Biosynthesis of Riboflavin. Studies on the Reaction Mechanism of 6,7-Dimethyl-8-ribityllumazine Synthase[†]

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ABSTRACT: The lumazine synthase/riboflavin synthase complex of *Bacillus subtilis* consists of an icosahedral capsid of 60 β subunits surrounding a core of 3 α subunits. The β subunits catalyze the condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione with (3*S*)-3,4-dihydroxy-2-butanone under formation of 6,7-dimethyl-8-ribityllumazine. This intermediate is converted to riboflavin by the α subunits via an unusual dismutation yielding 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione as second product. (3*R*)- and (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate were synthesized. Both enantiomers can serve as substrate for 6,7-dimethyl-8-ribityllumazine synthase. The reaction rate of the natural *S*-enantiomer is about 6-fold higher than that of the *R*-enantiomer. The K_m value for (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate is 130 μM, and the K_m value for the pyrimidine substrate is 5 μM. Diacetyl and 3,4-dihydroxy-2-butanone 3-phosphate do not serve as substrates for lumazine synthase. The enzyme-catalyzed condensation of the carbohydrate with the pyrimidine is strictly regiospecific. The enzyme does not catalyze the exchange of protons between (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate and solvent water in the absence of the pyrimidine cosubstrate. A reaction mechanism starting with the formation of a Schiff base followed by elimination of phosphate and cyclization is proposed. The lumazine synthase activities of the native enzyme complex and of reconstituted, hollow β_{60} capsids are virtually identical (about 12 000 nmol mg^{-1} h^{-1}).

Riboflavin (**4**) is biosynthesized from one molecule of GTP and two molecules of ribulose 5-phosphate via (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**) (Figure 1) (Bacher, 1990; Volk & Bacher, 1988, 1990). In *Bacillus subtilis*, the terminal two steps of the biosynthetic pathway are catalyzed by an enzyme complex consisting of 3 α subunits and 60 β subunits which has been designated as "heavy riboflavin synthase" [for a review, see Bacher and Ladenstein, (1990)].

The α subunits of the protein complex catalyze the dismutation of 6,7-dimethyl-8-ribityllumazine (**3**), resulting in the formation of riboflavin (**4**) and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**) (Bacher et al., 1980). The mechanism of this dismutation reaction has been studied in considerable detail by Plaut and his co-workers using the enzyme from *Saccharomyces cerevisiae* (Plaut, 1963, 1971; Plaut et al., 1974; Wacker et al., 1964).

The function of the β subunits of the enzyme remained unknown for a considerable period. Indirect evidence for a catalytic role in the biosynthesis of the lumazine **3** was obtained by ligand-binding studies indicating that the protein binds a variety of 8-ribityl-substituted lumazines and 5-ribitylaminopyrimidines (Bacher & Mailänder, 1978; Bacher & Ludwig, 1982). However, the precise enzymatic function of the β subunit could not be established because the nature of the putative 4-carbon substrate serving as a precursor for the pyrazine ring of the lumazine **3** was unknown. Numerous research groups involved in this problem over a period of four decades proposed a variety of candidate compounds such as diacetyl, acetoin, various carbohydrates, and the ribityl side chain of the pyrimidine derivative **1**. This work has been reviewed (Bacher, 1990).

More recent studies by Volk and Bacher (1988, 1991) unequivocally identified the elusive 4-carbon precursor as (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**). Using heavy riboflavin synthase of *Bacillus subtilis*, Neuberger et al. (1985) observed the enzyme-catalyzed formation of riboflavin from this carbohydrate and 5-amino-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (**1**). In light of these data, the β subunits can now be addressed as 6,7-dimethyl-8-ribityllumazine synthase (or "lumazine synthase").

The pyrimidine produced as the second product of the α subunits' catalysis serves as substrate for the β subunit and can thus be recycled in the catalytic process. The overall stoichiometry of the reaction catalyzed by the $\alpha_3\beta_{60}$ enzyme complex involves the formation of one molecule of riboflavin from one molecule of the pyrimidine **1** and two molecules of the carbohydrate **2**.

The structure of the lumazine synthase/riboflavin synthase complex has been studied in considerable detail [for a review, see Bacher and Ladenstein, (1990)]. The 60 β subunits form a capsid with icosahedral symmetry. A trimer of α subunits is enclosed in the central core. The enzyme complex can be dissociated to form α subunit trimers (Bacher et al., 1986) and large β subunits with the shape of hollow spheres consisting of more than 100 protomers. These β subunit aggregates are easily isolated in pure form. They can then be renatured in urea in the presence of substrate analogs such as 5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**5**, Figure 2) to yield hollow, icosahedral β_{60} capsids (Bacher et al., 1986).

The native $\alpha_3\beta_{60}$ complex has been crystallized (Ladenstein et al., 1983), and the structure of the icosahedral capsid

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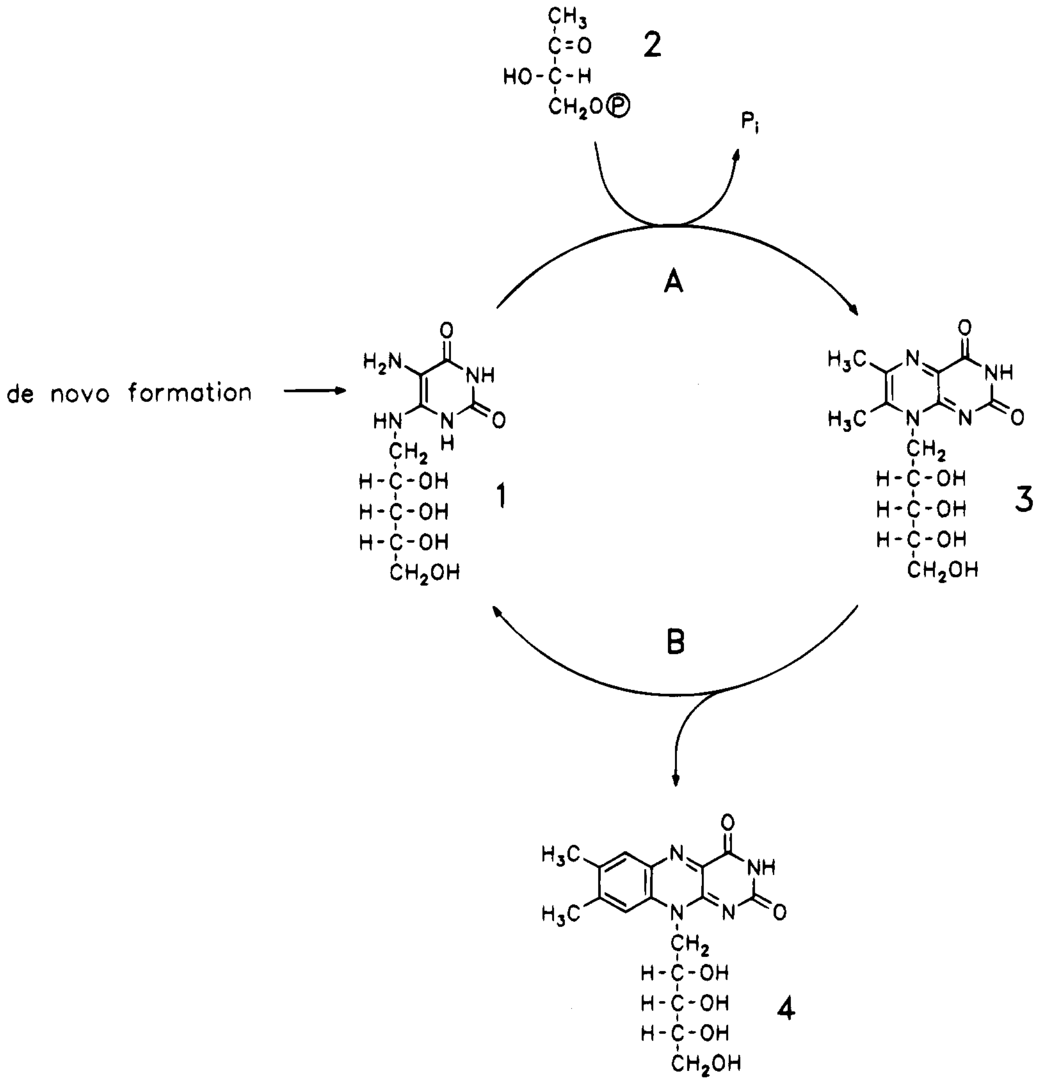

FIGURE 1: Biosynthesis of riboflavin: (A) 6,7-dimethyl-8-ribityllumazine synthase; (B) riboflavin synthase.

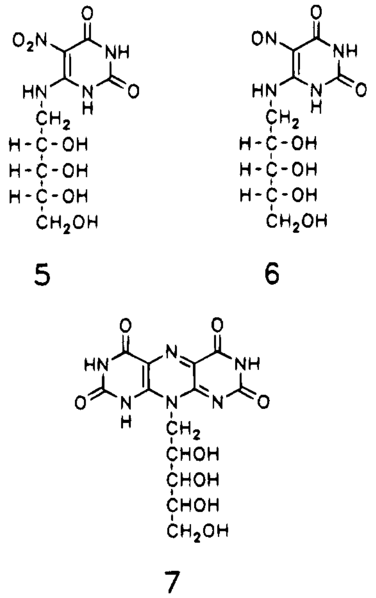

FIGURE 2: Substrate and product analogs.

has been determined at a resolution of 3.3 Å (Ladenstein et al., 1988), but no information was obtained on the α subunits located in the core. More recently, the artifactual, hollow β_{60} capsids obtained by ligand-driven reaggregation have also been crystallized (Schott et al., 1990a), and their structure has been determined at a resolution of 2.4 Å (Ladenstein et al., 1994; Ritsert et al., 1995).

The icosahedral β subunit capsid has 60 identical binding sites for the substrate **1**. Each of these sites is located at the interface of two respective β subunits, in close proximity of the inner surface of the capsid. The capsid structure is rather densely packed, and the routes for entry of substrates and exit of products are not clear. Channels running along the 5-fold axes of the icosahedron would allow the passage of **1** and **2** but appear too narrow for exit of enzymatically produced riboflavin (Ladenstein et al., 1988, 1994).

The reaction mechanism of lumazine synthase has not been studied up to now. We describe the synthesis of the enzyme substrate, (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**) and its utilization for kinetic and spectroscopic studies of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. L-Gulonolactone was a generous gift of Hoffmann-La Roche AG, Basel, Switzerland. [1-$^{13}C_1$]Ribose 5-phosphate was a gift of Prof. H. Floss, Seattle. The preparation of [6,7α,8α,9-$^{13}C_4$]riboflavin has been described elsewhere (Sedlmaier et al., 1987). 5-Nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**5**) (Cresswell et al., 1960), 5-nitroso-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**6**) (Plaut & Harvey, 1971), and 6,7-dimethyl-8-ribityllumazine (**3**)

(Bacher, 1986) were prepared by published procedures. The pyrimido-[5,4-*g*]-pteridine **7** was isolated from a sample of **1** which was exposed to the air. The spectroscopic properties of purified **7** were identical with those reported by Cresswell et al. (1960). Solvents were distilled before use. Dibenzyl phosphite was used without further purification. Potassium carbonate and magnesium sulfate were dried under reduced presure at 120 °C. Silica gel was activated by drying at 120 °C for 2 days. Activated silica gel was stored over phosphorous pentoxide.

Proteins. The lumazine synthase/riboflavin synthase complex (heavy riboflavin synthase) was purified from the derepressed *B. subtilis* mutant H94 (Schott et al., 1990a). β Subunits were isolated by treatment of the enzyme with 0.1 M Tris hydrochloride, pH 8, followed by gel permeation chromatography (Schott et al., 1990a). The solution of isolated β subunits was dialyzed against 0.1 M potassium phosphate, pH 7.0. Lumazine synthase with a stoichiometry of β_{60} was isolated from a recombinant strain of *B. subtilis* (described elsewhere). Riboflavin synthase ("light riboflavin synthase") was isolated as described (Bacher et al., 1980). The purification of (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate synthase from a recombinant strain of *Escherichia coli* has been described (Richter et al., 1992).

Alkaline phosphatase (EC 3.1.3.1) from calf intestine was purchased from Boehringer. Pentose phosphate isomerase (phosphoriboisomerase, EC 5.3.1.6) from spinach was purchased from Sigma.

5-Amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**1**). The compound was freshly prepared by hydrogenation of 5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**5**) over palladium on charcoal in aqueous solution (Sedlmaier et al., 1987).

Assay of Riboflavin Synthase Activity. Riboflavin synthase was determined as described earlier (Bacher, 1986).

Synthesis of 3,4-Dihydroxy-2-butanone 4-phosphate (**2**) *(i) (2S)-1,2-O-Isopropylidene-1,2,3-butanetriol* (*S*-**11**). 5,6-*O*-Isopropylidene-L-gulonolactone (**9**) [19 mmol, 4.1 g, prepared as described by Hubschwerlen 1986)] was suspended in a mixture of 250 mL of dichloromethane and 40 mL of absolute pyridine. Dry K_2CO_3 (0.3 mol, 41 g) was added, and the mixture was cooled to 0 °C. Lead tetraacetate (81 mmol, 36 g) was added in small portions under vigorous stirring. After 3 h, the excess of lead tetraacetate was destroyed by the addition of ethylene glycol. The suspension was filtered, and the residue was washed twice with ether. The combined solutions were dried over $MgSO_4$. The solvent was evaporated under reduced pressure. The residue [(2*S*)-2,3-*O*-isopropylideneglyceraldehyde, *S*-**10**] was dissolved in absolute ether. The solution was added to a cooled solution of 60 mmol of methyl magnesium iodide (prepared from 1.5 g of Mg and 4.4 mL of methyl iodide) in 30 mL of ether. After 2 h, the solution was poured on ice, and a saturated solution of ammonium chloride (20 mL) was added. The water phase was extracted 3 times with ether. The ether solutions were combined, washed with a small volume of water, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure. The residue contained small amounts of pyridine which were removed by azeotropic distillation with water under reduced pressure.

^{1}H NMR ($CDCl_3$, 360 MHz): δ (*threo*/*erythro* compounds) 3.57−3.95 (m, 4H), 2.71 (m, 1H), 1.29 (s, 3H), 1.10 (d, J = 6.4 Hz, 3H), 1.07 (d, J = 6.4 Hz, 3H). ^{13}C NMR ($CDCl_3$, 90 MHz): δ (*threo*/*erythro* compounds) 109.4, 109.0, 80.3, 79.4, 68.6, 66.9, 65.9, 64.8, 26.5, 26.3, 25.3, 25.0, 18.6, 18.4.

*(ii) (2R)-1,2-O-Isopropylidene-1,2,3-butanetriol (R-**11**).* The compound was prepared as described above using (2*R*)-2,3-*O*-isopropylideneglyceraldehyde (*R*-**10**) according to Dumont and Pfander (1983).

*(iii) (3R)- and (3S)-3,4-O-Isopropylidene-3,4-dihydroxy-2-butanone (R-**12**, S-**12**).* The compounds were prepared by a modification of the procedure reported by Hagen et al. (1979). (2*R*)- or (2*S*)-1,2-*O*-isopropylidene-1,2,3-butanetriol (*R*-**11**, *S*-**11**) (which must be free of pyridine) was dissolved in 30 mL of CCl_4. Sodium carbonate (25 mmol, 2.3 g) and a catalytic amount (200 mg) of water-soluble ruthenium dioxide were dissolved in 30 mL of water. The solutions were combined, and the mixture was cooled with ice/water. Small portions of sodium metaperiodate were added. The reaction was monitored by HPLC in order to follow the progress of oxidation. 2-Propanol was added to destroy the excess of ruthenium tetroxide. The aqueous layer was washed three times with $CHCl_3$. The combined organic solutions were dried over $MgSO_4$, and the solvent was removed under reduced pressure.

^{1}H NMR ($CDCl_3$, 360 MHz): δ 4.33 (dd, J = 5.2, 7.8 Hz, 1H), 4.11 (dd, J = 7.8, 8.4 Hz, 1H), 3.91 (dd, J = 5.2, 8.4 Hz, 1H), 2.17 (s, 3H), 1.41 (s, 3H), 1.31 (s, 3H). ^{13}C NMR ($CDCl_3$, 90 MHz): 209.0 (C-2), 110.0 (C_{quart}), 80.5 (C-3), 66.4 (C-4), 26.3 (C-1), 26.1 and 25.1 (isopropylidene methyl groups).

*(iv) (3R)- and (3S)-3,4-Dihydroxy-2-butanone (R-**13**, S-**13**).* A solution of 3,4-*O*-isopropylidene-3,4-dihydroxy-2-butanone (**12**) (*R* or *S*) (5.8 mmol, 840 mg) in 3 mL of 12% acetic acid was heated to 75 °C (Fischer et al., 1937). The reaction was monitored by HPLC and was terminated when about 80% of the starting material was deprotected. Toluene (3 × 5 mL) was added. Acetic acid and water were removed by azeotropic destillation under reduced pressure. The colorless oil was purified by chromatography on a column of activated silica gel (2 × 10 cm) which was developed with a mixture of hexane/ethyl acetate (1:4, v/v).

*(v) (3R,S)-3,4-Dihydroxy-2-butanone (RS-**13**).* The racemic compound was prepared by a modification of the procedure reported by Fischer et al. (1937). Freshly distilled methyl vinyl ketone (**15**) (0.57 mol, 40.3 g) was added to a solution of sodium chlorate (0.40 mol, 43 g) in 550 mL of water. The mixture was cooled with ice/water, and 200 mg of osmium tetroxide was added. The solution was kept at 0 °C for 2 h and then at room temperature for 4 days. The solution was filtered, and water was removed by evaporation under reduced pressure. The green residue was extracted with ethanol. The filtrate was evaporated to dryness under reduced pressure. The residue was crystallized from a mixture of hexane/ethyl acetate (1:4, v/v). The colorless crystals which formed in the cold were harvested at 4 °C and were washed with cold hexane. Traces of solvent were removed under reduced pressure at 0 °C. Yield: 45.6 g (0.44 mol, 77%).

^{1}H NMR (D_2O, 360 MHz): δ 4.41 (t, J = 3.8 Hz, 1H), 3.92 (dd, J = 12.0, 23.0 Hz, 1H), 3.91 (dd, J = 12.0, 23.0 Hz, 1H), 2.27 (s, 3H); (DMSO-d_6) δ 5.18 (d, J = 5.4 Hz, 1H), 4.77 (t, J = 5.8 Hz, 1H), 3.96 (q, J = 4.7 Hz, 1H), 3.54 (m, 2H), 2.14 (s, 3H). ^{13}C NMR (DMSO-d_6, 90 MHz): δ 210.5 (C-2), 78.0 (C-3), 63.2 (C-4), 26.3 (C-1).

(vi) Determination of Enantiomeric Excess. 3,4-Dihydroxy-2-butanone (**13**) was converted to the (2*S*)-acetylman-

delic acid ester according to the procedure of Parker (1983). The product was purified by column chromatography (column of activated silica gel 40, 70−230 mesh, ASTM, 2 × 10 cm, hexane/ethyl acetate, 1:4, v/v). ^{1}H NMR spectra were recorded in benzene-d_6. The mandelate signals of the resulting *R,S* and *S,S* diastereomers had chemical shifts of 6.05 and 5.90, respectively.

(vii) 3,4-Dihydroxy-2-butanone 4-Phosphate (**2**). Dibenzylphosphochloridate was prepared by the addition of *N*-chlorosuccinimide (2.6 mmol, 351 mg) to a solution of dibenzyl phosphite (1.4 mmol, 524 μL) in 9 mL of dry toluene (Atherton, 1957). 3,4-Dihydroxy-2-butanone (**13**) (2.4 mmol, 250 mg) and 2,6-lutidine (4 mL) were added, and the mixture was kept at 4 °C overnight (Atherton, 1948). Lutidine hydrochloride was filtered off, and the solvent was removed by evaporation under reduced pressure. The bulk of lutidine was removed by azeotropic distillation with water under reduced pressure. The residue was dissolved in water/methanol (1:1, v/v) and was hydrogenated over palladium on charcoal. Toluene was removed by partial evaporation under reduced pressure. The aqueous solution was neutralized by the addition of saturated barium hydroxide solution. The precipitate of inorganic barium phosphate was removed by filtration. The solution was concentrated, and acetone was added to a final concentration of 90% (v/v). The precipitate was collected, washed with acetone, and dried. The overall yield was 30%. The product contained about 90% 3,4-dihydroxy-2-butanone 4-phosphate and 10% 3,4-dihydroxy-2-butanone 3-phosphate.

3-Phosphate: ^{1}H NMR (D_2O, pH 1, 360 MHz): δ 3.80−4.00 (dq, 2H), 3.70 (m, 1H), 2.30 (s, 3H). ^{31}P NMR (10% D_2O, pH 1, 145 MHz, 10 mM, H-decoupled): δ 3.0 (s).

4-Phosphate: ^{1}H NMR (D_2O, pH 1, 360 MHz): δ 4.51 (m, 1H), 4.27 (m, 1H), 4.14 (m, 1H), 2.28 (s, 3H). ^{31}P NMR (10% D_2O, 100 mM, pH 1, H-decoupled): δ 3.5 (S).

Assay of Lumazine Synthase Activity. Assay mixtures contained 100 mM phosphate buffer, pH 7.0, 2 mM EDTA, 1 mM 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**), 1 mM 3,4-dihydroxy-2-butanone 4-phosphate (*S*-**2** or *RS*-**2**), 2 mM dithiothreitol, and protein. The assay mixtures were incubated at 37 °C, and protein was precipitated by the addition of trichloroacetic acid. 6,7-Dimethyl-8-ribityllumazine (**3**) and riboflavin (**4**) were determined by reversed-phase HPLC. The total amount of product formed by 6,7-dimethyl-8-ribityllumazine synthase was calculated as the molar yield of the lumazine **3** plus two times the molar yield of riboflavin.

Estimation of Protein Concentration. Protein concentration was monitored photometrically using the absorbance coefficients $\epsilon_{1\mathrm{cm},280\mathrm{nm}} = 7.0$ for the lumazine synthase/riboflavin synthase complex (Bacher et al., 1980) and $\epsilon_{1\mathrm{cm},280\mathrm{nm}} = 8.0$ for isolated β subunits (Bacher et al., 1986).

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at room temperature with AM 360, AC 250, and AC 200 spectrometers from Bruker Instruments, Karlsruhe, Federal Republic of Germany. Signals were referenced to TMS.

Deuterium-Exchange Experiments. Samples in D_2O as solvent contained 36 mM (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (*S*-**2**), 90 mM potassium phosphate, pH 7.0, 21.8 mM sodium acetate as internal standard, and protein as indicated. ^{1}H NMR spectra were recorded at intervals at room temperature.

Enzymatic Dephosphorylation of 3,4-Dihydroxy-2-butanone 4-Phosphate (**2**). 3,4-Dihydroxy-2-butanone 4-phosphate (60 mg) was dephosphorylated using 100 μg of alkaline phosphatase in 200 μL of 100 mM Tris hydrochloride, pH 7.0, containing 10 mM $MgCl_2$ and 10 mM $ZnSO_4$. The mixture was incubated for 35 min at 37 °C. Water was evaporated under reduced pressure. Methanol was added, the precipitate was filtered off, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of hexane/ethyl acetate (1:4, v/v; 1 mL) and was purified by column chromatography on silica gel (see above).

Assay for Racemase Activity. Assay mixtures contained 381 μM (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (*S*-**2**), 130 mM potassium phosphate, pH 7.0, 1.1 mM EDTA, and protein as indicated. The samples were incubated at 37 °C. Aliquots of 300 μL were retrieved at intervals. Circular dichroism was measured using a J500A instrument from Jasco.

Regiochemistry of Lumazine Synthase. A mixture containing 50 mM potassium phosphate, pH 7.0, 9.9 mM $MgCl_2$, 250 μM [1-$^{13}C_1$]ribose 5-phosphate, 9.9 mM dithiothreitol, 300 μM 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**), 4.1 mg of pentose phosphate isomerase, 3 mg of heavy riboflavin synthase, 0.6 mg of light riboflavin synthase, and 1.5 mg of (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate synthase in a total volume of 6 mL was incubated for 1 h at 37 °C and was subsequently heated to 95 °C for 5 min. The mixture was centrifuged at 20 000 rpm for 15 min. Riboflavin was isolated by reversed-phase HPLC (column of Nucleosil RP18, 4 × 240 mm; eluent, 25% methanol; flow rate, 2 mL/min). The effluent was monitored fluorometrically (excitation, 445 nm; emission, 516 nm). Fractions were collected and lyophilized.

High-Performance Liquid Chromatography. Preparative HPLC separation of 3,4-dihydroxy-2-butanone 4-phosphate (**2**) was performed with an SB10 anion-exchange column (16 × 250 mm) or a reversed-phase RP18 column (16 × 250 mm). The effluent was monitored photometrically (280 nm). The eluent used for anion-exchange chromatography contained 1 M formic acid. The flow rate was 9 mL/min, and the retention volume was 300−380 mL. Water was used as eluent for reversed-phase chromatography. The flow rate was 7 mL/min, and the retention volume was 230−290 mL.

Analytical HPLC was performed with a reversed-phase column of Nucleosil 10C18 (4 × 250 mm). 3,4-*O*-Isopropylidene-3,4-dihydroxy-2-butanone (**12**) and 3,4-dihydroxy-2-butanone (**13**) were monitored photometrically (280 nm). The eluent contained 15% methanol and 100 mM ammonium formate. The flow rate was 2 mL/min. Retention volumes were 18 and 3.2 mL, respectively. 6,7-Dimethyl-8-ribityllumazine (**3**) and riboflavin (**4**) were monitored fluorometrically. For the analysis of **3**, an eluent containing 7% methanol and 30 mM formic acid was used (excitation, 408 nm; emission, 487 nm). The retention volume was 8.4 mL. For the analysis of **4**, the eluent contained 40% methanol and 100 mM ammonium formate (excitation, 445 nm; emission, 516 nm). The retention volume was 6.0 mL.

RESULTS

Synthesis and Properties of 3,4-Dihydroxy-2-butanone 4-Phosphate (**2**). Small amounts of (3*S*)-3,4-dihydroxy-2-

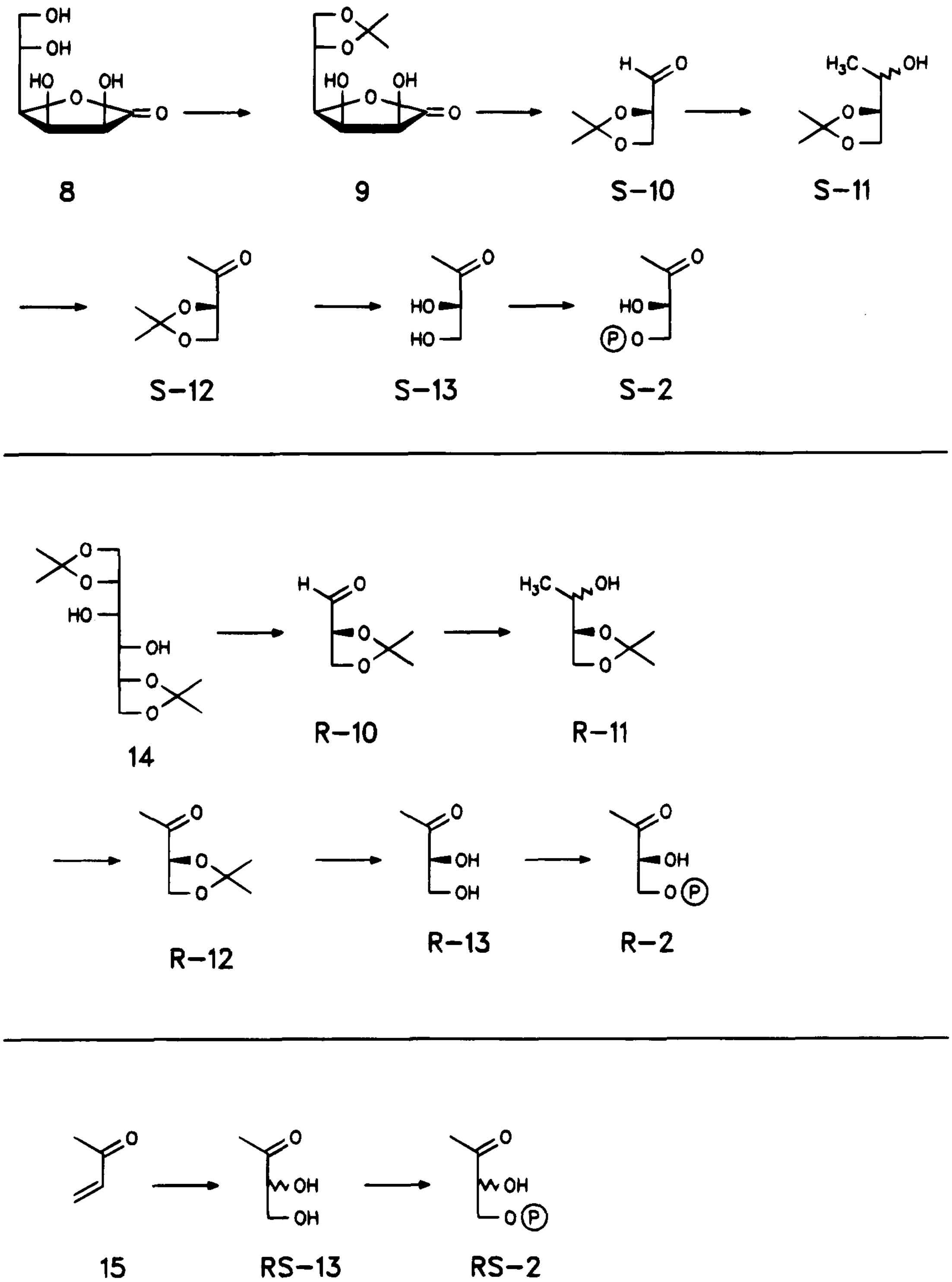

FIGURE 3: Preparation of (*R*)-, (*S*)-, and racemic 3,4-dihydroxy-2-butanone 4-phosphate (*R*-**2**, *S*-**2**, and *RS*-**2**).

butanone 4-phosphate (*S*-**2**) have been obtained enzymatically from ribulose 5-phosphate (Volk & Bacher, 1990). However the chemical synthesis of batch quantities was desirable as a prerequisite for studies on the mechanism of 6,7-dimethyl-8-ribityllumazine synthase. Both enantiomers of the carbohydrate phosphate as well as the racemate were obtained by the following procedure.

The starting materials, enantiomeric and racemic 3,4-dihydroxy-2-butanone (**13**), were obtained by modifications of published proceedures which are described under Experimental Procedures. Briefly, *S*-**13** was obtained from L-gulonolactone (**8**), and *R*-**13** was obtained from diisopropylidene mannitol (**14**) (Figure 3). Both reaction sequences proceed via the isopropylidene derivatives of 1,2,3-butanetriol (**11**). The critical step for optical purity was the removal of the isopropylidene protecting group from 3,4-*O*-isopropylidene-3,4-dihydroxy-2-butanone (**10**). This reaction must be followed by HPLC and terminated after 80−90% of the starting material has been consumed. Under these reaction conditions, both enantiomers are obtained with an enantiomer excess of about 93% as determined by ^{1}H NMR spectroscopy of the respective (*S*)-acetylmandelic acid esters. Longer treatment with acetic acid results in higher yield but reduced optical purity. Racemic 3,4-dihydroxy-2-butanone can be easily prepared in large quantity by hydroxylation of methyl vinyl ketone.

Table 1: ^{13}C NMR Chemical Shifts (ppm) of 3,4-Dihydroxy-2-butanone 4-Phosphate and 3-Phosphate

	4-phosphate		3-phosphate	
	δ (ppm)	J_{CP} (Hz)	δ (ppm)	J_{CP} (Hz)
C-1	28.4		29.1	
C-2	213.8		212.6	4.0
C-3	78.5	8.4	84.0	5.3
C-4	69.4	5.1	64.6	5.1

3,4-Dihydroxy-2-butanone (**13**) (racemate or enantiomer) was reacted with dibenzylphosphochloridate. Removal of the benzyl protecting groups by catalytic hydrogenation over palladium on charcoal afforded **2**, which was obtained as a solid barium salt. The yield based on 3,4-dihydroxy-2-butanone was about 30%. The products contained about 10% of the respective 3-phosphate as shown by NMR spectrometry (Table 1).

The phosphorylation reaction was not accompanied by racemization. A sample of 3,4-dihydroxy-2-butanone obtained by enzymatic dephosphorylation of (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (*S*-**2**) had the same enantiomer excess as the starting material used for phosphorylation. The p*K* values of the 3- and 4-phosphates are 6.3 and 6.4, respectively, as determined by ^{31}P NMR spectrometry (Gassner, 1977).

3,4-Dihydroxy-2-butanone 4-phosphate is a rather unstable compound. Even at −20 °C, the barium salt shows marked decomposition during a period of several weeks. The major product is diacetyl formed by spontaneous phosphate elimination as shown by NMR spectroscopy. In order to avoid decomposition, the barium salt should be stored under liquid nitrogen. Diacetyl can be removed from partially decomposed solutions of **2** by lyophilization.

It should be noted that diacetyl reacts spontaneously with the pyrimidine **1** to form the lumazine **3**. This can lead to large experimental errors in enzyme kinetic studies, and care should be taken to avoid decomposition of **2**.

The methyl protons of **2** exchange spontaneously with the solvent as shown by NMR analysis (Volk, 1989, 1991). In order to monitor the exchange reaction more accurately, solutions of **2** in D_2O buffer were incubated at 20 °C and were monitored by ^{1}H NMR spectroscopy at intervals. The proton-exchange reaction was first order with a rate constant of 0.4 day^{-1} in D_2O containing 90 mM phosphate, pH 7.0 (half-life, 1.7 days). The deuterium exchange at the methyl group was accelerated by the second substrate **1** by a factor of about 20.

The rate constant for the decomposition of **2** to diacetyl (vanishing CH_2 signals) was 0.08 day^{-1} (half-life, 9 days) at 20 °C in 0.1 M phosphate, pH 7. Exchange of the position 3 H-atom with solvent was not observed. In light of the experimental sensitivity, the exchange rate constant must be less than 0.01 day^{-1}, which corresponds to a minimum half-life of about 70 days.

Richard (1991) has found that the phosphate elimination from the structurally similar 3-phosphoglyceraldehyde occurs via the enediolate as an intermediate. Since we could not observe the exchange of the position 3 proton of **2**, it appears that the formation of the position 3 carbanion of **2** irreversibly leads to the abstraction of phosphate and the formation of diacetyl.

Kinetic Studies. Using a partially pure preparation of enzymatically prepared *S*-**2**, Neuberger et al. (1985) obtained the first evidence that the compound can serve as substrate for heavy riboflavin synthase (i.e., the lumazine synthase/riboflavin synthase complex). It was also shown that isolated β subunits had at least some catalytic activity. However, a detailed investigation has not been performed.

For detailed kinetic studies with lumazine synthase, it is important to note the limited stability of **2**. Spontaneous elimination of phosphate yields diacetyl, which can yield **3** by spontaneous non-enzymatic reaction with **1**. It should also be noted that **2** can react spontaneously with **1** when present at high concentration, and blank values without enzyme should be obtained in all experiments.

The native lumazine synthase/riboflavin synthase complex catalyzes two consecutive reactions. The condensation of (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**) with 5-amino-6-ribitylamino-2,4(1*H*,3*H*)pyrimidinedione (**1**) yields 6,7-dimethyl-8-ribityllumazine (**3**). Dismutation of this product yields riboflavin. In order to calculate the total production of **3** by the β subunits, it must be considered that one molecule of riboflavin is formed from two molecules of the lumazine intermediate. The total rate of lumazine formation can therefore be given as $v_{\text{lumazine}} = v(\text{apparent})_{\text{lumazine}} + 2v_{\text{riboflavin}}$.

Kinetic analysis showed a linear Lineweaver−Burk plot for the total velocity of lumazine formation by the $\alpha_3\beta_{60}$ complex (Figure 4). The K_m for (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate had a value of 130 μM, and the K_m for the pyrimidine substrate **1** had a value of 5 μM. The overall specific activity for lumazine formation by the enzyme complex has a value of V_{max} = 12 000 nmol h^{-1} mg^{-1}. The turnover number is 0.06 s^{-1} per β subunit. The enzyme shows a broad pH optimum extending from about 6.5 to 8.0 for the formation of the lumazine **3**.

The lumazine synthase/riboflavin synthase complex can be dissociated under mild conditions, e.g., dialysis against 100 mM Tris hydrochloride, pH 8. Under these conditions, the β subunits form large aggregates with the shape of hollow spheres of diameters around 30 nm. These aggregates are structurally heterogeneous and may represent a variety of somewhat irregular icosahedral species with triangulation number $T = 3$ or $T = 4$. These β subunit aggregates catalyze the formation of the lumazine **3** but not of riboflavin, and the reaction velocity is similar to that of the native $\alpha_3\beta_{60}$ complex (specific activity, 12 000 nmol h^{-1} mg^{-1}; turnover number per β subunit, 0.06 s^{-1}). Kinetic analysis of the β subunit aggregates gave a linear Lineweaver−Burk plot indicating a value of K_m = 108 μM for *S*-**2** (Figure 4).

Recent studies have shown that the expression of the β subunit gene of *B. subtilis* in a recombinant *E. coli* strain yields icosahedral β_{60} capsids devoid of riboflavin synthase activity. Kinetic analysis of the recombinant protein also showed a linear Lineweaver−Burk plot with respect to substrate *S*-**2**. The K_m for *S*-**2** (59 μM) is smaller, and the specific activity (16 700 nmol h^{-1} mg^{-1}) is slightly higher, as compared with the other lumazine synthase species (Figure 4).

Unphosphorylated 3,4-dihydroxy-2-butanone does not serve as a substrate for lumazine synthase. Moreover, we found that 3,4-dihydroxy-2-butanone 3-phosphate and diacetyl are not substrates for lumazine synthase (data not shown).

Substrate Stability in Relation to Kinetic Analysis. As shown above, contamination of **2** with diacetyl interferes with the kinetic analysis of the lumazine synthase reaction. A

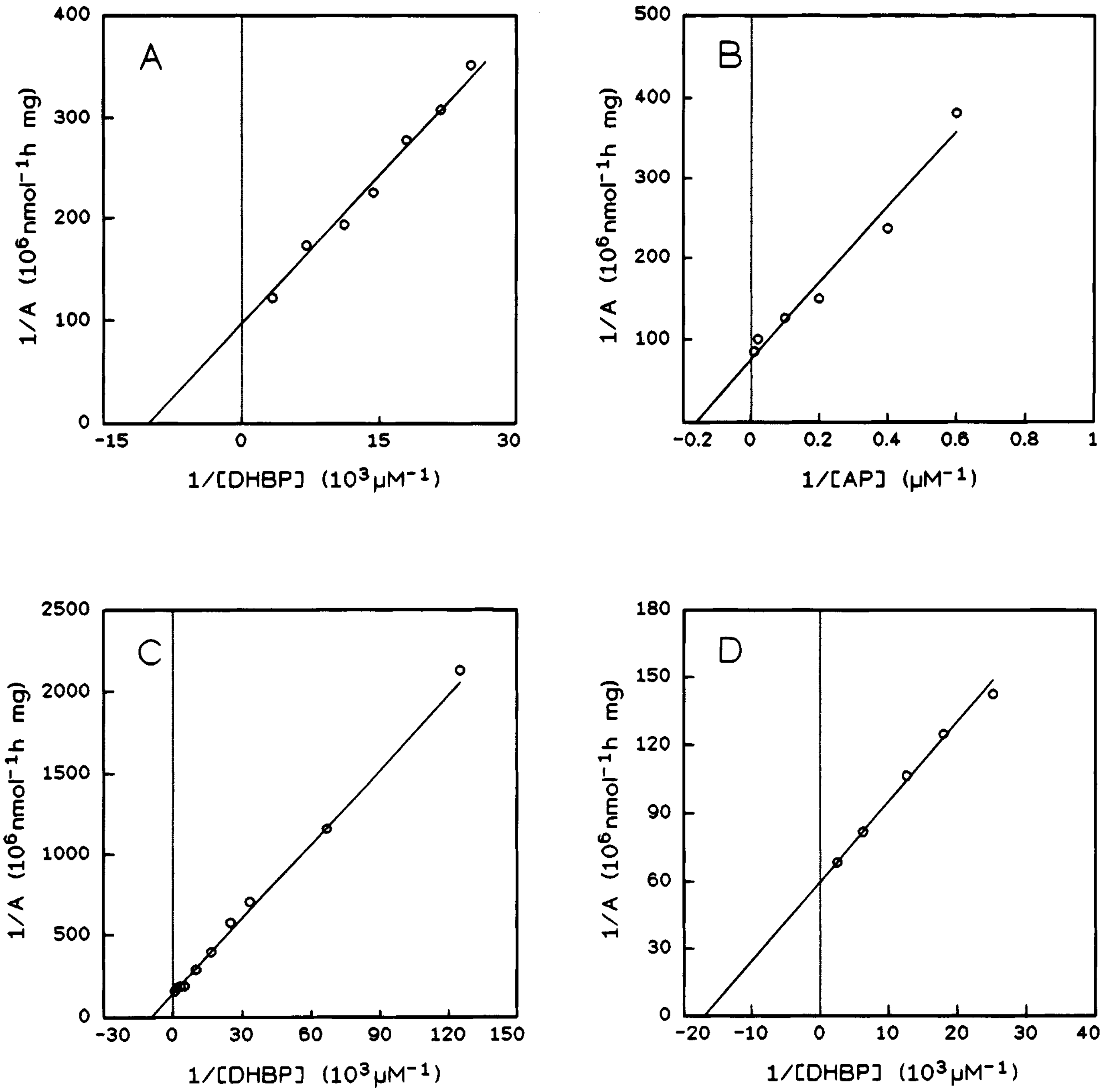

FIGURE 4: Steady-state kinetics of lumazine synthase (Lineweaver−Burk plots). Panels A and B, native $\alpha_3\beta_{60}$ complex; panel C, large β subunit aggregates; panel D, recombinant β_{60} aggregates. 1/*A*, reciprocal specific activity; AP, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**); DHBP, (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**).

second problem arises by the well-known oxidation sensitivity of the pyrimidine substrate **1**, which can decompose by self-condensation to form the pyrimido[5,4-*g*]pteridine **7** (Figure 2) even in the presence of the antioxidant dithiothreitol (Cresswell et al., 1960). Because the structure of **7** mimics the structure of riboflavin, we examined whether it can inhibit the reactions catalyzed by the lumazine synthase/riboflavin synthase complex. Using the lumazin synthase/riboflavin synthase complex and lumazine as substrate, we found that **7** inhibits the riboflavin synthase reaction competitively with a K_i of 9 μM. On the other hand, the reaction catalyzed by the β subunits using **1** and **2** as substrates showed only a very weak inhibition with a K_i of at least 13 mM.

Stereospecificity of Lumazine Synthase. As shown in Figure 5, the non-natural *R* enantiomer of 3,4-dihydroxy-2-butanone 4-phosphate can also serve as a substrate for lumazine synthase. The reaction velocity of the natural substrate, *S*-**2**, exceeds the reaction velocity of the *R*-enantiomer by a factor of 5−6, corresponding to a stereoselectivity of 71% with respect to the *S*-enantiomer. A rigorous kinetic analysis of the *R*-enantiomer was not feasible because the compound was only available with an enantiomer excess of 93%, and the contaminating *S*-enantiomer compromised the determination of initial rates.

Deuterium-Exchange Experiments. The enzyme-catalyzed condensation of the pyrimidine **1** with the carbohydrate **2** involves the loss of the hydrogen at position 3 of **2**. We have shown above that the enzyme accepts both enantiomers of **2** as substrate. We therefore checked (i) whether the enzyme can act as a racemase for the substrate **2** and (ii) whether the enzyme can catalyze the exchange of the position 3 H-atom with solvent water.

Preliminary experiments indicated that the catalytic rate for formation of the lumazine **3** from **1** and **2** in D_2O buffer is decreased by a factor of 1.5 at 37 °C and by a factor of 1.1 at 20 °C by comparison with H_2O buffer as solvent.

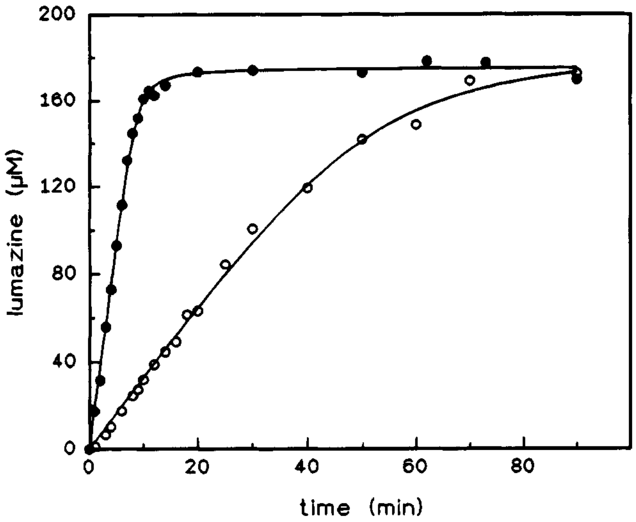

FIGURE 5: Formation of 6,7-dimethyl-8-ribityllumazine by the lumazine synthase/riboflavin synthase complex from *S*-**2** (●) and *R*-**2** (○).

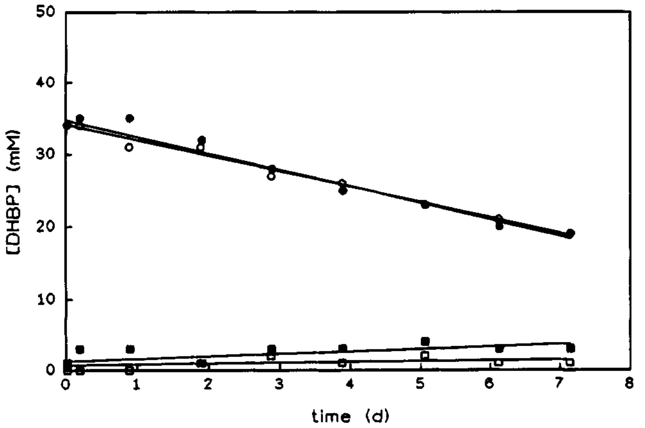

FIGURE 6: Deuterium exchange at position C-3 of *S*-**2**. ○ and ●, deuterium exchange at C-3 plus decomposition to diacetyl; □ and ■, deuterium exchange at C-3 of *S*-**2**; filled symbols, in the presence of 235 μg of lumazine synthase/riboflavin synthase complex; open symbols, without enzyme;. DHBP, (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**). For experimental details, see Experimental Procedures.

The carbohydrate **2** was incubated with the lumazine synthase/riboflavin synthase complex (0.23 mg) in 0.5 mL of 90 mM phosphate in D_2O at pH 7.0, and ^1H NMR spectra were recorded at intervals. The exchange of the methyl protons occurred at the same rate in the presence and absence of the enzyme. No exchange of the proton at C-3 was observed in any of the experiments. Any (undetected) exchange reaction of the position 3 H-atom of **2** would have been slower than the enzyme-catalyzed formation of the lumazine **3** by a factor of at least 300 (Figure 6).

In a similar experiment, a solution of **2** was incubated at 37 °C in 130 mM phosphate buffer, pH 7.0 (with or without enzyme, 0.20 mg), and circular dichroism was determined at intervals. No racemization was detected. If the reaction occurs at all, it would have been slower than the catalytic formation of the lumazine **3** from **2** by at least a factor of 60.

Regiospecificity. Earlier *in vivo* and *in vitro* studies had suggested that carbohydrate precursors are incorporated into the lumazine **3** with a regiospecificity of about 90% (Bacher et al., 1982, 1985; Le Van et al., 1985; Nielsen et al., 1986). It seemed likely that this incomplete regiospecificity could be a consequence of metabolic turnover of the precursor. In order to determine the regiospecificity of lumazine synthase more rigorously, we prepared (3*S*)-3,4-dihydroxy-2-[1-$^{13}C_1$]-butanone 4-phosphate from [1-$^{13}C_1$]ribose 5-phosphate by treatment with pentose phosphate isomerase and (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate synthase as described under Experimental Procedures. The compound was used as substrate for the lumazine synthase/riboflavin synthase complex, and the product, riboflavin, was isolated by HPLC. The ^{13}C NMR spectrum is shown in Figure 7. The spectrum of an authentic sample of [6,7α,8α,9-$^{13}C_4$]riboflavin is shown for comparison. ^{13}C NMR signals were only detected for C-6 and C-8α of riboflavin obtained from [1-$^{13}C_1$]*S*-**2**. Thus, the enzyme reaction is at least 98% regiospecific.

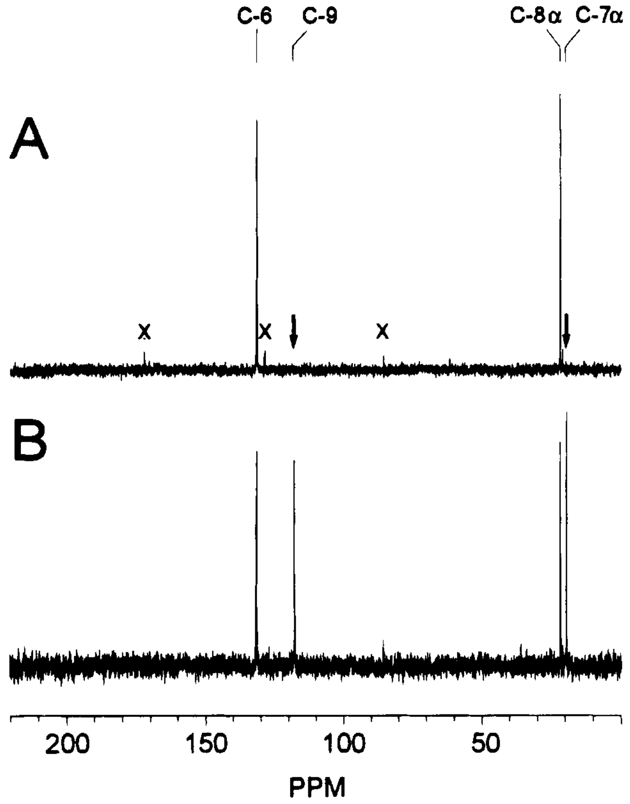

FIGURE 7: Regiospecificity of the lumazine synthase/riboflavin synthase complex. NMR spectra of (A) riboflavin obtained enzymatically from (3*S*)-3,4-dihydroxy-2-[1-$^{13}C_1$]butanone 4-phosphate and (B) authentic [6,7α,8α,9-$^{13}C_4$]riboflavin. The spectra were recorded in D_2O at room temperature. X, impurities.

DISCUSSION

Whereas the structure of lumazine synthase has been studied in considerable detail, the enzyme mechanism eluded analysis because the structure of the carbohydrate substrate, (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (*S*-**2**) was elucidated only relatively recently (Volk & Bacher, 1988, 1991). We have prepared both enantiomers of **2** with an enantiomer excess of about 93% and were surprised to find that both can serve as substrate for lumazine synthase. The reaction velocity of the naturally occurring *S*-compound exceeds that of the *R*-compound only by a factor of 5−6.

The K_m for the pyrimidine substrate **1** (5 μM) is in good agreement with the dissociation constants of the pyrimidine analogs **5** and **6** (Figure 2), which are in the range of 1 μM (Bacher & Ludwig, 1982). The K_m for the second substrate, **2**, exceeds that for substrate **1** by more than an order of magnitude. This may be indicative of an ordered bi-bi

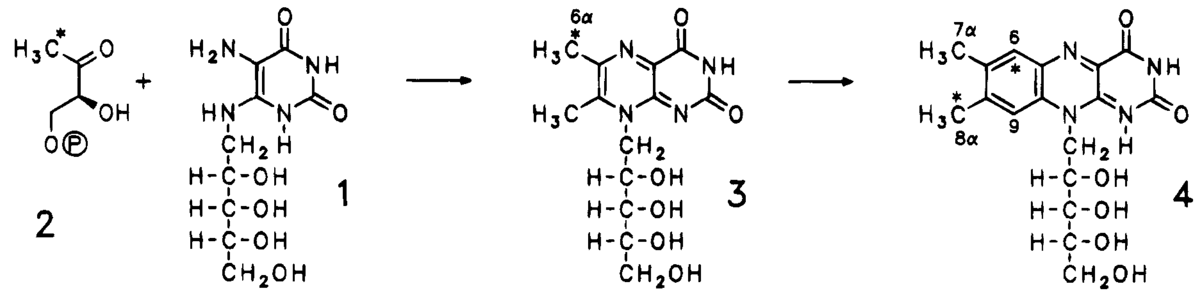

FIGURE 8: Stereospecific formation of 6α-[$^{13}C_1$]methyl-7-methyl-8-ribityllumazine by lumazine synthase and conversion into [6,8α-$^{13}C_2$]-riboflavin by riboflavin synthase. ^{13}C-labelled positions are designated with asterisks.

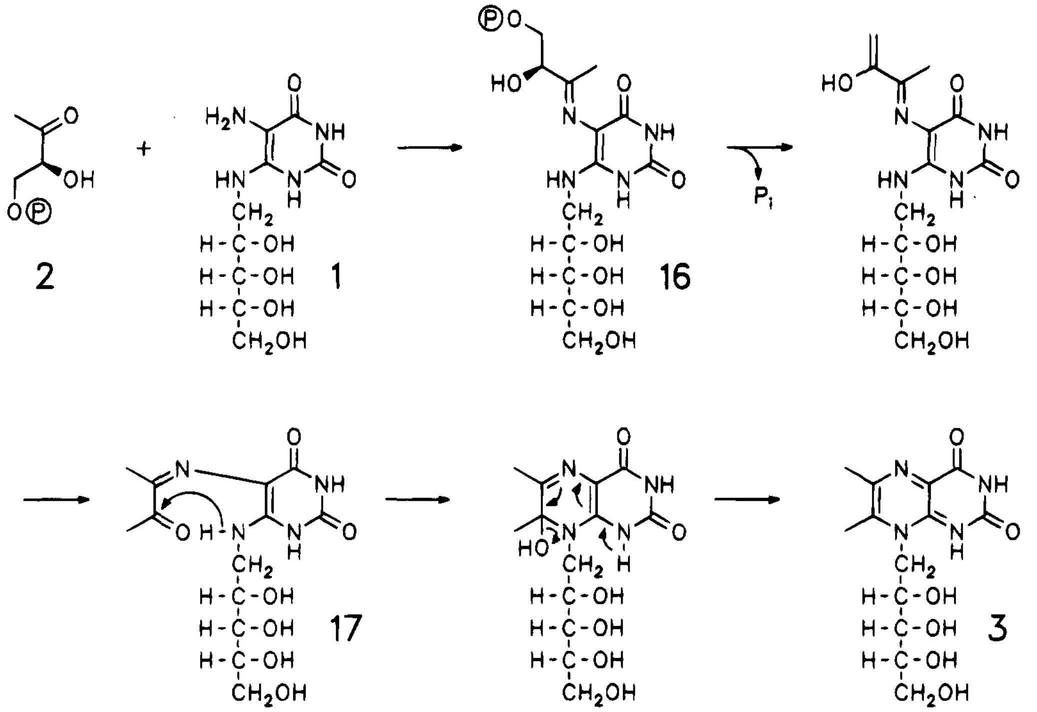

FIGURE 9: Hypothetical mechanisms for the condensation of (3*S*)-3,4-dihydroxy-2-butanone 4-phosphate (**2**) with 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**).

mechanism, but conclusive evidence for this hypothesis has not been obtained.

In the absence of the pyrimidine **1**, the enzyme does not react with the carbohydrate substrate **2**. More specifically, the enzyme complex does not catalyze the exchange of the proton at C-3 of **2** with solvent water, nor does it act as a racemase. The spontaneous proton exchange at the methyl group of **2** is not accelerated by the enzyme. It was also shown that the enzyme does not catalyze the formation of diacetyl from **2**. We conclude from these negative results that the initial step of the enzyme-catalyzed reaction requires the presence of the second substrate, i.e., the pyrimidine **1**, at the active site.

Earlier studies had indicated the lumazine synthase reaction was at least partially regiospecific. Thus, label from [1-$^{13}C_1$]-ribose was predominantly incorporated into positions 6 and 8α of riboflavin by growing cultures of *Ashbya gossypii* (Bacher et al., 1982, 1985; Le Van et al., 1985) with an observed regiospecificity of about 90%. *In vitro* experiments using crude preparations of [2,3,5-$^{13}C_3$]ribose 5-phosphate had also suggested an apparent regiospecificity of about 90% (Nielsen et al., 1986). However, it appeared possible that this was due to the non-enzymatic formation of diacetyl from the labeled carbohydrate phosphate. For this reason, we repeated the experiment with [1-$^{13}C_1$]*S*-**2** prepared *in situ* from [1-$^{13}C_1$]ribose 5-phosphate. A large amount of the lumazine synthase/riboflavin synthase complex was used to keep the incubation time as short as possible. Under these conditions, a ^{13}C NMR experiment showed only the formation of [6,8α-$^{13}C_2$]riboflavin. In light of the experimental sensitivity, we conclude that the regiospecificity of the enzyme reaction is at least 98%. This adds additional weight to the conclusion that the enzyme does not catalyze the formation of diacetyl by elimination of phosphate from the substrate **2** (Figure 8). If the enzyme had appreciable diacetyl synthase activity, a lowering of the regiospecificity would be expected.

In summary, we believe that the enzyme reaction is initiated by formation of the Schiff base **16** due to the reaction of the more reactive 5-amino group of the pyrimidine substrate **1** with the carbonyl group of **2** (Figure 9). This reaction step could be followed by the elimination of inorganic phosphate. The resulting double bond would be favored by conjugation with the pyrimidine system. The 6-amino group of the pyrimidine could then directly attack the newly formed double bond. Alternatively, the intermediate **17** could tautomerize under formation of a carbonyl group, which could then be attacked by the 6-amino group. The release of water from the intermediate could terminate the reaction. This step would be thermodynamically favored by the resulting conjugation in the heterocyclic moiety.

The inability of the enzyme to catalyze the abstraction of the position 3 proton from **2** in the absence of **1** could imply that the release of this proton is facilitated by protonation of the imine nitrogen of the Schiff base **16**. This is supported by the catalytic influence of **1** on the rate of deuterium exchange at the C-1 proton of **2**.

As mentioned above, the enzyme reaction can occur with both enantiomers of the substrate **2**, and the reaction velocities differ only by a factor of about 5. If the elimination of phosphate is preceded by the removal of the proton at C-3 with formation of an anion as proposed above, this would imply that the enzyme has the potential to remove this proton irrespective of the configuration at C-3.

Earlier studies using partially purified **2** seemed to indicate that artifactual β subunit aggregates have at best very low lumazine synthase activities (Neuberger & Bacher, 1985). Contrary to these initial observations, we have now observed that all the molecular species studied here catalyze the formation of **3** with similar velocities and have similar K_m affinities for the substrate **1**. This is not surprising in the case of the hollow β_{60} capsids, which have a molecular structure virtually identical with that of the β subunit capsid in the native $\alpha_3\beta_{60}$ molecules (Ladenstein et al., 1994; Ritsert et al., 1995). However, it is surprising that the large, heterogeneous aggregates are also catalytically active. This may be due, at least in part, to the perturbation of the equilibrium between different aggregates by the substrates. Earlier studies have shown that β_{60} capsids can be formed from the large aggregates in the presence of the substrate analog **6**, although at a low rate. It is conceivable that the simultaneous presence of the substrates **1** and **2** can induce this reaggregation at a much faster rate.

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