was verified by sequencing and cloned into YEp352 to yield pJR627.

Nucleic Acid Manipulations—Transformation of Escherichia coli, Southern blot analysis and other routine DNA and RNA manipulations were carried out as described by Sambrook et al. (18). Yeast cells were transformed by the method of Ito et al. (19). Plasmids were recovered from yeast cells by the rapid protocol described previously (20).

DNA sequencing reactions were performed using the dideoxy chain termination method (21) using the T7 sequencing system (Pharmacia). Primer extension analyses were done as described (18) using the synthetic oligonucleotide RIB6, which is complementary to nucleotides 57–80 of the RIB4 ORF.

6,7-Dimethyl-8-ribityllumazine Synthase Purification and Enzyme Assay—Aj 106 yeast cells transformed with pJR627 were grown to late-log phase (A_{600nm} = 1.5–2) in SD medium lacking riboflavin to select transformant-containing cells. All procedures described below were done at 4°C unless otherwise stated. Cells were harvested by centrifugation, washed once with ice-cold water, and resuspended in 1/50 volume of extraction buffer (20 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride). Glass beads (0.5 mm) were added, and the cells were disrupted in a Braun homogenizer. Glass beads, unbroken cells and cell wall components were removed by centrifugation at 4000 × g for 20 min. The crude extract was then centrifuged at 100,000 × g for 60 min to obtain the supernatant cytosolic fraction. Methyl alcohol (final concentration, 30%, v/v) was added to the cytosolic fraction, incubated at 0°C for 12 h and centrifuged at 15,000 × g for 60 min. The supernatant fraction was then lyophilized, resuspended in 20 mM Tris-HCl, pH 7.5, and passed through a 0.45-μm sterile filter prior to the lyophilization chromatographic step. In consecutive runs, the filtered protein fraction (5 mg/ml) was loaded onto a MonoQ column (0.5 × 5-cm, HR 5/5, fast performance liquid chromatography system, Pharmacia) that had been equilibrated with 20 mM Tris-HCl, pH 7.5. After sample passage, the column was washed with 5 ml of the same buffer, and the chromatogram was developed with a 25-ml linear gradient of 0.05–0.5 M NaCl in 20 mM Tris-HCl, pH 7.5. Fractions (0.5 ml) were analyzed by SDS-PAGE and enzyme activity. Lumazine synthase eluted at about 300 mM NaCl in the gradient. Fractions from MonoQ chromatographies containing enzyme activity were pooled, concentrated by lyophilization, resuspended in 20 mM Tris-HCl, pH 7.5, and loaded onto a SuperDex 200 column (Pharmacia) that had been equilibrated with 20 mM Tris-HCl, pH 7.5, 90 mM NaCl. Peak fractions (0.25 ml, fractions 28–30) for lumazine synthase activity eluted from the SuperDex 200 chromatography appeared apparently pure as judged by SDS-PAGE analysis. Lumazine synthase activity was determined by the procedure described by Neuberger and Bacher (22). Enzyme activity is expressed as nanomoles of 6,7-dimethyl-8-ribityl-lumazine formed per h.

Other Protein Methods—The method of Laemmli (23) was used for SDS-PAGE, and proteins were detected by silver staining. Protein content was determined by the method of Lowry et al. (24). The apparent native molecular mass of purified riboflavin synthase was determined by both gel filtration chromatography and nondenaturing pore limit PAGE. Nondenaturing pore limit PAGE was done as described previously in a 4–20% polyacrylamide gradient gel (Bio-Rad). Computer-aided Analysis of Sequence Data—The search for protein homologies was performed through the National Center for Biotechnol-
characterization of RIB4 and the purification and preliminary analysis of the encoded protein.

Cloning of RIB4—The RIB4 gene was isolated by complementation of the riboflavin auxotrophy of the rib4-1 mutant strain AJ 21. This strain was transformed with a yeast genomic DNA library constructed in the yeast shuttle vector YEp352 (15). One of the complementing plasmids, designated pJR R66, was chosen for further analyses. A series of subclones of pJR R66 were constructed into vector YEp352 to delineate the end points of the RIB4 gene (Fig. 2A). One of these subclones, pJR R497, contained a 4.2-kb BglII–Xba fragment that complemented the rib4 mutation of AJ 21. When a 2.1-kb Hpal–Xba fragment was removed from pJR R497 to generate pJR R633, the smallest subclone complementing the rib4 auxotrophy was generated. Comparison of the restriction maps of pJR R633 and pJR R609, the latter of which does not complement the mutation, indicates that the RIB4 gene should span a cluster of BamHI, HindII, Sall, Clal, and PstI restriction sites.

Based on the phenotype of the rib4-1 mutation, disruption of the RIB4 locus was expected not to be lethal but should result in riboflavin auxotrophy. To inactivate RIB4 function, the RIB4 gene of the haploid, wild-type TD30 strain was disrupted by the one-step gene disruption method (13). A deletion/substitution of the 0.5-kb SspI–PstI fragment, spanning the cluster of restriction sites, by a 1.1-kb fragment containing the URA3 gene was created as described under “Experimental Procedures” (Fig. 2B). Ura+ prototroph (Ura+) transformants were riboflavin auxotrophs (Rib−) as determined by replica-plating onto complete media lacking riboflavin. Moreover, these transformants showed the rib4 phenotype (i.e. accumulation of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and the ability to grow on diacetyl but not on 3,4-dihydroxy-2-butanoate-supplemented minimal medium). A Southern hybridization analysis of genomic DNA of the parental strain TD30 and one Rib−Ura+ transformant, designated AJ 106 (rib4Δ11::URA3), were used to confirm genomic integration and deletion at the correct site (Fig. 2B). Strain AJ 106 was crossed with strain AJ 22; the resulting diploid had an Rib− phenotype, suggesting that the two mutations are allelic. Finally, the diploid was sporulated, and 15 tetrads were dissected. As expected, all tetrads analyzed segregated four Rib− spores, showing the identity of the cloned gene and RIB4.

Nucleotide Sequence Analysis of RIB4—The nucleotide sequence of the 2.1-kb BglII–Hpal fragment of pJR R633 was determined on both strands. The sequence contains a single complete open reading frame, capable of encoding a protein of 169 amino acids with a calculated molecular mass of 18,661Da (Fig. 3). An in-frame stop codon is located 9 base pairs upstream from the putative initiating methionine codon. The yeast intron splice signal TACTAAC (27) was not found. The upstream flanking region of the RIB4 open reading frame reveals the presence of a conserved TATA-box sequence (28, 29) at position −105 with respect to the ATG initiation codon. A striking feature of the upstream region is the repeated decanucleotide AGTATAACGC, which occurs in a perfect tandem repeat of four copies immediately preceding the initiation codon. At the 3' end of the gene, the open reading frame is terminated by two consecutive stop codons. Consensus sequences for polyadenylation, ATATAT (30), and transcription termination in yeast, TGATATGTTT (31), were detected at positions 508 and 532, respectively.

The predicted encoded product is a slightly charged protein with a calculated pI of 6.61. Hydrophathy analysis (32, 33) predicts a moderately hydrophilic, globular protein with no sequence of sufficient length and hydrophobicity to be considered a membrane-spanning domain. From the distribution of the codons used, a codon adaptation index of 0.271 can be calculated (34), which suggests a weak or moderate level of gene expression.

Direct evidence for the expression of the RIB4 open reading frame came from Northern blot analysis of S. cerevisiae mRNA using the 0.3-kb BamHI–PstI containing part of RIB4 as a probe. In the experiments of Fig. 4A, mRNA transcripts of the anticipated size for RIB4, approximately 700 nucleotides, were readily detected even in the presence of saturating concentra-
RIB4 Gene Encoding 6,7-Dimethyl-8-ribityllumazine Synthase

Fig. 3. Nucleotide sequence and predicted amino acid sequence of the RIB4 gene. Amino acids are shown in three-letter codes. The numbering adopted for both nucleotides and amino acids is initiated at the first ATG of the RIB4 ORF. The consensus TATA element is underlined, and the four repeats are indicated by open circles. Two downward arrowheads indicate the major transcription initiation sites. In the 3'-flanking region, sequences matching the transcription termination and polyadenylation signals are indicated by open circles and filled circles, respectively.

Fig. 4. Analysis of the RIB4 transcript. A. Northern analysis. Total RNA (50 μg) from wild-type strain X2180-1A grown on different culture media was purified, electrophoresed, transferred to a nylon membrane, and probed with a 32P-labeled fragment containing part of the RIB4 ORF. Lane 1, minimal medium; lane 2, minimal medium supplemented with 200 μM riboflavin; lane 3, rich medium supplemented with 200 μM riboflavin. The ACT1 transcript serves as an internal control. B. Primer extension analysis. A synthetic 24-mer oligonucleotide (RIB4 primer), complementary to nucleotides 25–80 of the RIB4 ORF, was annealed to 25 μg of poly(A) RNA from the wild-type strain X2180-1A (lane 1) or 25 μg of yeast tRNA (lane 2) and extended with avian myeloblastosis virus reverse transcriptase. The extension products were run on a denaturing polyacrylamide gel next to a sequencing reaction using the same oligonucleotide as sequencing primer. The positions of the major extension products are indicated on the right.

Box-like sequence CATATAAA and therefore occur well within the region predicted for a S. cerevisiae TATA box (35–37).

Physical Mapping of RIB4—In order to determine the chromosomal location of RIB4, the 2.1-kb BglII–Hpal fragment from pl R633 was used as a probe to hybridize to yeast chromosomes from the strain YNN295 that had been separated by pulse-field gel electrophoresis. A signal was detected at the position of the comigrating chromosomes VII and XV. The experiment was therefore repeated with chromosomes from strain YPH149, in which chromosome XV is clearly separated by gel electrophoresis from two small chromosomal fragments derived from chromosome VII. In this case, the RIB4 probe hybridized only to chromosome XV (data not shown). A more precise physical location of RIB4 was determined by hybridization of the RIB4 probe to a set of overlapping λ and cosmid clones representing ~96% of the S. cerevisiae genome (American Type Culture Collection 76269; Ref. 38). The RIB4 probe only hybridized to ATCC clone 70642, which possesses a genomic insert from the left arm of chromosome XV. The probe did not hybridize to a set of flanking, partially overlapping clones 70573 and 70589; thus, according to the EcoRI–HindII restriction map provided by ATCC for these clones, the location of the RIB4 gene is delimited to a 6.1-kb EcoRI–HindII fragment near (roughly 50-kb apart) the left telomere of chromosome XV.

Lumazine Synthase Activity of rib4 Mutants and Transformant Strains—Because accumulation and nutritional tests suggested that RIB4 encodes a structural component of 6,7-dimethyl-8-ribityllumazine synthase, we tested the in vitro activity of lumazine synthase in wild-type and rib4 strains. Although the wild-type strain X2180-1A and the parental strain TD30 showed comparable levels of lumazine synthase specific activity, almost at the limit of the detection level, none of the mutant strains, AJ21(rib4–1), AJ22(rib4–2), and AJ106(rib411:URA3), showed detectable activity. Furthermore, lumazine synthase activity was restored to about 30 times higher than that of the wild-type level in rib411:URA3 mutants transformed with a 2 μm-based high copy number plasmid harboring the RIB4 gene (pl R497). To further increase the lumazine synthase-specific activity of yeast cells, a gene fusion was constructed between the 635-base pair upstream promoter sequence of the highly expressed TEF1 gene, which encodes the translation elongation factor EF-1α (19, 39), and the complete coding and terminator sequences of RIB4 (see “Experimental Procedures”). A 2.3-kb XbaI–XbaI fragment bearing the TEF1–RIB4 gene fusion was cloned into the multicopy plasmid YEP352 to generate pl R627. Yeast strain AJ106 was transformed with the recombinant plasmid pl R627 to uracil prototrophy, and lumazine synthase activity was determined. Compared with wild-type cells, disrupted rib4 cells carrying plasmid pl R627 showed an approximately 90-fold increase in lumazine synthase activity. These data indicate that the RIB4 gene encodes lumazine synthase. Furthermore, overexpression of lumazine synthase in yeast was associated with a moderate increase (2.1-fold) in the amount of free riboflavin accumulated by the cells.

Purification and Subunit Structure of Lumazine Synthase—To facilitate the purification of lumazine synthase, the protein was overproduced in yeast cells transformed with the TEF1–RIB4 gene fusion containing plasmid pl R627. The specific activity of lumazine synthase in the total cell lysate of strain TD30 transformed with pl R627 was 126 units/mg of protein, as compared with 1.35 units/mg of protein in the non-transformed TD30 strain. The procedure to generate highly purified lumazine synthase is summarized in Table I. The protein patterns in SDS-polyacrylamide gels of the crude extract and eluted fractions containing lumazine synthase ac-
tivity from the successive purification steps are depicted in Fig. 5. In SDS gels after the MonoQ step, the purified protein displayed only one major polypeptide band (Fig. 5, lane Q). Some faint bands of higher molecular masses could be removed by chromatography on a SuperDex 200 column (Fig. 5, lane S). From the total cell lysate, an approximately 120-fold enrichment was achieved with a yield of about 4% (Table II). The final preparation had a specific activity of 8.09 mmol/h/mg and was used as the source of enzyme for further structural studies.

Analysis of the purified lumazine synthase in SDS-polyacrylamide gels revealed a single protein band with an apparent molecular mass of 91 kDa (Fig. 6, lane S), which is in good agreement with the predicted molecular mass of Rib4p deduced from its amino acid composition.

Two methods were used to estimate the native subunit structure of lumazine synthase (Fig. 6). Gel filtration chromatography indicated that the molecular mass of the native protein was 95 kDa using a column calibrated with typical globular proteins (Fig. 6A). Nondenaturing pore limit polyacrylamide gels were used to verify the molecular mass determined by gel filtration chromatography. The migration of lumazine synthase in 4–20% gradient nondenaturing polyacrylamide gel compared with the position of standards proteins indicated a molecular mass of 91 kDa (Fig. 6B). Based on these two results, we concluded that lumazine synthase was a homopentamer.

Alignment with β Subunits of Riboflavin Synthase from Prokaryotes—Comparison of the deduced primary amino acid sequence of Rib4p with available data bases showed that Rib4p is distantly related to the β subunits of riboflavin synthase from Bacillus subtilis (ribB), E. coli (ribH), and Photobacterium leiognathi (ribH) (7), as shown in Fig. 7. Pairwise comparisons among these sequences revealed that the yeast Rib4p is the most highly divergent protein, displaying approximately 26–29% amino acid sequence identity to bacterial β subunits, compared with 48–64% identities between the bacterial proteins. Analysis of these data indicated that three regions of these enzymes were most conserved. In the first region (residues 22–65 of the yeast lumazine synthase), there was a 34–46% sequence identity, which was extended to 78–82% similarity when conserved amino acids were included, to residues 15–62 of the E. coli enzyme and residues 14–61 and 15–61 of the P. leiognathi and B. subtilis enzymes, respectively. In the second region (amino acid residues 83–97 of the S. cerevisiae enzyme), 93.7% of the amino acid residues were similar and 44% were identical for all four enzymes. Moreover, in this region the yeast protein displays a marked similarity with the proposed donor, C-terminal domain of yeast riboflavin synthase (40).

Finally, in the third region (amino acid residues 120–139 of the yeast enzyme) 55% of the amino acids were identical and 80% were similar for all four proteins.

**DISCUSSION**

In this report we describe the isolation, sequence, and expression of a S. cerevisiae gene, RIB4, which encodes lumazine synthase. Nutritional tests using chemically synthesized 3,4-dihydroxy-2-butanoate suggested that rib4 mutants would be

**TABLE II**

| Description          | Total protein | Total activity | Specific activity | Recovery | Purification factor |
|----------------------|---------------|----------------|-------------------|----------|--------------------|
| Cell extract         | 276           | 18,699         | 68                | 100      | 1.20               |
| 30% methanol         | 31            | 3,260          | 117               | 17       | 1.72               |
| precipitation        |               |                |                   |          |                    |
| MonoQ chromatography | 1.20          | 928            | 774               | 5        | 11.38              |
| SuperDex 200         | 0.093         | 752            | 8089              | 4        | 119.10             |
The heavy enzyme, characterized by a molecular mass of 1 MDa, consists of an α subunit trimer enclosed within an icosahedral structure composed of 60 β subunits of 6,7-dimethyl-8-ribityllumazine synthase (5, 6, 8, 41).

The complete sequence of the B. subtilis β subunit was first established by direct sequencing of the protein (42). Subsequently, the amino acid sequence of lumazine synthase was confirmed by sequencing the rib4 open reading frame of the B. subtilis riboflavin operon (43). A computer prediction of the secondary structure indicated the presence of approximately 34% α helix, 30% β sheet, and 18% β turn in the β subunit. Most α helices and β strands predicted by a modified Chou-Fasman/Robson algorithm (44) in yeast lumazine synthase (data not shown) matched the observed secondary elements in the Bacillus enzyme surprisingly well, thus suggesting a similar chain folding for the enzyme in both organisms. However, completely different quaternary structures, pentamers in yeast and icosahedral capsids in Bacillus, are formed.

The atomic structure of the Bacillus β subunit has been deduced from electron density maps to 3.3 Å resolution by the group of Bacher (9). The main chain of the β subunit folds into a structure of four parallel β-sheet flanked on both sides by two α-helical segments. Due to the symmetry relations occurring in the β-60 icosahedral capsid, intersubunit contacts are those of dimers, trimers, and pentamers. In this context, it should be noted that most amino acid residues contributing to the stability of pentamers in the Bacillus lumazine synthase (Met3, Ile49, Arg5, Met4, Val53, Pro10, Phe13, Glu142, Arg27, Tyr91, Lys46, Pro54, Gin123, and Thr146) are conserved in the yeast enzyme. By contrast, a large proportion of amino acids involved in dimer (Arg50, His41, Gin5, Asp39, and Val) or trimer (Asp39, Lys78, Asp24, Glu126, Phe92, and Phe22, and Thr130) contacts in the icosahedral Bacillus enzyme are not conserved in yeast. Therefore, it is tempting to speculate that the discrepancy in the quaternary structures shown by the Bacillus and yeast enzymes is the result of different levels of complexity in the same pathway of protein assembly, the pentamer form corresponding to an intermediate level and the β-60 icosahedral enzyme being the most complex stage of organization. Finally, it is assumed that in Bacillus the His52 may take part in the catalytic reaction as a proton-abstracting base. In agreement with this assumption, this residue is conserved in all four organisms considered.

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