

## Presteady State Kinetic Analysis of Riboflavin Synthase\*

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Boris Illarionov‡, Ilka Haase, Adelbert Bacher, Markus Fischer, and Nicholas Schramek§

From the Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München,  
Lichtenbergstrasse 4, D-85747 Garching, Germany

**Riboflavin synthase catalyzes a mechanistically complex dismutation affording riboflavin and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione from 6,7-dimethyl-8-ribityllumazine. The kinetics of the enzyme from *Escherichia coli* were studied under single turnover conditions. Stopped flow as well as quenched flow experiments documented the transient formation of a pentacyclic reaction intermediate. No other transient species were sufficiently populated to allow detection. The data are best described by a sequence of one second order and one first order reaction.**

Riboflavin synthase catalyzes a complex dismutation affording riboflavin (8) and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (9) from 6,7-dimethyl-8-ribityllumazine (1) (1). The enzyme-catalyzed dismutation can be described as an exchange of a 4-carbon unit between one substrate molecule acting as donor and a second substrate molecule acting as acceptor (typically, however, dismutation reactions involve the exchange of simple particles, e.g. hydride anions) (1, 2). The regiochemical features of the reaction require an antiparallel arrangement of the two identical substrate molecules at the active site of the enzyme (3–5).

Riboflavin synthases of *Bacillus subtilis* and *Escherichia coli* are homotrimers of 23.4-kDa subunits (6, 7). Each subunit folds into two closely similar domains (7, 8). The homotrimeric enzymes have six substrate-binding sites (one binding site each on each N-terminal and C-terminal domain, respectively, of each subunit) (9–11). Surprisingly, the crystal structure of riboflavin synthase from *E. coli* shows an inherently asymmetric molecule where only a single N-terminal domain and a single C-terminal domain are appropriately oriented for catalysis of a two-substrate reaction (8).

Recently, we reported the formation of the pentacyclic lumazine dimer 6 from 6,7-dimethyl-8-ribityllumazine by the S41A mutant of *E. coli* riboflavin synthase (12). The native enzyme can generate 6,7-dimethyl-8-ribityllumazine (backward reaction) as well as a mixture of riboflavin and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (forward reaction) from that adduct, which fulfills the criteria for a kinetically competent reaction intermediate. The hypothetical reaction mechanism shown in Fig. 1 combines the earlier mechanistic sugges-

tions of Rowan and Wood (13) and Beach and Plaut (14) with the more recent findings.

This paper describes presteady state kinetic studies on this mechanistically complex enzyme-catalyzed reaction.

### EXPERIMENTAL PROCEDURES

**Materials**—6,7-Dimethyl-8-ribityllumazine and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione were synthesized by published procedures (15). A recombinant strain of *E. coli* engineered for overexpression of the *ribC* gene specifying riboflavin synthase of *E. coli* has been described elsewhere (16).

**Protein Purification**—All procedures were performed at 4 °C unless otherwise stated. Frozen cell mass (5 g) was thawed in 25 ml of 50 mM Tris hydrochloride, pH 7.2, containing 0.5 mM EDTA and 0.5 mM dithiothreitol (buffer A). The suspension was subjected to ultrasonic treatment and was then centrifuged. The supernatant was dialyzed against 10 volumes of buffer A and centrifuged. The solution was placed on top of a column of Q-Sepharose Fast Flow (2 × 18 cm) that had been pre-equilibrated with buffer A. The column was washed with 100 ml of buffer A and was then developed with a linear gradient of 0–0.5 M NaCl in buffer A (total volume, 280 ml; flow rate, 1 ml min<sup>-1</sup>). Riboflavin synthase was eluted from 200 to 240 ml. Fractions were combined, concentrated, dialyzed against 50 mM potassium phosphate, pH 7.0, and centrifuged. The supernatant was passed through a Superdex 200 HiLoad 26/60 column (2.6 × 60 cm) that was developed with 50 mM potassium phosphate, pH 7. Riboflavin synthase was eluted from 210 to 225 ml. Fractions were combined, concentrated by ultrafiltration, and stored at –80 °C. The protein concentration was determined photometrically ( $\epsilon_{280} = 47,700 \text{ M}^{-1} \text{ cm}^{-1}$ ).

**Stopped Flow Measurements**—Experiments were performed with an SFM4/QS apparatus from Bio-Logic (Claix, France) equipped with a linear array of three mixers and four independent syringes. The content of a 1.5-mm light path quartz cuvette behind the last mixer was monitored by a Tidas diode array spectrophotometer (200–610 nm) equipped with a 15-watt deuterium lamp as light source (J&M Analytische Mess- und Regeltechnik, Aalen, Germany). The reaction buffer contained 150 mM potassium phosphate, pH 6.9, and 2 mM dithiothreitol. The enzyme solution was mixed with substrate solution at a ratio of 1:1 at a temperature of 25 °C and a total flow rate of 4 ml s<sup>-1</sup>. The calculated dead time was 7.6 ms. Spectra integrated over 96 ms were recorded at intervals of 100 ms.

**Quenched Flow Experiments**—The mixing apparatus described above was equipped with a computer-controlled valve instead of the optical cuvette. A delay loop of 230- $\mu$ l nominal volume was filled with reactants at a temperature of 25 °C and a total flow rate of 4 ml s<sup>-1</sup>. At the times indicated, the reaction mixtures in the delay loop were mixed at a 1:1 ratio with 0.3 M hydrochloric acid at a flow rate of 4 ml s<sup>-1</sup>. Trichloroacetic acid was added to a final concentration of 50 mM, and the samples were centrifuged (15,000 × g, 10 min). The supernatant was stored at –80 °C for further analysis.

**High Performance Liquid Chromatography**—Experiments were performed with a column of Hypersil RP 18 (4 × 250 mm, Schambeck, Bad Honnef, Germany) at a flow rate of 1.5 ml min<sup>-1</sup>. An eluent containing methanol/formic acid/water (25:1:288, v/v/v) was used for determination of 6,7-dimethyl-8-ribityllumazine (retention volume, 6.5 ml) and compound 6 (retention volume, 7.4 ml). An eluent containing 0.1 M ammonium formate and 40% methanol (v/v) was used for determination of riboflavin (retention volume, 2.2 ml). The effluent was monitored by a diode array photometer.

**Global Analysis of Data from Stopped Flow Experiments**—Prior to data analysis, the absorbance background caused by the enzyme was

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‡ On leave of absence from the Institute for Biophysics, Krasnoyarsk, Russia.

§ To whom correspondence should be addressed: Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany. Tel.: 49-89-289-13361; Fax: 49-89-289-13363; E-mail: nicholas.schramek@ch.tum.de.

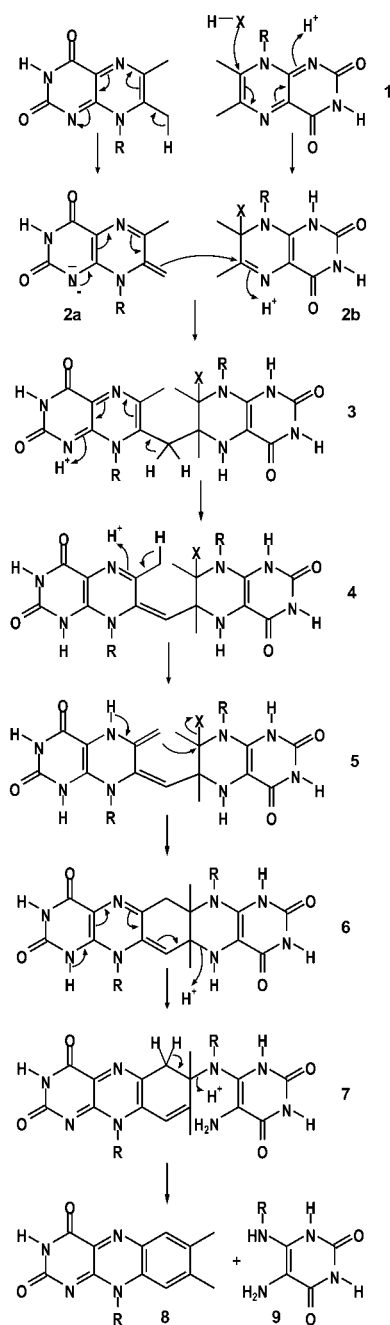


FIG. 1. Hypothetical reaction mechanism of riboflavin synthase (12, 27–29). R, ribityl; X, unknown nucleophile.

removed by subtraction of a blank data set obtained without addition of substrate. Data reduction and stronger weighting of early spectra were achieved by extracting 300 spectra on a pseudo-logarithmic time base from the difference data sets. These data sets were then analyzed using the program SPECFIT/32 3.0.30 (Spectrum Software Associates, Marlborough, MA).

**Analysis of Data from Quenched Flow Experiments**—The data sets were analyzed using the program DynaFit (17).

## RESULTS

For presteady state kinetics studies, substrate and protein were mixed at 25 °C. After rapid mixing, the solution typically contained 56  $\mu\text{M}$  enzyme subunits and 92  $\mu\text{M}$  6,7-dimethyl-8-ribityllumazine (*i.e.* slightly less than one substrate molecule per binding site). Subsequent to mixing, the samples were monitored at 25 °C by multiwavelength photometry. Alternatively, they were incubated for a predetermined period at

25 °C, and the reaction was then terminated by rapid mixing with 0.3 M hydrochloric acid.

Optical spectra from stopped flow experiments were corrected for the absorbance contribution of the protein by subtraction of a blank data set acquired without addition of substrate. A series of absorption spectra from a stopped flow experiment extending over a period of 100 s is shown in Fig. 2; the complex landscape reveals major absorption changes over the entire spectral range from 240 to 500 nm.

For easier inspection, selected spectra from Fig. 2 are shown in Fig. 3. The first spectrum was acquired by data accumulation during the initial 100 ms after mixing. Although the spectrum is qualitatively similar to that of 6,7-dimethyl-8-ribityllumazine in aqueous solution at pH 7 (Fig. 4A), there are some significant differences, which will be discussed below.

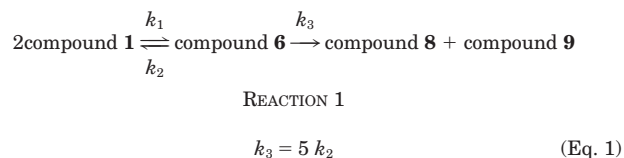
The absorption at 272 nm reaches a shallow minimum at 1.2 s. Notably, that decrease is the fastest process observed (Fig. 3A and Fig. 5). The spectrum acquired 2.2 s after mixing (Fig. 3A) indicates a decrease of absorption at 408 and 256 nm and an increase of absorption in the range of 300–320 and in the range above 445 nm.

In the later phase of the experiment, the long wavelength region of the spectra (Fig. 4B) is similar to the characteristic spectrum of riboflavin. The absorption at 480, which is almost exclusively due to enzymatically formed riboflavin (see below for details), increases monotonously with a markedly sigmoidal initial phase (Fig. 5).

Numerical deconvolution of the spectra in Fig. 2 afforded three components (Fig. 6A). The reconstructed spectrum A is similar to but not identical with the spectrum of free 6,7-dimethyl-8-ribityllumazine (Fig. 4A) and will be discussed in more detail below. Transient B is similar to the spectrum of compound 6 (Fig. 4A). Transient C (Fig. 6A) is similar to a mixture of riboflavin and the compound 9 (Fig. 4B). The subtle differences between the reconstructed transient spectra and the spectra of compounds 1, 6, 8, and 9 in aqueous solutions without enzyme are relevant and will be addressed under the “Discussion.”

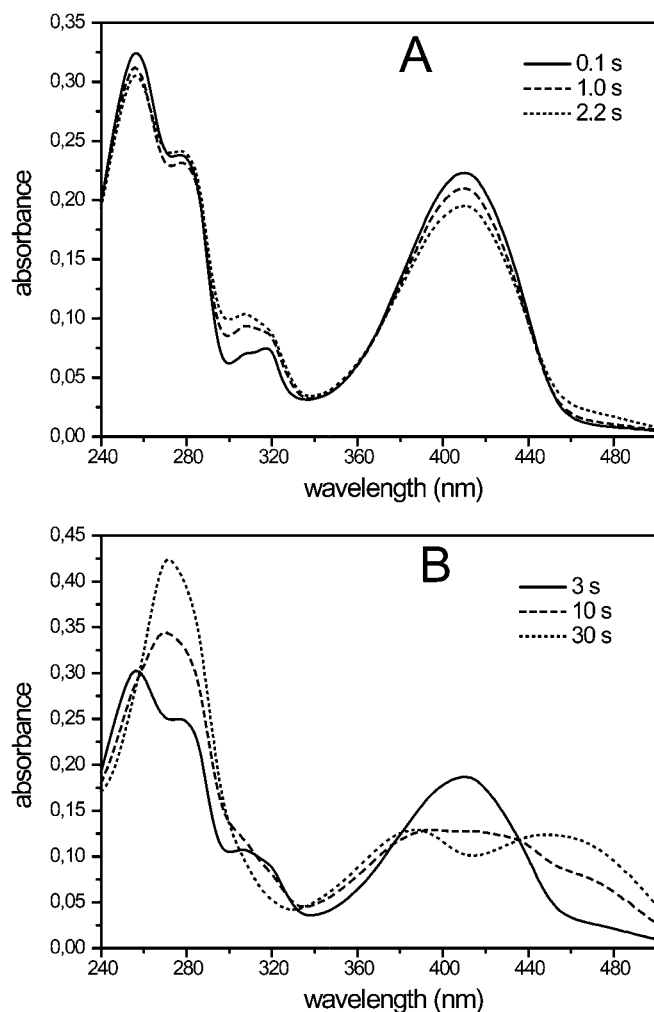
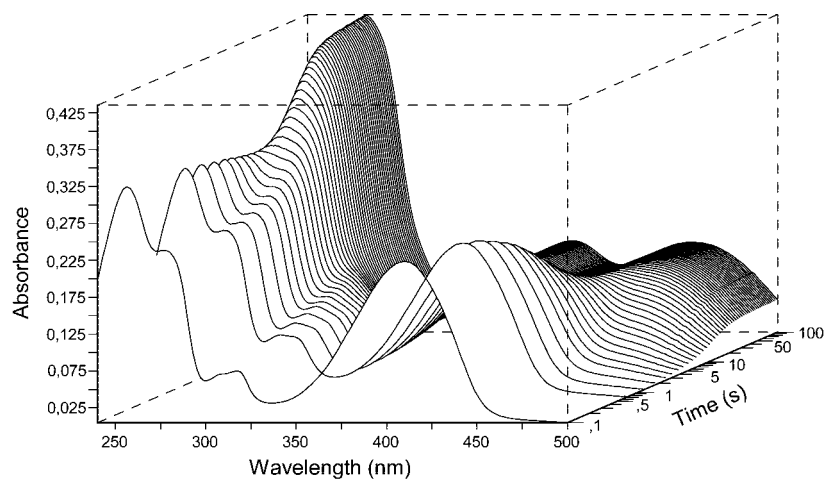
The concentrations of the transient species as a function of time were extracted from the data in Fig. 2 by linear deconvolution (Fig. 5). The concentration of the transient species A (enzyme-bound substrate, *cf.* “Discussion”) shows a monotonous decrease. The concentration of the transient species B (*i.e.* the pentacyclic intermediate, compound 6) goes through a maximum at about 3 s. The transient species C (*i.e.* the sum of riboflavin and 9 formed in a 1:1 ratio) shows a monotonous increase with a sigmoidal initial phase. For the interpretation of the kinetic data, it is important to note that the formation of compound 6 from two molecules of lumazine (1) is a reversible reaction. Our earlier studies have shown that the partitioning factor for the transformation of compound 6 into compound 1 (reverse reaction) and to riboflavin (8) (forward reaction) by riboflavin synthase of *E. coli* has a value of about 5 (12). That ratio was included in the numerical analysis described below.

The data in Fig. 2 were numerically fitted to Reaction 1 and Equation 1.



The forward reaction affording 6 from 1 is best described as a second order reaction, whereas the subsequent decay of 6 affording two molecules of 1 or one molecule each of riboflavin (8) and compound 9 is best described as a first order reaction.

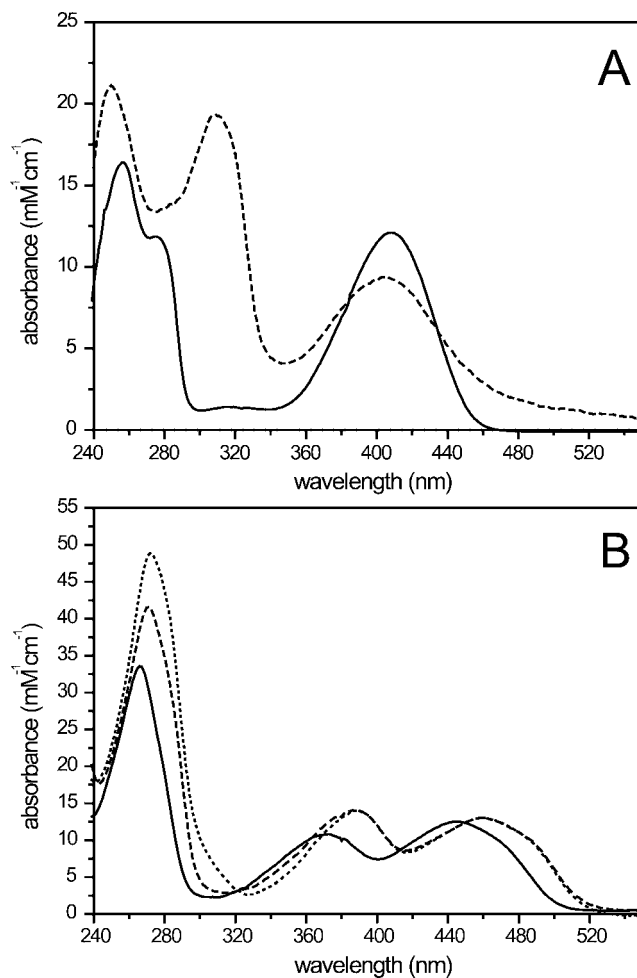
**FIG. 2. Optical spectra from a single turnover stopped flow experiment.** A solution of riboflavin synthase in 150 mM potassium phosphate, pH 6.9, containing 2 mM dithiothreitol, was rapidly mixed with a solution of 6,7-dimethyl-8-ribityllumazine in the same buffer at 25 °C. The concentrations after mixing were 56  $\mu\text{M}$  enzyme subunits and 92  $\mu\text{M}$  6,7-dimethyl-8-ribityllumazine. Absorption spectra were recorded at intervals of 100 ms.



**FIG. 3. Selected optical spectra from the single turnover experiment shown in Fig. 2.** A, early reaction phase; B, later reaction phase. For details see legend to Fig. 2.

The best fit was obtained with the rate constants shown in Table I. The calculated absorption of the samples at different wavelength as a function of time affords the lines shown in Fig. 5. The experimental data (*symbols* in Fig. 5) agree well with the results of the numerical simulation.

In order to confirm unequivocally the identity of all transient species, we performed quenched flow experiments under experimental conditions closely similar to the stopped flow experiments. After rapid mixing of enzyme solution and substrate



**FIG. 4. Optical spectra of compounds supposed to occur in the reaction trajectory of riboflavin synthase.** A, 6,7-dimethyl-8-ribityllumazine in aqueous solution (*solid line*); compound 6 in aqueous solution (*dashed line*). B, riboflavin in aqueous solution (*solid line*); enzyme-bound riboflavin (*dashed line*); enzyme-bound mixture of riboflavin and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (*dotted line*).

solution and a subsequent incubation period, the reaction was terminated by rapid mixing with hydrochloric acid. The samples were then analyzed by high pressure liquid chromatography, which indicated three components with significant absorbance in the spectral range above 320 nm. Based on the retention volumes and the optical spectra, these components could be easily assigned as riboflavin (8), 6,7-dimethyl-8-ri-

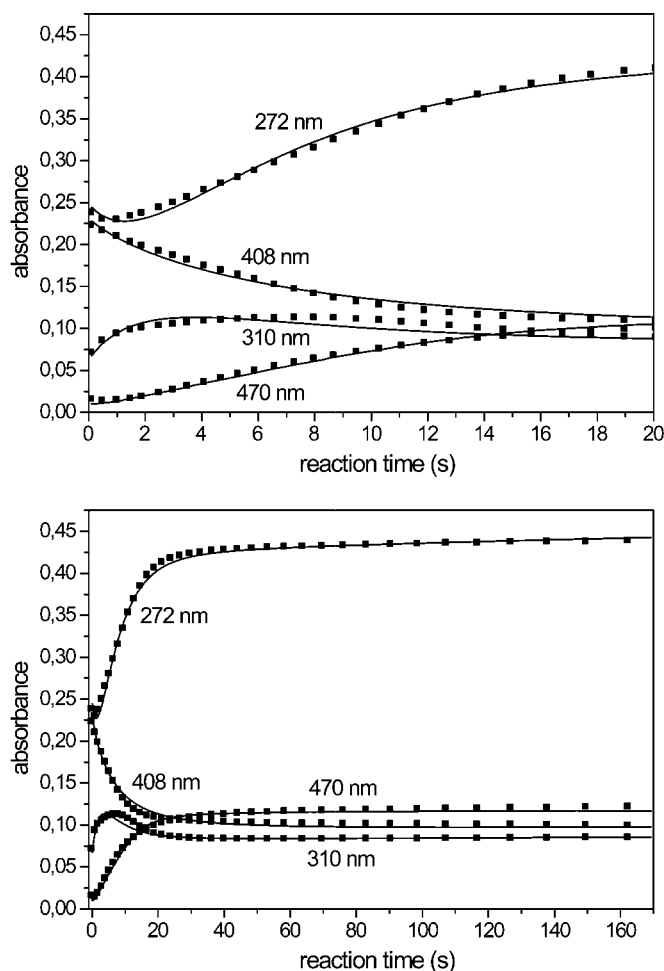


FIG. 5. Absorbance changes observed during a single turnover stopped flow experiment. Symbols represent experimental data from Fig. 2. Solid lines represents the numerical simulation using the kinetic constants in Table I.

tyllumazine (1), and the pentacyclic intermediate (6).

The second enzyme product, compound 9, migrates with the solvent front on the reversed phase high pressure liquid chromatography column and is relatively unstable. However, that compound did not require individual monitoring because riboflavin and compound 9 are formed in a 1:1 molar ratio. When the experimentally determined concentrations of compounds 1, 6, and 8 were added up under consideration of the reaction stoichiometry (2 equivalents of compound 1 are equivalent to compound 6 and compound 8, respectively), the sum was found to be constant within the experimental limits throughout the time course of the quenched flow experiment (Fig. 7). This suggests that no significantly populated transient species had gone undetected. Stopped flow as well as quenched flow experiments indicate that the pentacyclic intermediate (compound 6) is the only transient species that is significantly populated.

In Fig. 6B, the concentration of compound 6 observed in a typical quenched flow experiment is shown together with concentrations calculated from optical stopped flow data. The results from the two methods are in reasonable agreement.

The quenched flow data were again fitted to Reaction 1 and Equation 1. The curves in Fig. 7 give a fit obtained with the parameters in Table I. The kinetic constants derived from stopped flow and quenched flow experiments differ by factors of ~2.

The approximate concentration of riboflavin formed in stopped flow experiments can be gleaned easily from the ab-

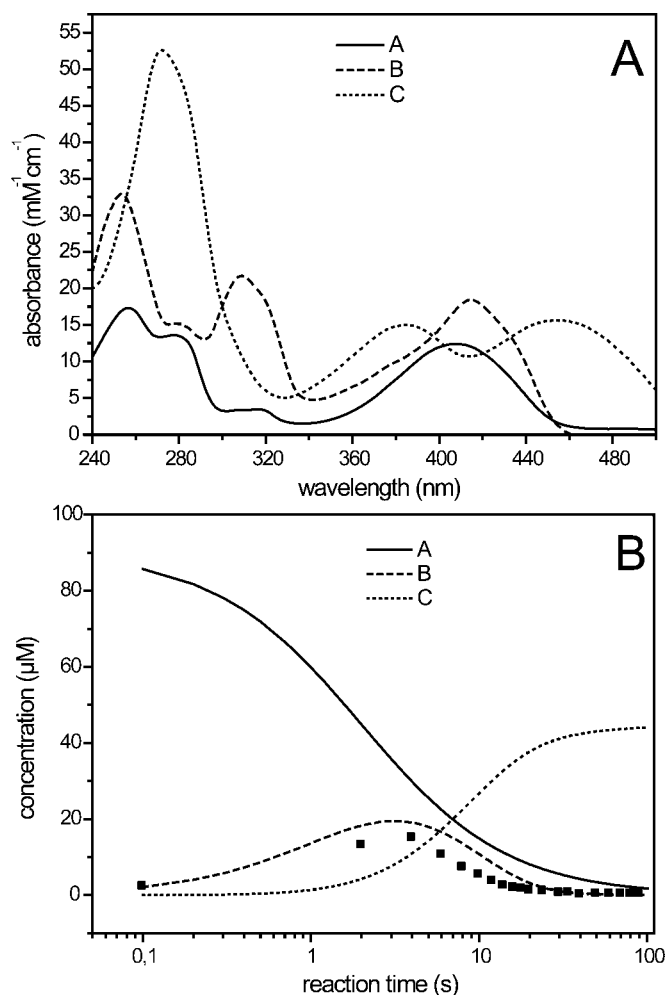


FIG. 6. Numerical simulation of stopped and quenched flow data. A, reconstructed absorbance spectra of transient chromophores. Trace A, lumazine; trace B, compound 6; trace C, mixed spectrum of riboflavin and compound 9. B, concentrations of transient species afforded by regression analysis (■, compound 6 from quenched flow data).

TABLE I  
Kinetic constants obtained from single turnover stopped flow and quenched flow experiments

	Rate constants, quenched flow	Rate constants, stopped flow
$k_1, \mu\text{M}^{-1} \text{s}^{-1}$	$0.0048 \pm 0.0008$	$0.0023 \pm 0.0003$
$k_2, \text{s}^{-1}$	$0.060 \pm 0.007$	$0.036 \pm 0.002$
$k_3, \text{s}^{-1}$	$0.29 \pm 0.03$	$0.18 \pm 0.01$

sorption at 480 nm. More specifically, riboflavin has an absorbance coefficient of  $7140 \text{ M}^{-1} \text{ cm}^{-1}$ , and compound 6 in the enzyme-bound form has an absorbance coefficient of about  $1100 \text{ M}^{-1} \text{ cm}^{-1}$  at that wavelength. Moreover, we know from the analysis described above that the fraction of 6 is always low and negligible in the terminal reaction phase.

In order to determine how riboflavin synthase responds kinetically when the amount of substrate is insufficient to load all six binding sites on the protein trimer in single turnover experiments, we performed a series of stopped flow experiments with different enzyme/substrate ratios (0.5–2 substrate molecules per enzyme subunit). The formation of riboflavin was monitored photometrically at 480 nm. In Fig. 8, these data have been normalized to the initial substrate concentration. The normalized absorption curves are closely similar at the higher substrate concentrations used but differ at the lower substrate concentrations used in the experiment.

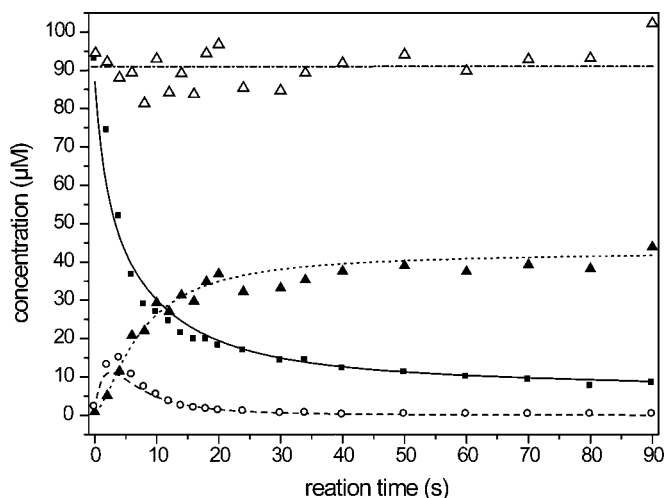


FIG. 7. Numerical simulation of quenched flow data. Symbols represent experimental data. ■, compound 1; ○, compound 6; ▲, riboflavin (8); △, molar sum. Lines represent the numerical simulation using the kinetic constants in Table I.

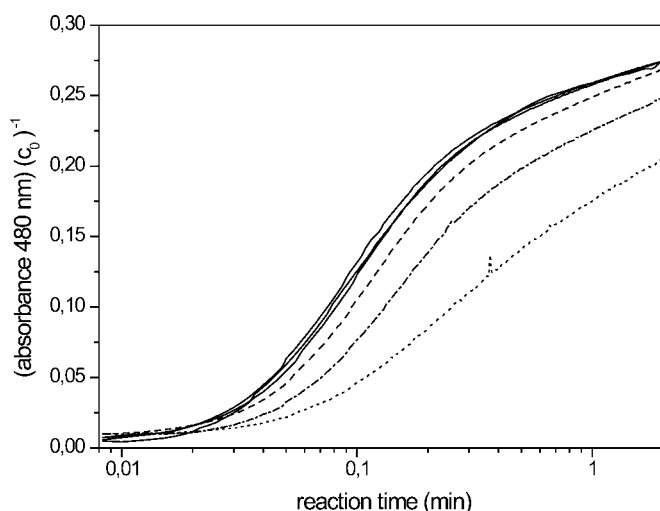


FIG. 8. Stopped flow experiments performed with different enzyme to substrate ratios, normalized to the initial substrate concentration. The concentration of enzyme subunits was constant for all experiments (56  $\mu\text{M}$ ). The concentrations of 6,7-dimethyl-8-ribityllumazine were (from top to bottom) 92, 77, 61, 46, 31, and 15  $\mu\text{M}$ .

#### DISCUSSION

Rowan and Wood (18) found that riboflavin is formed upon boiling of 6,7-dimethyl-8-ribityllumazine in neutral phosphate buffer without a catalyst. Later, Rowan and Wood (13) and Plaut and Beach (14) extended these observations and showed that the uncatalyzed reaction can proceed in neutral as well as in acidic aqueous solution. The uncatalyzed and the enzyme-catalyzed reactions were shown to proceed with the same regioselectivity (5, 19).

The enzyme does not require cofactors or metal ions for activity, and systematic mutagenesis failed to identify amino acid residues that could be assigned a specific role in enzyme catalysis (16). Although certain modifications of the N-terminal amino acid sequence are conducive to the loss of catalytic activity, the respective amino acids are not in direct contact with the substrate and must exert their influence by indirect effects on the active site topology. The replacement of serine 41 in the *E. coli* enzyme or cysteine 48 in the enzyme from *Schizosaccharomyces pombe* is conducive to the loss or substantial reduction of catalytic activity (11, 16), but neither amino acid residue appears likely to be involved directly in catalysis on the

basis of x-ray crystallographic analysis. Hence, the enzyme may primarily exert its catalytic activity by establishing a favorable topologic relationship of the two-substrate molecules.

The riboflavin synthase substrate 1 has rather unusual properties. Deprotonation ( $\text{p}K_a$  8.3) affords a complex mixture of monoanions (Fig. 9) (20). A minor anionic species characterized by a position 7 exomethylene group (compound 10) results from deprotonation of the unusually acidic position 7 methyl group, but the equilibrium mixture is dominated by the tricyclic species 11 and 12, which arise by the addition of the 2'- or 3'-hydroxy groups of the ribityl side chain to the ring carbon atom 7. Rowan and Wood (13, 18) and Beach and Plaut (14) suggested that the initial reaction step in the uncatalyzed as well as the enzyme-catalyzed reaction could involve the electrophilic attack of an exomethylene anion by a second substrate molecule (Fig. 1). Later, Beach and Plaut (3) suggested the involvement of tricyclic anion species but that hypothesis appears unlikely in light of recent protein structure data (11, 21), which show the substrate to be bound with the ribityl side chain of 6-carboxyethyl-7-oxo-8-ribityllumazine, respectively, riboflavin in extended conformations at the binding sites of the N-terminal as well as the C-terminal domain of the enzyme.

The hypothetical species 2b suggested to be attacked by 2a has not yet been shown to be present in the mixture of 6,7-dimethyl-8-ribityllumazine anions, and its abundance must be assumed to be less than 1%, but the hexafluoro analog, 6,7-bis(trifluoromethyl)-8-ribityllumazine (compound 13, Fig. 9), has been characterized in detail and is known to exist in two stable diastereomeric forms (22, 23).

With this information in hand, we can now address the interpretation of the transient optical spectra in Fig. 6A in closer detail. The major absorption maxima in the reconstructed spectrum of transient A are similar to those of the neutral form of 6,7-dimethyl-8-ribityllumazine (1); the observed wavelength shifts (Table II) may arise by modulation of the lumazine chromophore by the protein environment. The minor absorption maxima of transient A in Fig. 6A at 308 and 318 nm are believed to represent a species of 6,7-dimethyl-8-ribityllumazine bound to the enzyme, possibly compound 2b. A comparison of transient A with the spectrum of compound 1 in aqueous solution at pH 6.9 (Fig. 4A) suggests that the  $\text{p}K_a$  of the substrate is decreased to some extent upon binding to the enzyme.

The first spectrum in Fig. 3, which was accumulated during a period of about 100 ms after termination of the mixing process, is virtually identical with the reconstructed transient A spectrum in Fig. 6B. This suggests that binding of the substrate to the enzyme is too rapid to be resolved under our experimental conditions. Hence,  $k_{\text{on}}$  cannot be determined from the present data.

The spectrum of transient B (Fig. 6A) closely resembles that of compound 6 in aqueous solution (Fig. 4A). The concentration of that transient species passes through a maximum at about 4 s after mixing, as shown in Fig. 6B. The data from quenched flow experiments agree with the data from stopped flow within the experimental limits. Hence, transient B in Fig. 6B can be assigned as enzyme-bound compound 6.

The reconstructed spectrum C in Fig. 6A is similar to the spectrum of the enzyme-bound stoichiometric mixture of riboflavin and compound 9 (Fig. 4B), but the long wavelength maxima are shifted from 445 to 453 nm and from 370 to 385 nm. This is similar to bathochromic shifts observed upon binding of flavins to a variety of flavoproteins (24).

The failure to detect other reaction intermediates in the presteady state experiments could be due to the following reasons. (i) The reaction mechanism may involve fewer interme-

FIG. 9. Anionic species of 6,7-dimethyl-8-ribityllumazine (1) and 6,7-bis(trifluoromethyl)-8-ribityllumazine (13) (3, 20).

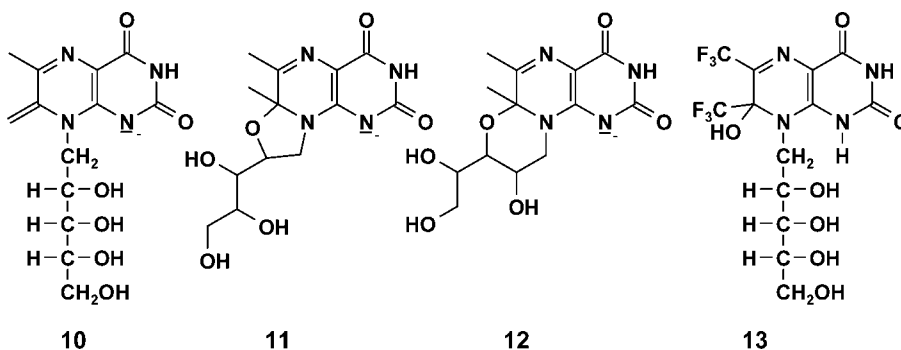


TABLE II  
Optical absorption maxima of compounds occurring  
in the reaction trajectory of riboflavin synthase (150 mM  
potassium phosphate, pH 6.9)

The data for transient spectra from stopped flow experiments are shown for comparison.

	$\lambda_1$	$\lambda_2$	$\lambda_3$	$\lambda_4$
	<i>nm</i>			
Compound 1	256	276		408
Transient A	257	279	318	409
Compound 6	256	279	306	415
Transient B	254	280	308	415
Compound 8	266	370	445	
Compound 8, enzyme-bound	272	385	453	
Compound 9	284			
Compound 9, enzyme-bound	278			
Transient C	272	385	454	

diates than suggested in Fig. 1. (ii) With the exception of compound 6, the transient concentrations of all intermediates may be too low for optical detection. (iii) The spectra of certain intermediates may be too closely similar to those of compounds 1, 2, or 3 to permit detection by numerical deconvolution.

The hypothetical exomethylene anion 2a in Fig. 1 should absorb around 360 nm, based on comparison with spectroscopic data of 6,7,8-trimethylumazine (25). That species, if sufficiently populated, would probably not have escaped detection. Similarly, the hypothetical intermediate 4 in Fig. 1 would be expected to absorb around 360 nm and should have been detected if the transient concentration had been sufficiently large. The hypothetical intermediate 7 is structurally similar to a transient species of the reaction catalyzed by 6,7-dimethyl-8-ribityllumazine synthase (26), which shows strong absorbance at 455 nm but not around 370 nm. Again, such a transient spectrum would probably have been picked up by the deconvolution program if the concentration had been sufficient enough for detection.

In attempts to simulate the experimental data using a variety of reaction schemes, Reaction 1 in conjunction with Equation 1 gave the best fit for the stopped flow as well as for the quenched flow data. The theoretical curves in Fig. 5 generated by numerical simulation using the rate constants from Table I are in good agreement with the observed optical absorption (symbols in Fig. 5).

The mechanism shown in Reaction 1 can also explain the rapid decrease of the riboflavin formation rate in the late phase of the experiment (Fig. 7). As shown in Fig. 8, the normalized absorption curves are identical when the molar ratio of substrate to substrate-binding sites was in the range of 6:6 to 4:6. This suggests pseudo-first order kinetics under these conditions. At lower ratios of substrate to binding sites, the normalized absorption curves become dependent on the substrate concentration, suggesting a higher order reaction. Closer examination of the reaction parameters (Table I) has revealed that in the early phase of the experiment, the normalized

velocity of compound 6 formation from 1<sup>1</sup> is around 0.5 s<sup>-1</sup> (quenched flow data) and is higher than the normalized velocity of riboflavin formation from 6,<sup>2</sup> 0.29 s<sup>-1</sup>. In line with this, the rapid accumulation of compound 6 is observed during the first few seconds after the reaction starts (Figs. 6 and 7). After an initial reaction period of about 4 s when approximately half of the substrate has been consumed, the formation of compound 6 becomes slower than its cleavage, indicating that the first step becomes a rate-limiting step. In the next 10 s compound 6 cannot be observed in the quenched flow experiment anymore.

In summary, the stopped flow and the quenched flow data are well in line with the hypothesis that only the pentacyclic intermediate (compound 6) is significantly populated in the trajectory of the reaction catalyzed by riboflavin synthase. As a cautionary note, it should be added that the signal from compound 6 detected by high pressure liquid chromatography analysis could in reality consist of a mixture of compound 6 plus one or several minor transient species, on the assumption that the latter were converted to compound 6 by means of the acid quench. Although that cannot be ruled out formally, it should be noted that the compound 6 fulfills the criteria of a kinetically competent reaction intermediate as shown earlier (12).

X-ray diffraction of the homotrimeric *E. coli* enzyme afforded a structure, which is devoid of trigonal symmetry. The N-terminal domain of one subunit and the C-terminal domain of one adjacent subunit are related by pseudo-c<sub>2</sub> symmetry, and the substrate binding cavities of the two domains are closely adjacent (8). Recent modeling studies based on structural data of riboflavin synthases of *E. coli* and *S. pombe* showed that two substrate molecules bound to these two domains are ideally placed for the dismutation reaction to occur (11). The ligand-binding sites of the other four domains of the *E. coli* enzyme are remote from each other, and it is not immediately obvious how they could participate in the dismutation of the substrate. That could imply that riboflavin synthase has only a single catalytic site, and that four substrate-binding sites are not involved at all in catalysis. Alternatively, we have proposed that riboflavin synthase undergoes major conformational fluctuations during catalysis, with the result that each N-terminal domain can temporarily form a pseudo-c<sub>2</sub> symmetric pair with the C-terminal domain of an adjacent subunit.<sup>3</sup> As a result of that dynamic fluctuation, each of the domains could participate, at intervals, in the formation of riboflavin from two substrate molecules. Because the turnover number of riboflavin synthase is very low (0.5 per s and per subunit), there could be ample

<sup>1</sup> The normalized velocity of compound 6 formation from compound 1:  $\partial[6]/\partial t \times 1/[1] = k_1 \times [1]$ . At the start of the quenched flow experiment,  $[1] = 100 \mu\text{M}$ .

<sup>2</sup> The normalized velocity of compound 8 formation,  $\partial[8]/\partial t \times 1/[6] = k_3$ , and is independent on concentration of any reactant.

<sup>3</sup> M. Fischer, A.-K. Schott, K. Kemter, R. Feicht, G. Richter, B. Illarionov, W. Eisenreich, S. Gerhardt, M. Cushman, R. Huber, and A. Bacher, submitted for publication.

time for catalysis, even on the basis of relatively slow conformational motions of the protein.

A firm conclusion whether one pair of subunits would build one constitutive catalytic site or whether each domain can participate in catalysis in a sequential mode cannot be reached on the basis of the present data. However, the dynamic model intuitively fits our observations. The simulated data (Fig. 6) show  $\sim 19 \mu\text{M}$  compound **6** which agrees with the suggestion that two of the six subunits ( $56 \mu\text{M}$ ) form a catalytic site, *i.e.* at 4 s after the start of the experiment most active sites are occupied by compound **6**. If the enzyme had six substrate-binding sites but only one catalytically active site, one would expect that one-third of the proffered substrate, at best, can be converted to product in the first round of a single turnover experiment. The conversion of additional substrate to product could only occur by dissociation of substrate from non-productive substrate-binding sites followed by secondary binding at the one and only true catalytic site. Even if the dissociation as well as the rebinding were to occur at high velocity, it would seem rather unlikely that more than one molecule of the dimeric intermediate **6** per subunit homotrimer is present at any time in the reaction sequence.

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