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Riboflavin synthase of *Schizosaccharomyces pombe*. Protein dynamics revealed by ^{19}F NMR protein perturbation experiments

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Abstract

Background: Riboflavin synthase catalyzes the transformation of 6,7-dimethyl-8-ribityllumazine into riboflavin in the last step of the riboflavin biosynthetic pathway. Gram-negative bacteria and certain yeasts are unable to incorporate riboflavin from the environment and are therefore absolutely dependent on endogenous synthesis of the vitamin. Riboflavin synthase is therefore a potential target for the development of antiinfective drugs.

Results: A cDNA sequence from *Schizosaccharomyces pombe* comprising a hypothetical open reading frame with similarity to riboflavin synthase of *Escherichia coli* was expressed in a recombinant *E. coli* strain. The recombinant protein is a homotrimer of 23 kDa subunits as shown by sedimentation equilibrium centrifugation. The protein sediments at an apparent velocity of 4.1 S at 20°C. The amino acid sequence is characterized by internal sequence similarity indicating two similar folding domains per subunit. The enzyme catalyzes the formation of riboflavin from 6,7-dimethyl-8-ribityllumazine at a rate of 158 nmol mg^{-1} min^{-1} with an apparent K_M of 5.7 microM. ^{19}F NMR protein perturbation experiments using fluorine-substituted intermediate analogs show multiple signals indicating that a given ligand can be bound in at least 4 different states. ^{19}F NMR signals of enzyme-bound intermediate analogs were assigned to ligands bound by the N-terminal respectively C-terminal folding domain on basis of NMR studies with mutant proteins.

Conclusion: Riboflavin synthase of *Schizosaccharomyces pombe* is a trimer of identical 23-kDa subunits. The primary structure is characterized by considerable similarity of the C-terminal and N-terminal parts. Riboflavin synthase catalyzes a mechanistically complex dismutation of 6,7-dimethyl-8-ribityllumazine affording riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. The ^{19}F NMR data suggest large scale dynamic mobility in the trimeric protein which may play an important role in the reaction mechanism.

Background

Flavocoenzymes derived from riboflavin (vitamin B_2) act as cofactors for a variety of redox reactions and are indispensable in all cells. Whereas plants and many microorganisms obtain riboflavin by endogenous synthesis, animals depend on nutritional supply of the vitamin. Certain bacteria (e.g. Enterobacteriaceae) and yeasts are unable to incorporate riboflavin from the environment and are therefore absolutely dependent on the endogenous synthesis of the vitamin [1-4], and the enzymes of the riboflavin biosynthetic pathway are therefore potential targets for antiinfective drug design.

The absence of the riboflavin biosynthetic pathway in the human host is advantageous with regard to drug development. The final step in the riboflavin biosynthetic pathway is catalyzed by riboflavin synthase. The mechanistically unusual dismutation of the substrate, 6,7-dimethyl-8-ribityllumazine (Compound **3**, Fig. 1) affording riboflavin (Compound **4**) and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (Compound **1**) requires the presence of two substrate molecules serving as donor resp. acceptor of a 4-carbon moiety at the active site of the enzyme [5-7]. The regiochemistry of the enzyme-catalyzed reaction predicts an antiparallel arrangement of the two substrate molecules [8-10]. A pentacyclic dimer of 6,7-dimethyl-8-ribityllumazine (Compond **5**, Fig. 2) has been identified as a kinetically competent intermediate in the complex reaction sequence [11]. The riboflavin synthases of *Escherichia coli* and *Bacillus subtilis* are homotrimeric proteins [12-14].

Intramolecular sequence similarity suggested that each subunit folds into two topologically similar domains, each of which can accommodate one substrate molecule. The active site was proposed to be located at the interface of adjacent domains; two substrate molecules were assumed to be sandwiched between adjacent subunits [14]. The structure of riboflavin synthase from *E. coli* as determined by X-ray crystallography at a resolution of 2.0 Å confirmed the predicted similarity in the folding patterns of the N-terminal and C-terminal domain (rms = 0.8 Å) [15] but gave no direct information on enzyme substrate interaction. The N-terminal domain of the *E. coli* enzyme (residues 1 – 97) could be expressed as a stable protein with a relatively high affinity for riboflavin.

Surprisingly, this artificial protein is a c_2 symmetric homodimer, whereas the full length riboflavin synthase subunit forms a homotrimer [16]. The structure of the artificial N-terminal domain dimer in complex with riboflavin has been determined by NMR spectroscopy [17]. Protein perturbation studies monitored by ^{19}F NMR with fluorine-substituted intermediate analogs had suggested that the binding sites of the homotrimeric riboflavin synthase are not topologically equivalent [18-20].

More specifically, these experiments showed that certain ligands could be bound to the protein in at least four different binding states, whereas only two different states (one for the N-terminal and one for the C-terminal domain) would have been expected in a c_3 symmetric homotrimer. These observations are well in line with the absence of trigonal molecular symmetry revealed by the X-ray structure analysis of riboflavin synthase of *E. coli* [15]. The structure of riboflavin synthase from *S. pombe* in complex with the substrate analog, 6-carboxyethyl-7-oxo-8-ribityllumazine has been determined at 2.1 Å resolution. In contrast to the homotrimeric solution state of native riboflavin synthase, the enzyme was found to be monomeric in the crystal structure. Structural comparison of the riboflavin synthases of *S. pombe* and *E. coli* suggested oligomer contact sites and delineated the catalytic site for dimerization of the substrate and subsequent fragmentation of the pentacyclic intermediate [21]. This paper reports ^{19}F NMR protein perturbation studies using wild type and mutant riboflavin synthase of *S. pombe* in complex with fluorinated intermediate analogs. The data suggest large scale domain mobility which appear relevant for the complex reaction mechanism.

Results

A putative *rib5*$^+$ gene of *S. pombe* (NCBI accession number, T40995) predicted a protein of 208 amino acid residues with similarity to riboflavin synthase (50 % identity with riboflavin synthase of *S. cerevisiae* and 36 % identity with riboflavin synthase of *E. coli*).

The open reading frame was amplified from *S. pombe* cDNA and was cloned into the plasmid vector pNCO113. The cDNA sequence as determined by dideoxynucleotide sequencing was identical with the chromosomal sequence reported earlier and has been deposited in the GenBank data base (accession number AF505789).

A recombinant *E. coli* strain carrying a plasmid with the *S. pombe rib5*$^+$ gene under control of the T5 and the *lac* operator expressed a protein with an approximate mass of 23 kDa as judged by SDS electrophoresis. The recombinant protein accounting for about 20 % of cell protein was purified to apparent homogeneity by three chromatographic steps as described under Methods. Partial Edman degradation afforded the N-terminal sequence MFTGLVEAIGVVKDVQGTID in agreement with the open reading frame.

The protein shows internal sequence similarity. Twenty one amino acid residues are identical in the N-terminal and C-terminal section (Fig. 3).

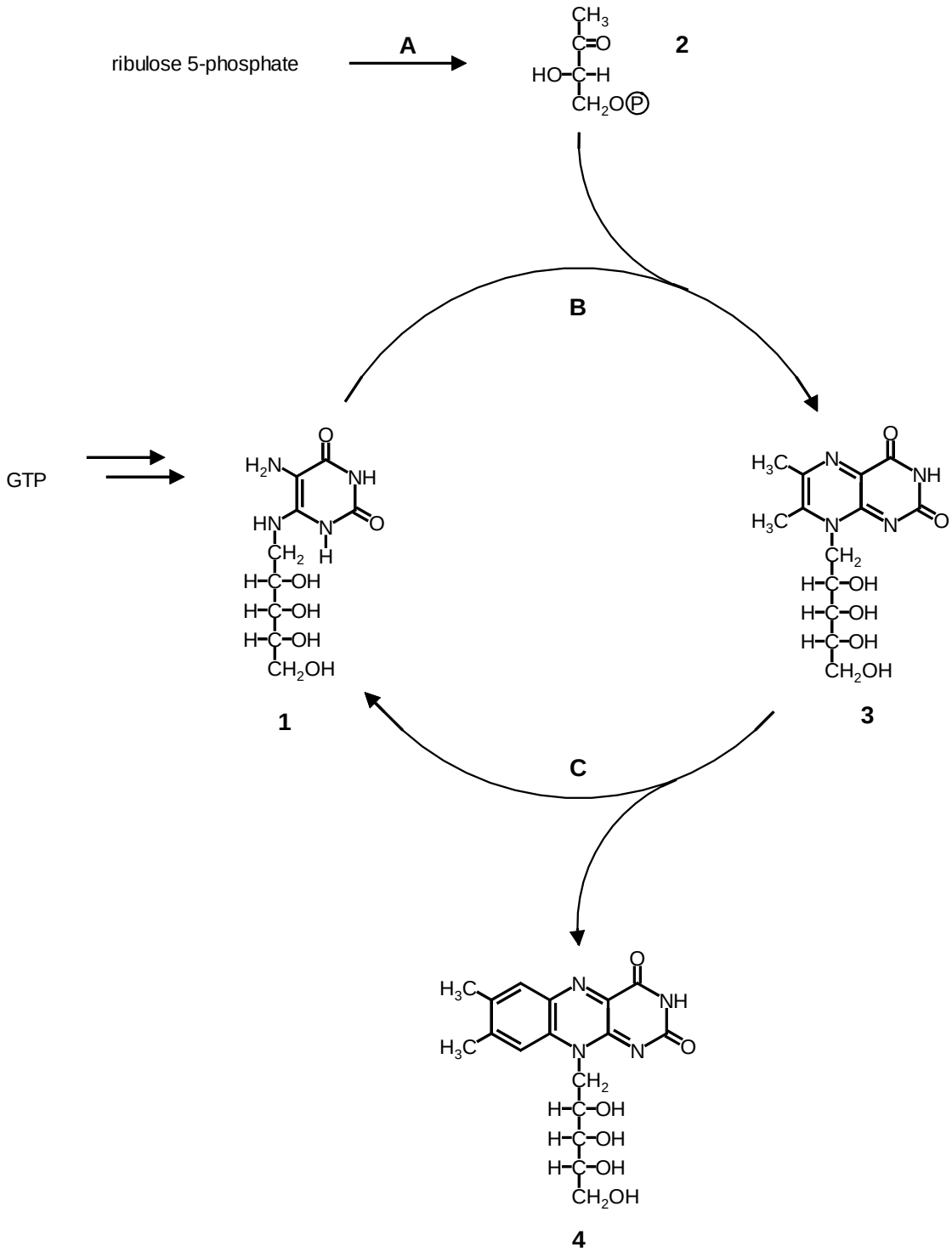


Figure 1
Terminal reactions in the pathway of riboflavin biosynthesis. A, 3,4-dihydroxy-2-butanone 4-phosphate synthase; B, 6,7-dimethyl-8-ribityllumazine synthase; C, riboflavin synthase.

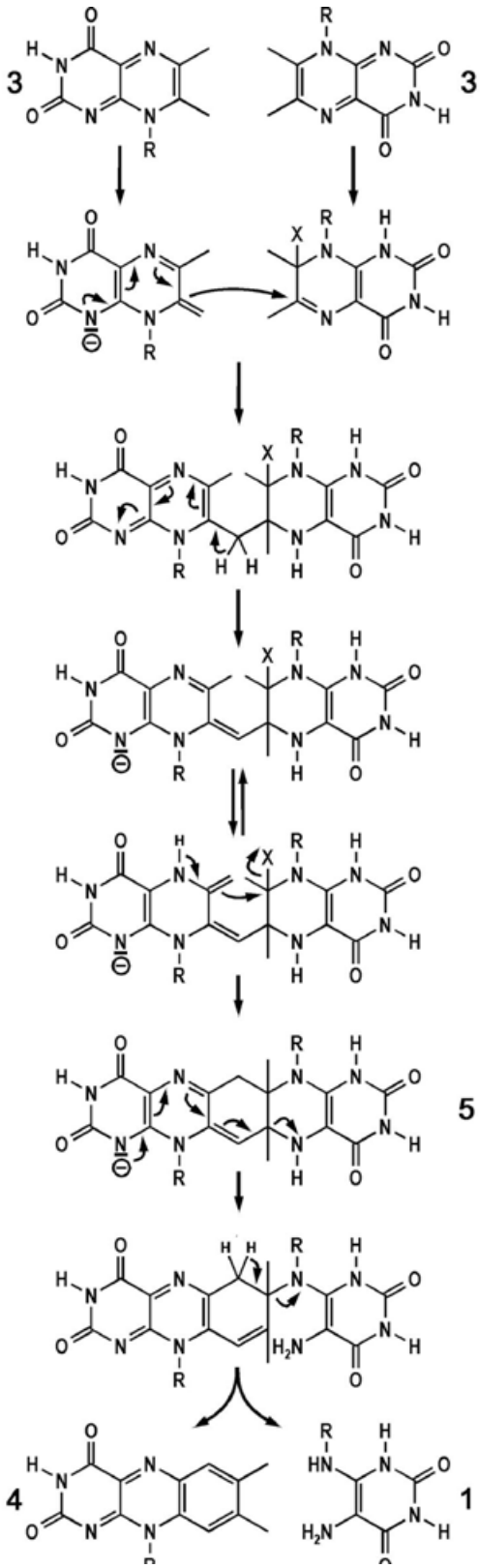


Figure 2
Hypothetical reaction mechanism of riboflavin synthase. X, unkown nucleophile; R, ribityl. [11]

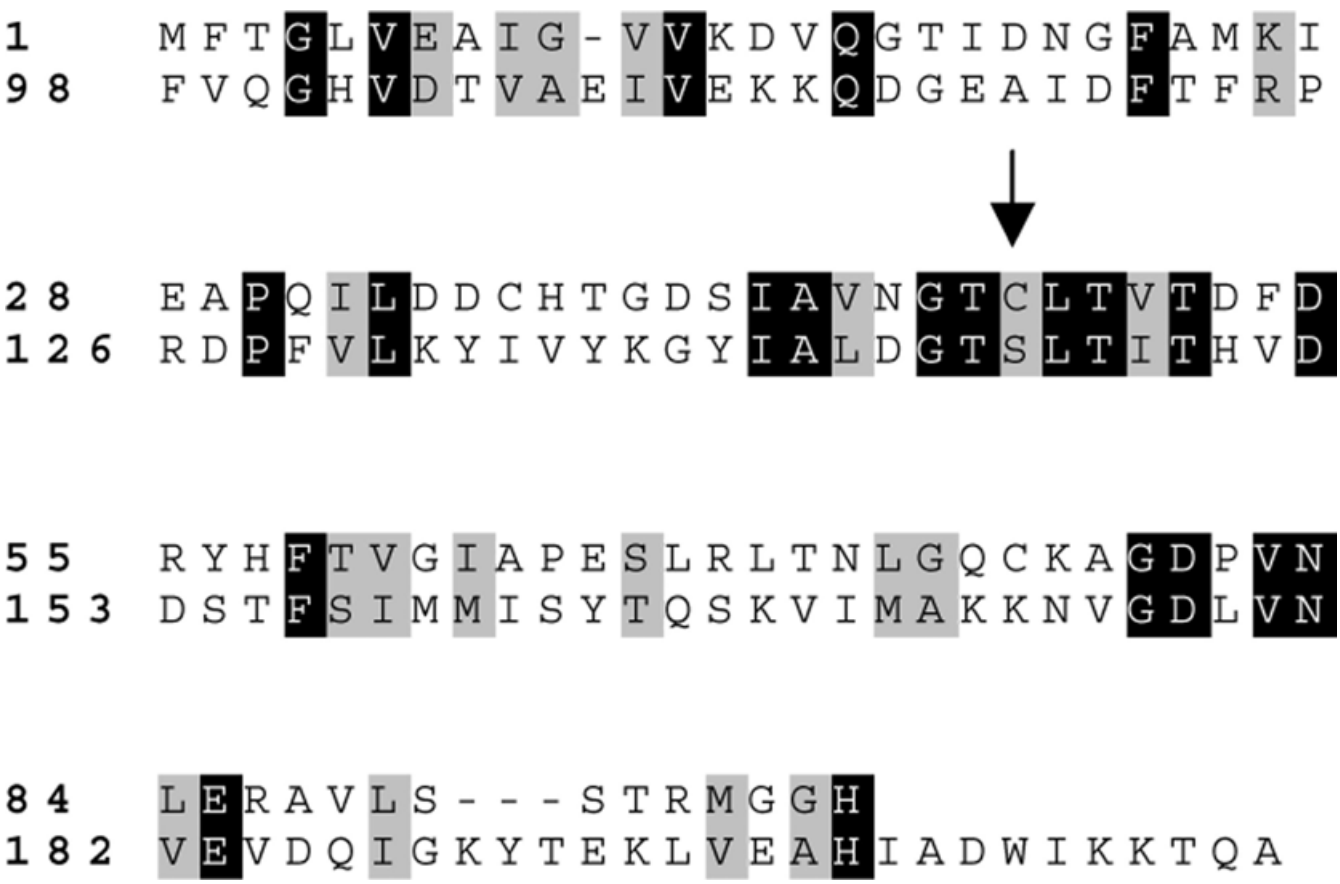


Figure 3
Intramolecular sequence similarity of *S. pombe* riboflavin synthase. N-terminal part, amino acids 1–97; C-terminal part, amino acids 98–208. Identical residues are coloured black. The residues subjected to mutagenesis in this study are marked by an arrow.

The recombinant protein had a specific activity of 158 nmol mg^{-1} min^{-1} and an apparent K_M of 5.7 μM at pH 7.2 and 37°C (Table 1). Electrospray mass spectrometry afforded a relative mass of 22,863 Da in good agreement with the predicted mass of 22,861 Da. The recombinant protein sediments as a single, symmetrical boundary with an apparent velocity of 4.1 S. Sedimentation equilibrium experiments indicate a molecular mass of 74 kDa using an ideal monodisperse model for simulation. Sedimentation equilibrium analysis in the presence of 5 mM 6-carboxyethyl-7-oxo-8-D-ribityllumazine (Compound **8**; Fig. 4) afforded an apparent molecular weight of 76 kDa. It follows that the enzyme is homotrimer like the riboflavin synthases of *B. subtilis* and *E. coli* [13,22] and that the quaternary structure is not affected by binding of the substrate analog.

Protein perturbation studies monitored by ^{19}F NMR had been performed earlier with riboflavin synthases from *B. subtilis* and *E. coli* and had shown surprising multiplicities of signals for enzyme-bound ligands [18-20,23]. Thus, studies with the intermediate analog, 6-trifluoromethyl-7-oxo-8-ribityllumazine (Compound **7**, Fig. 4) had suggested at least four different binding states characterized by different ^{19}F signals spread over a range of about 9 ppm, and the covalent hydrate of 6,7-bis(trifluoromethyl)-8-ribityllumazine (Compound **6a**, epimer A) could be bound in at least two different states. On the other hand, lumazine protein from *Photobacterium phosphoreum*, a monomeric paralog of riboflavin synthase without enzyme activity which serves as an optical transponder in bioluminescence showed single signals for each trifluoromethyl group of lumazine analogues [24,25]. Similarly, an artificial N-terminal domain of *E. coli* riboflavin synthase which forms a c_2 symmetric homodimer showed single lines for each trifluoromethyl group of trifluoromethylsubstituted lumazine derivatives [16].

Table 1: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
E. coli strain		
XL-1-Blue	recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F', proAB, $lacI^qZ\Delta$ M15, Tn10(tet^r)]	34
M15 [pREP4]	lac, ara, gal, mtl, $recA^+$, uvr^+, [pREP4: lacI, kan^r]	43
BL21 (DE3)	F^-, ompT, hsdSB, (r^-B, m^-B), gal, dcm (DE3)	44
Plasmids		
pNCO113	expression vector	36
pT7-7	expression vector	45
pNCO-SSP-Rosy-WT	vector expressing the wild type SSP-Rosy	this study
pT7-SSP-Rosy-C48S	vector expressing a C48S mutated SSP-Rosy	this study
pT7-SSP-Rosy-C48A	vector expressing a C48A mutated SSP-Rosy	this study
pT7-SSP-Rosy-C48M	vector expressing a C48M mutated SSP-Rosy	this study
pT7-SSP-Rosy-S146A	vector expressing a S146A mutated SSP-Rosy	this study
pT7-SSP-Rosy-S146C	vector expressing a S146S mutated SSP-Rosy	this study

Figs. 5 and 6 show comparative protein perturbation experiments with various orthologs and paralogs of the *S. pombe* enzyme including lumazine protein (Figs. 5A, 6A), the N-terminal domain of *E. coli* riboflavin synthase (Figs. 5B, 6B) and full length riboflavin synthases of different organisms (Figs. 5C,5D,5E, 6C,6D,6E). All spectra were recorded in the present study under identical experimental conditions.

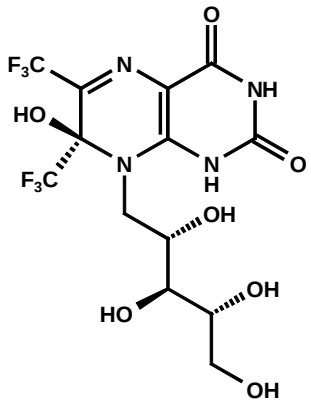
The monotrifluoromethyl derivative, Compound **7**, in complex with riboflavin synthase of *S. pombe* shows four signals at 8.8, 10.9, 15.6 and 16.0 ppm (Fig. 5E). The signal of the free ligand resonates at 7.7 ppm (indicated by a dashed line in Fig. 5) with a line width of 56 Hz, whereas the signals of the ligand bound in different states had apparent line widths extending from 90 to approximately 540 Hz. The riboflavin synthases of *E. coli* (Fig. 5C) and *B. subtilis* (Fig. 5D) show similar signal multiplicities, but the chemical shifts and line widths are markedly different. In stark contrast, lumazine protein (Fig. 5A) and the c_2 symmetric N-terminal domains of *E. coli* riboflavin synthase (Fig. 5B) show single and much narrower lines for the protein bound ligand. It is immediately obvious that a given ligand can be bound in multiple, significantly different states by all riboflavin synthases studied, but only in a single state by lumazine protein or the artificial, c_2 symmetric N-terminal domain.

A similar set of experiments with the bistrifluoromethyl analog **6a** of 6,7-dimethyl-8-ribityl-lumazine is shown in Fig. 6. Due to the presence of six fluorine atoms, the lumazine derivative forms two stable diastereomeric hydrates **6a** and **6b** which differ by their configuration at the ring carbon atom 7 and are not subject to racemization or dehydration. The stereochemical assignments shown in Fig. 4 were suggested on basis of solid-state NMR studies [26,27].

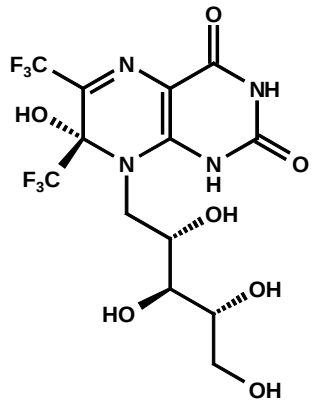
As shown in Fig. 6A,6B, lumazine protein and the dimeric N-terminal domain of *E. coli* riboflavin synthase show single lines for each trifluoromethyl group of bound Compound **6a**. In contrast to that, the riboflavin synthases (Figs. 6C,6D,6E) studied show multiple signals, and the signal patterns are highly variable for enzymes from different species. The enzyme of *S. pombe* (Fig. 6E) shows the most complex picture with three major signals for the position 7 trifluoromethyl group and two major signals for the position 6 trifluoromethyl group of the ligand in the bound state. The signals of the free epimer A appeared broadened to 80 Hz – 100 Hz, whereas the signals of the ligand bound in different states had apparent line widths of 170 – 300 Hz.

In an earlier study, we reported ^{19}F protein perturbation studies obtained with a series of mutants of *E. coli* riboflavin synthase [18]. In that study, it was not possible to express mutant proteins characterized by replacement of the conserved cysteine 48 which is located close to the bound riboflavin in the artificial N-terminal domain dimer [17]. Since a catalytic role had been proposed for that residue [17], a renewed mutagenesis attempt was made with the *S. pombe* protein. In contrast to our earlier findings with the *E. coli* enzyme, cystein 48 of the *S. pombe* enzyme could be replaced by various amino acids, and the proteins could be expressed to high levels. Similarly, mutant proteins with the topologically equivalent S146 residue in the C-terminal domain (cf. Fig. 3) could be expressed.

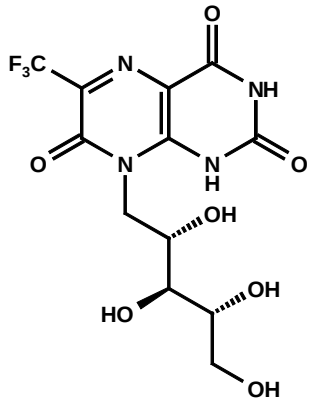
The catalytic properties of the mutants are summarized in Table 3. Replacement of cystein 48 by serine reduced the catalytic rate of the enzyme by a factor of about 6; C48A and C48M mutants had no detectable enzyme activity. The replacement of serine 146 had only a minor influence on the catalytic rate, in close similarity with our earlier



6a
(epimer A)



6b
(epimer B)



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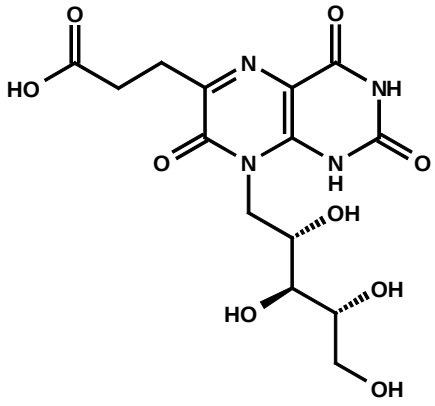

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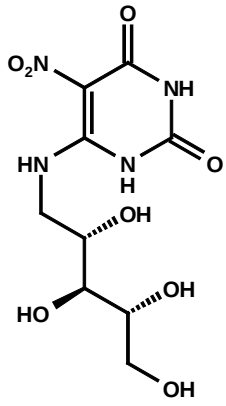

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Figure 4
Intermediate analogs used for protein perturbation studies. 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrate, epimer A (Compound **6a**) and epimer B (Compound **6b**). 6-trifluoromethyl-7-oxo-8-ribityllumazine (Compound **7**). 6-carboxyethyl-7-oxo-8-ribitylluamzine (Compound **8**), 5-nitro-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (Compound **9**).

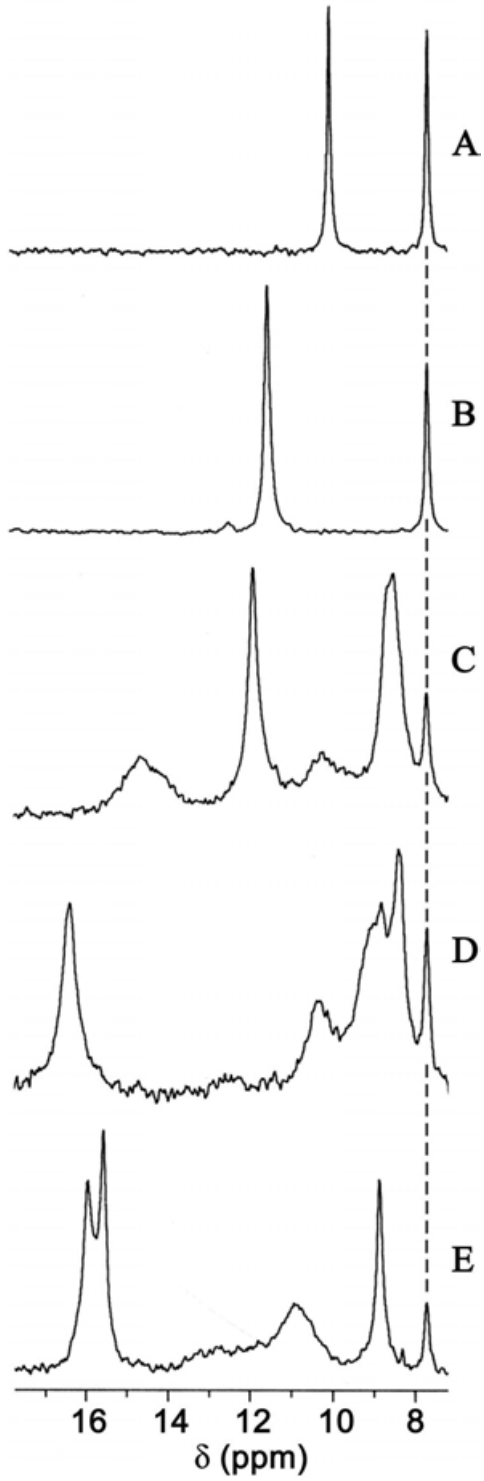

Figure 5
^{19}F NMR spectra of 6-trifluoromethyl-7-oxo-8-ribityllumazine (Compound 7). A, lumazine protein of *P. leiognathi* (0.13 mM protein, 0.25 mM **7**); B, recombinant N-terminal domain of *E. coli* riboflavin synthase (0.80 mM protein, 0.98 mM **7**); C, riboflavin synthase of *E. coli* (0.37 mM protein, 1.43 mM **7**); D, riboflavin synthase of *B. subtilis* (0.10 mM protein, 0.75 mM **7**); E, riboflavin synthase of *S. pombe* (0.28 mM protein, 1.22 mM **7**). All samples contained 20 mM phosphate buffer, 100 mM KCl and 10% D_2O, pH 7. Signals of the free ligand are indicated by a dashed line. The data were processed with a line broadening of 20 Hz.

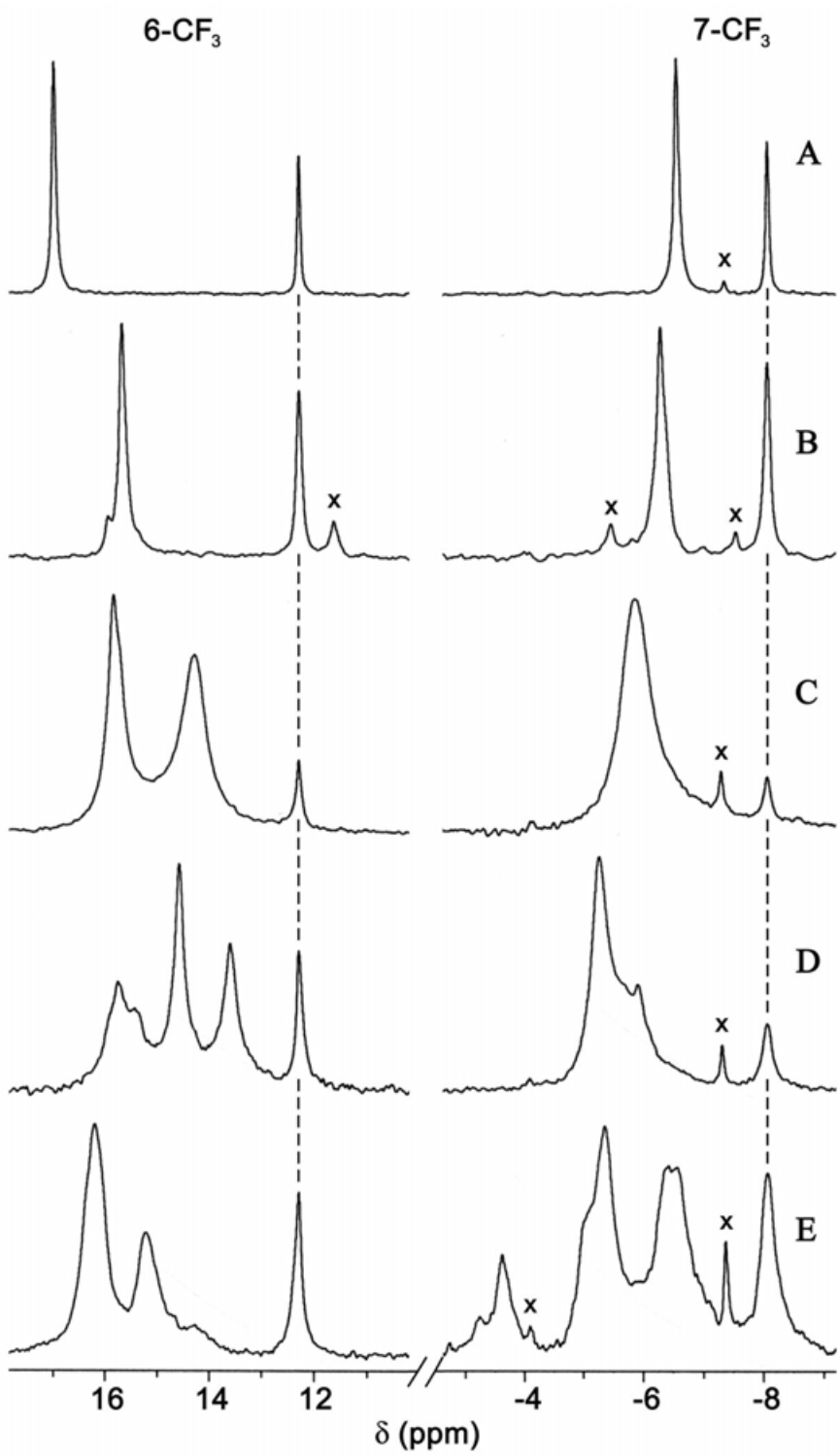

Figure 6
^{19}F NMR signals of 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrate (epimer A; Compound 6a). A, lumazine protein of *P. leiognathi* (0.14 mM protein, 0.23 mM **6a**); B, recombinant N-terminal domain of *E. coli* riboflavin synthase (0.75 mM protein, 0.56 mM **6a**); C, riboflavin synthase of *E. coli* (0.5 mM protein, 3.34 mM **6a**); D, riboflavin synthase of *B. subtilis* (0.13 mM protein, 0.41 mM **6a**); E, riboflavin synthase of *S. pombe* (0.26 mM protein, 0.60 mM **6a**); x, impurities. For other details see legend to Fig. 5.

Table 2: Oligonucleotide primers used for construction of *rib5*⁺ expression plasmid and for site-directed mutagenesis

Primer	Primer orientation	Amino acid replacement	Novel restriction site	Sequence (5'– 3')
SSP-RIB5-1	-	none	EcoRI	ATAATAGAATTCATTAAAGAGGAGAAATTAACCATGTTTACC GGTCTTGTTGAAGCCATTGGTGTTG
SSP-RIB5-2	+	none	BamHI	TATTATGGATCCTCAAGCTTGGGTTTTCTTAATCCAGTC
1	-	none (flanking primer)		GGTCTAGATCACACAGAATTCATTAAAGAGG
2	+	none (flanking primer)		GACTGCAGTCAAGCTTGGGTTTTCTTAA
3	+	C48 → S	HpaI	TCTGTCACTGTAAG**agA**AGTACCGTTaACGGCGATACT
4	+	C48 → A	HpaI	TCTGTCACTGTAAG**agc**AGTACCGTTaACGGCGATACT
5	+	C48 → M	HpaI	TCTGTCACTGTAAG**cat**AGTACCGTTaACGGCGATACT
5	-	S146 → C	KpnI	ATAGCCCTGGACGGTACc**Tgt**CTGACCATTACTCAT
6	-	S146 → A	KpnI	ATAGCCCTGGACGGTACc**gCG**CTGACCATTACTCAT

Oligonucleotides were designed to hybridize to the sense (+) and to the antisense (-) strand of the *rib5*⁺. Mutated bases are indicated in lower case. Codons specifying modified amino acid residues are shown in bold type. Novel restriction sites are underlined.

findings with *E. coli* riboflavin synthase [18]. The replacement of cystein 48 had major impact on ligand perturbation spectra (Fig. 7) with 6-trifluoromethyl-7-oxo-8-ribityllumazine (Compound **7**, Fig. 4). When cystein was replaced by less bulky residues (alanine, serine), no ^{19}F NMR signals were noted in the range of 14 – 17 ppm, whereas the wild type protein shows two intense, closely spaced signals at 15.6 and 16.0 ppm. When cystein 48 was replaced by the more bulky methionine, intense signals were observed at 13.8 and 14.0 ppm. Notably, the mutation in the N-terminal domain affects the entire spectrum rather than individual signals. Although a signal is consistently observed at about 8.8 ppm, at least its relative intensity is modulated substantially by the replacement of cystein 48.

In the C-terminal domain, serine 146 is the topological equivalent of cystein 48. Although this residue appears to be absolutely conserved, its replacement by the less bulky alanine or the more bulky cystein has relatively little influence on the catalytic rate and the Michaelis constant of the enzyme. However, the replacement of serine 146 had major impact on the protein perturbation spectra (Fig. 7). The signal at 8.8 ppm which is present in the wild type protein and all cystein 48 mutants has an increased intensity in case of the S146A mutant but is absent or shifted in the S146C mutant protein. Again, it should be noted that the replacement of serine 146 affects the entire spectrum rather than individual signals.

In line with the other proteins described in this study, riboflavin synthase of *S. pombe* binds epimer A believed to represent the 7-R diasteromer, whereas epimer B (7-S) showed no detectable interaction (data not shown). The 6-trifluoromethyl groups of 6-trifluoromethyl-7-oxo-8-ribityl-lumazine and 6,7-bis(trifluoromethyl)-8-ribityl-lumazine hydrate (epimer A) are topologically equivalent. The ^{19}F NMR signals assigned to that group in the bistrifluoromethyl compound **6a** in Figs. 6 and 8 are spread out over a range of only about 4 ppm, as compared to a range of 9 ppm in case of the 7-oxo compound **7**, but the impact of mutations is similar. Again, mutation of either cystein 48 or serine 146 affects all signals attributed to bound ligands (Fig. 8).

6,7-Bis(trifluoromethyl)-8-ribityllumazine hydrate (epimer A) could be displaced from *S. pombe* protein by addition of riboflavin or the product analog, 5-nitro-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (Compound **9**, Fig. 4), thus suggesting that each of the signals, assigned to bound ligand, represented binding sites with specificity for substrate/intermediate analogs rather than non-specific protein surface interactions (data not shown). In the mixture, epimer A displaced from the enzyme by the competing ligands had a linewidth of about 20 Hz.

The complexity of the interaction of the proteins with the ligands is further illustrated by the titration experiments shown in Figs. 9 and 10. In the spectra of the wild type enzyme as well as the C48A mutant, the protein/ligand ratio affects not only the intensity and the line width of the different bands assigned to compound **7** in the protein bound state, but also the chemical shifts of individual signals. Remarkably, some of the peaks appeared with smaller line widths in later stages of the titration experiment (cf. peak at about 9 ppm in Fig. 9). This effect could indicate a combination of substrate induced conformational changes affecting the dynamics of the protein.

Discussion

Lumazine proteins are paralogs of riboflavin synthase occurring in luminescent marine bacteria where they are believed to serve as optical transponders which absorb energy from bacterial luciferase by Förster transfer and are conducive to enhanced luminescence quantum yield as well as modulation of the emission frequency [28,29]. In

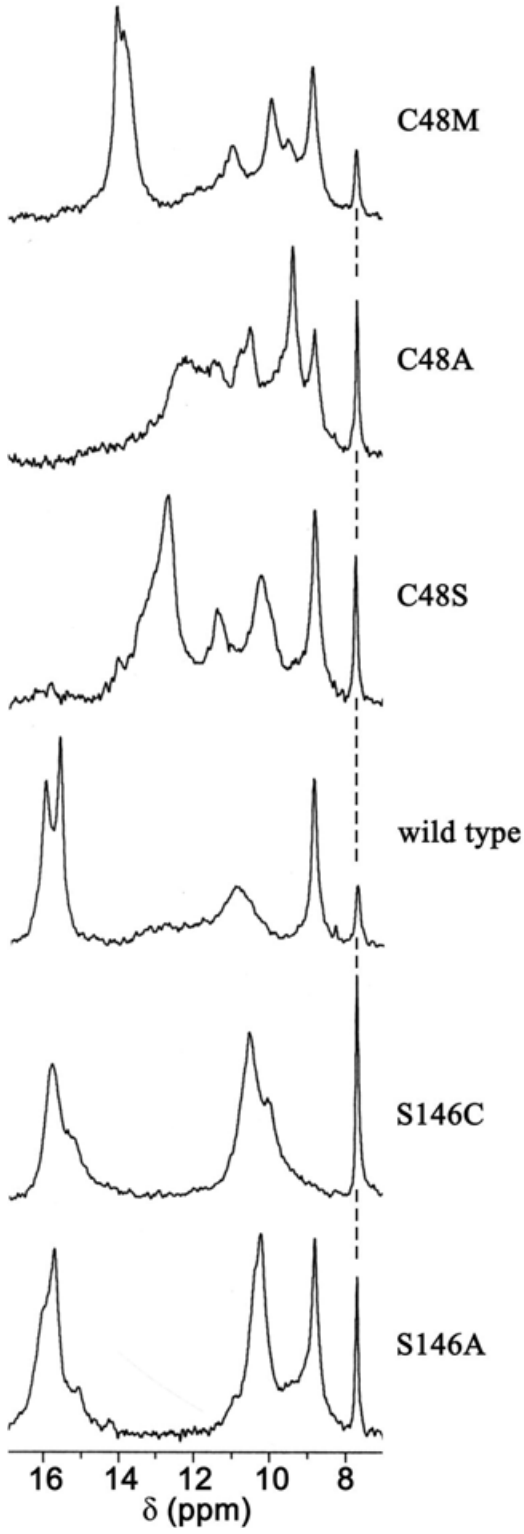

Figure 7
^{19}F NMR spectra of 6-trifluoromethyl-7-oxo-8-ribityllumazine (Compound 7) in the presence of riboflavin synthase mutants of *S. pombe*. Concentrations in the NMR samples were as follows: C48M, 0.28 mM protein, 1.10 mM **7**; C48A, 0.15 mM protein, 0.57 mM **7**; C48S, 0.15 mM protein, 0.80 mM **7**; wild type, 0.28 mM protein, 1.22 mM **7**; S146C, 0.20 mM protein, 0.92 mM **7**; S146A, 0.26 mM protein, 1 mM **7**. For other details see legend to Fig. 5.

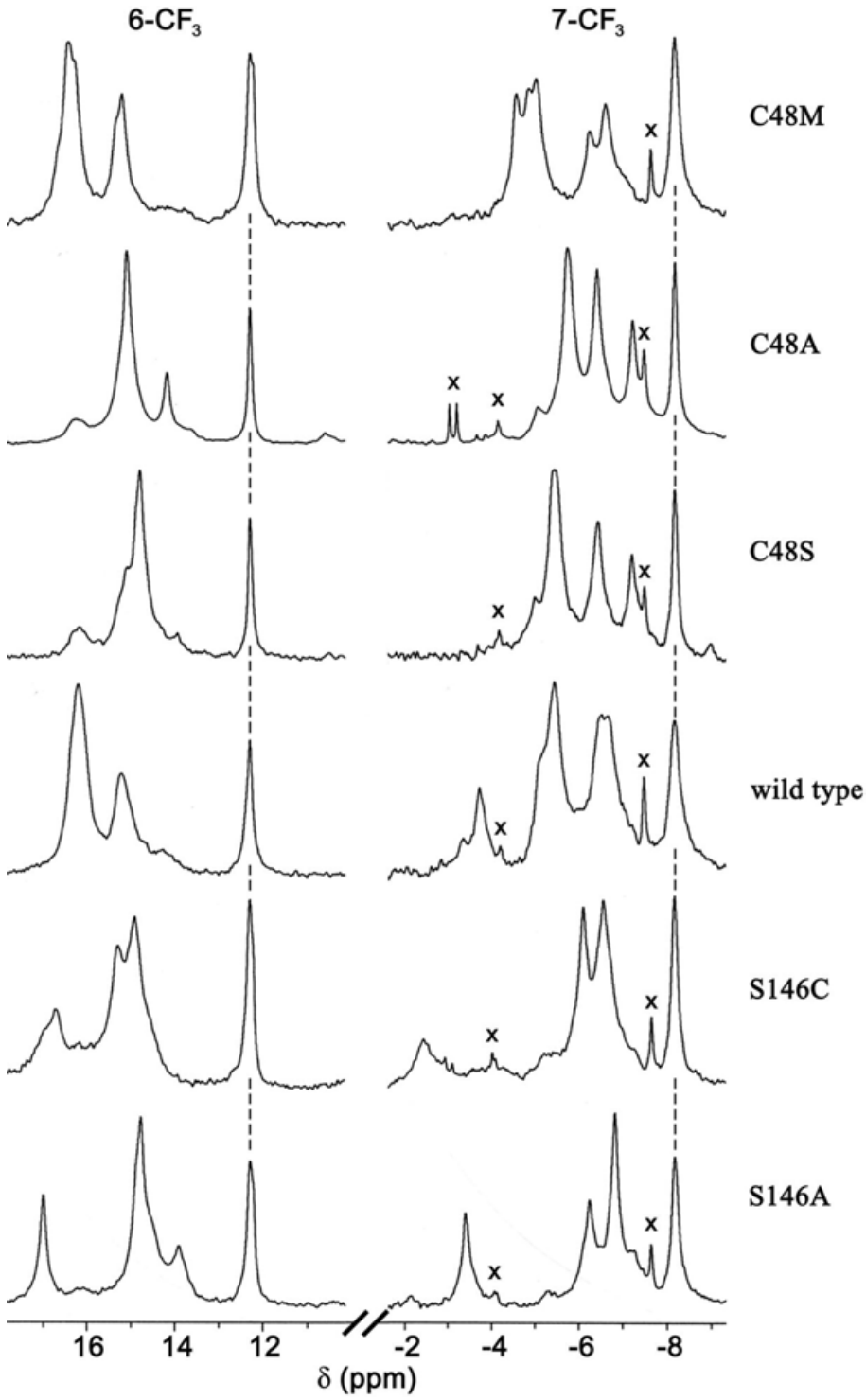

Figure 8
^{19}F NMR spectra of 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrate (epimer A, Compound 6a) in the presence of riboflavin synthase mutants of *S. pombe*. Concentrations in the NMR samples were as follows: C48M, 0.30 mM protein, 0.94 mM **6a**; C48A, 0.19 mM protein, 0.33 mM **6a**; C48S, 0.20 mM protein, 0.42 mM **6a**; wild type, 0.26 mM protein, 0.60 mM **6a**; S146C, 0.26 mM protein, 0.65 mM **6a**; S146A, 0.34 mM protein, 1 mM **6a;** x, impurities. For other details see legend to Fig. 5.

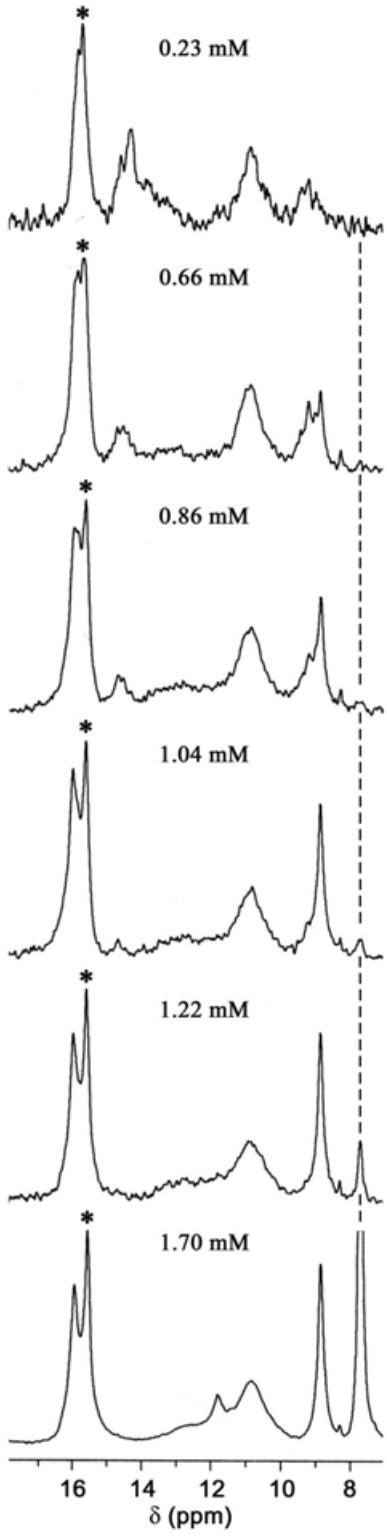

Figure 9
^{19}F NMR spectra of 6-trifluoromethyl-7-oxo-8-ribityllumazine (Compound 7) in titration experiments with riboflavin synthase of *S. pombe* (wild type). The initial concentration of the protein was 0.36 mM. Compound **7** was added to the concentrations indicated. The spectra are displayed with equal intensities of the signals at 15.6 ppm (indicated by *). For other details see legend to Fig. 5.

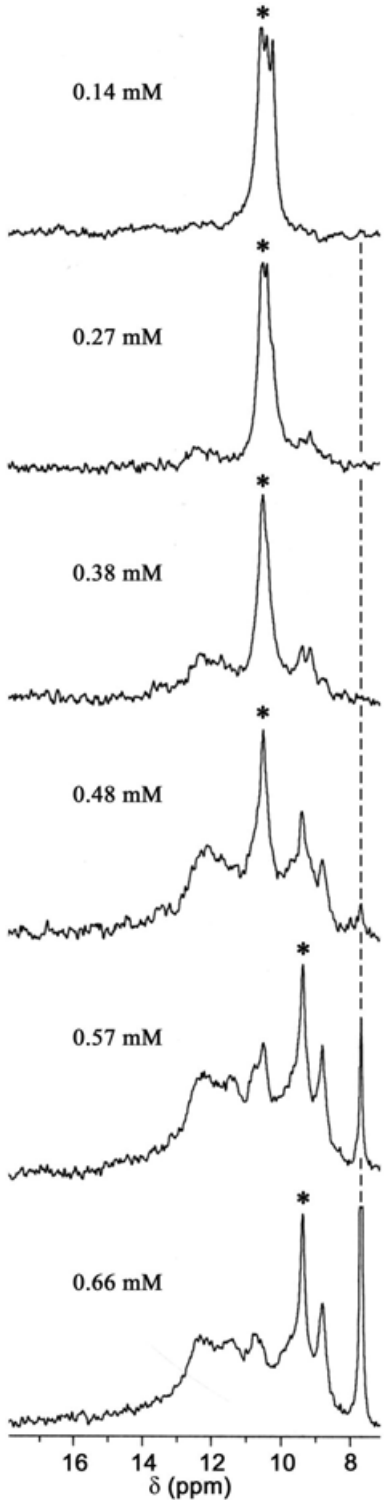

Figure 10
^{19}F NMR spectra of 6-trifluoromethyl-7-oxo-8-ribityllumazine (Compound 7) in titration experiments with C48A mutant of riboflavin synthase of *S. pombe*. The initial concentration of the protein was 0.24 mM. Compound 7 was added to the concentrations indicated in the spectra. The spectra are displayed with equal intensities of the signals as indicated by *. For other details see legend to Fig. 5.

contrast to the homotrimeric riboflavin synthase which can bind up to 6 substrate molecules, lumazine proteins have been reported to be monomers which bind only one molecule of 6,7-dimethyl-8-ribityllumazine. Although they are likely to share the two-domain architecture of the riboflavin synthase subunit, it appears that only one domain engages in ligand binding. Notably, all riboflavin synthases studied as well as lumazine protein are strictly stereoselective and bind epimer A (Compound **6a**, Fig. 4) but not epimer B (Compound **6b**) of the diastereomeric 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrate.

Not surprisingly, when the fluorinated lumazines **6a** or **7** are bound to lumazine protein, a single ^{19}F NMR signal is observed for each respective trifluoromethyl group (Figs. 5A, 6A). Similarly, the c_2 symmetric dimer obtained by recombinant expression of the N-terminal domain of riboflavin synthase shows a single ^{19}F NMR signal for each trifluoromethyl group of a bound ligand (Figs. 5B, 6B).

Since both domains of riboflavin synthase can bind ligand molecules, one would expect to observe separate sets of signals for ligands bound to each respective domain. Beyond that expectation, however, all riboflavin synthases studied in this paper show multiple signals for each ligand studied (Figs. 5C,5D,5E, 6C,6D,6E). Moreover, the signal patterns depend on the ratio of protein and ligand in the solution in unexpected ways (Figs. 9, 10), and the replacement of an amino acid at the C-terminal or N-terminal domain ligand binding site affects virtually all signals attributed to bound ligands. It follows that riboflavin synthase is not c_3 symmetric in solution. The asymmetry revealed by the X-ray structure of the *E. coli* enzyme [15] is thus an inherent property of the enzyme and is not due to crystal contacts.

Based on the NMR experiments with the mutant proteins obtained by replacement of cystein 48 or serine 146, it is now possible to assign ^{19}F signals of the bound ligand in Fig. 7 to the N- or C-terminal domain. Comparison of the spectra in Fig. 7 suggests that the ^{19}F signals shifted to lower field represent ligand molecules which are bound to the N-terminal domain and are therefore predominantly affected by the modification of cystein 48, whereas the signals shifted to higher field represent molecules bound to the C-terminal domain which are more susceptible to the modification of serine 146. However, it must be emphasized that all signals are affected at least to some extent by the mutations.

The X-ray structure of the *E. coli* enzyme suggests that direct contact exists only between the ligand binding sites of one N-terminal domain and the C-terminal domain of the adjacent subunit [15], whereas the ligand binding sites of two N-terminal and two C-terminal domains are exposed to solvent but not to an adjacent protein subunit. Since a number of studies has indicated that riboflavin synthase can bind certain ligands at a stoichiometry of 1:6 [18,20,22], we assume that each N-terminal and each C-terminal domain can participate in ligand binding.

The major difference is a cysteine residue (position 48 in the N-terminal domain) being replaced by a serine residue (position 146 in the C-terminal domain). Both amino acids are absolutely conserved in all putative riboflavin synthase paralogs. Nevertheless, serine 146 of the *S. pombe* enzyme can be replaced by alanine with only a minor impact on enzyme activity. Replacement of cysteine 48 by serine reduces the activity by a factor of five, but replacement by alanine affords a soluble protein whose activity, if any, is below the level of detection. It should also be noted that mutant genes specifying *E. coli* riboflavin synthase mutants carrying alanine or serine instead of cysteine 48 (corresponding to cysteine 48 of the *S. pombe* enzyme) could not be expressed in recombinant *E. coli* strains [18]. As seen in the X-ray structure of riboflavin synthase of *S. pombe* [21] the closest neighbor of the thiol group at position 48 is the 6-methylene group of the substrate analog, 6-carboxyethyl-7-oxo-8-ribityllumazine with a distance of 3.7 A° indicating an essential role of C48 in the catalytic mechanism of riboflavin synthase.

The riboflavin synthase subunit folds into two domains with closely similar folding topology. This had been anticipated on the basis of sequence arguments [14] and has been confirmed by X-ray structure analysis of riboflavin synthase of *E. coli* (without bound ligand) [15] and of *S. pombe* (with bound ligand). The monomeric *S. pombe* riboflavin synthase model arranged in a distinct N-terminal barrel (residues 1–90), an almost identically folded distinc C-terminal barrel (residues 91–184), and a C-terminal α-helix [21].

On basis of these structural arguments, one could expect that ligands bound to C-terminal domains without close interaction with N-terminal domains should not be influenced by conservative changes in the N-terminal domain. More specifically, the replacement of sulfur by oxygen in the C48S mutant should not influence the ^{19}F resonance of ligands bound at topologically remote C-terminal domain. The same argument holds *vice versa* for the influence of mutations in the C-terminal domain, e.g. the replacement of oxygen by sulfur in the C-terminal domain on ligands bound to distant N-terminal domains.

On basis of the finding that, contrary to that hypothesis, minor modifications of either N-terminal or C-terminal binding sites can modulate all aspects of the ^{19}F signatures of the bound ligands, we propose that riboflavin synthase is subject to large scale dynamic motions occurring on the

time scale of the NMR experiments. More specifically, we propose that the pair of N-terminal and C-terminal domain in direct contact as observed in the crystal structure is not permanently associated in solution where the respective domains of different subunits can temporarily assume that special relationship by major domain motions. Previous structural analysis [15] and molecular modeling revealed a markedly nonsymmetric trimeric state of riboflavin synthase. The modeling experiments, based on the inhibitor-bound protein [21], strongly suggest that the close intersubunit contact of the trimer as seen in the crystals of the *E. coli* protein is indeed in an active conformation and capable of supporting the dismutation reaction. Therefore, this trimer very likely represents a distinct functional state. As there is no reason to suggest a static asymmetric ensemble, it appears likely that thermal fluctuation or substrate binding converts the three different intersubunit contacts into one another, retaining an asymmetric trimer.

Such a dynamic model would fit the wide variation of line widths found for the different protein bound states as well as the unexpected dependence of the ^{19}F NMR spectra on the molar ratio between protein and ligand as revealed in Figs. 9 and 10.

Conclusions

The observations on protein dynamics are highly relevant for the reaction mechanism. Conceivably, the ligand binding sites of N- and C-terminal domains can be loaded with substrate while they are out of physical contact with each other. In order for the dismutation to occur, the domains could then form the local N-terminal/C-terminal domain complex by motion of the entire protein. The products could be unloaded after that domain pair has been separated again by major protein motions, and contacts between other domains could be established which could also result in product formation. In other words, all six domains would participate in the dismutation of the substrate, but they would do so at different times.

A dynamic model involving configuration changes of riboflavin synthase in the context of the catalytic reaction has been proposed earlier on basis of competitive ligand binding [23]. Since the reaction catalyzed by riboflavin synthase occurs on the time of seconds (the turnover number of the *S. pombe* enzyme is 11 min^{-1} per trimer), relatively slow dynamic motions occurring on the millisecond time scale would amply suffice the dynamic requirements of that slow catalytic reaction.

Methods

Materials

6,7-Dimethyl-8-ribityllumazine [30], 6-trifluoromethyl-7-oxo-8-ribityllumazine [19], 6-carboxy-ethyl-7-oxo-8-ribityllumazine [31,32] and 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrate (epimers A and B) [19] were prepared by published procedures. Oligonucleotides were custom synthesized by Interactiva, Ulm, Germany. Restriction enzymes were from New England Biolabs, Schwalbach, Germany. T4 DNA ligase and reverse transcriptase (SuperScriptII) were from Gibco BRL, Eggenstein, Germany. Taq Polymerase was from Finnzyme, Epsoo, Finnland. Vent DNA po-lymerase was purchased by New England Biolabs, Schwalbach, Germany. DNA fragments were purified with the QIAquick PCR Purification Kit from Qiagen, Hilden, Germany.

Microorganisms and plasmids

Microorganisms and plasmids used in this study are summarized in Table 1.

Proteins

Recombinant riboflavin synthase of *E. coli* and *B. subtilis* were prepared as described earlier [18,23].

Preparation of cDNA

The isolation of RNA from *S. pombe var. pombe* Lindner (ATCC 16491) and the preparation of cDNA was performed as described earlier [33].

Construction of the rib5+ expression plasmid

A hypothetical open reading frame (accession number T40995) was amplified by PCR using *S. pombe* cDNA as template and the oligonucleotides SSP-RIB5-1 and SSP-RIB5-2 (Table 2) as primers. The amplificate was digested with *Eco*RI and *BamH*I and was ligated into the expression vector pNCO113 which had been digested with the same enzymes yielding the plasmid designated *pNCO-SSP-Rosy-WT*.

Site directed mutagenesis

A procedure described earlier was used for site-directed mutagenesis [18]. Plasmid *pNCO-SSP-Rosy-WT* was used as template. The primers used for mutagenesis are shown in Table 2.

Transformation of E. coli *cells*

E. coli XL-1 Blue cells were transformed with ligation mixtures by published procedures [34]. Transformants were selected on LB agar plates supplemented with ampicillin (170 mg/l). All plasmid constructs were sequenced by the automated dideoxynucleotide method [35] using a 377 Prism DNA sequencer from Applied Biosystems (Weiterstadt, Germany).

pNCO113 type plasmids reisolated from XL-1 Blue cells were transformed into *E. coli* M15 [pREP4] [36] cells carrying the pREP4 repressor plasmid for the overexpression of *lac* repressor protein. Kanamycin (15 mg/l) and ampi-

Table 3: Steady state kinetic analysis of wild type and mutant riboflavin synthases

enzyme	v_{max} [nmol mg^{-1} min^{-1}]	K_M [μM]
wild type	158 ± 2.9	5.7 ± 0.24
S146A	183 ± 9.5	3.8 ± 0.40
S146C	179 ± 7.0	8.9 ± 1.06
C48S	27 ± 1	1.1 ± 0.13
C48A	< 0.1	
C48M	< 0.1	

cillin (170 mg/l) were added to secure the maintenance of both plasmids in the host strain. In the pNCO113 expression plasmids, the riboflavin synthase variants are under control of the T5 promotor and the *lac* operator.

pT7-7 type plasmids reisolated from XL-1 Blue cells were transformed into *E. coli* BL21 (DE3) cells. The host strain contains a T7 RNA polymerase gene under the control of a *lac* operator, whereas the riboflavin synthase genes are under control of the T7 promotor.

Protein purification

Recombinant *E. coli* strains were grown in LB medium containing ampicillin (170 mg/l) and kana-mycin (20 mg/l) at 37°C with shaking overnight. Erlenmeyer flasks containing 500 ml of medium were inoculated at a ratio of 1:50 and were incubated at 37°C with shaking. At an optical density of 0.6 (600 nm), IPTG was added to a final concentration of 2 mM, and incubation was continued for 5 h. The cells were harvested by centrifugation, washed with 0.9 % NaCl and stored at -20°C. Frozen cell mass (6 g) was thawed in 35 ml of 20 mM potassium phosphate, pH 7.0. The suspension was subjected to ultrasonic treatment and was then centrifuged (20 min, 15,000 rpm, 4°C). The supernatant was placed on top of a Q-Sepharose column (2.0 × 7.5 cm, Amersham Phar-macia Biotech, Freiburg, Germany) (flow rate, 5 ml min^{-1}) which had been equilibrated with 200 mM potassium phosphate, pH 7.0. The column was developed with a linear gradient of 0–1.0 M potassium chloride in 20 mM potassium phosphate, pH 7.0 (total volume, 700 ml). Fractions were combined and concentrated by ultrafiltration using Amicon 10 kDa membranes. The solution was placed on top of a Superdex-200 column (2.6 × 60 cm, Amersham Pharmacia Biotech, Freiburg, Germany) (flow rate, 3 ml min^{-1}) which was developed with 20 mM potassium phosphate, pH 7.0, containing 70 mM potassium chloride. Fractions were combined and placed on top of a column of Phenyl-Sepharose CL4B (1.5 × 5.5 cm, Amersham Pharmacia Biotech, Freiburg, Germany) (flow rate, 2 ml min^{-1}) which had been equilibrated with 20 mM potassium phosphate, pH 7.0, containing 500 mM ammonium sulfate. The column was developed with a linear gradient of 0–10 % glycerol containing 20 mM potassium phosphate, pH 7.0 (total volume, 100 ml).

Isolation and purification of lumazine protein

Inclusion bodies were isolated from the recombinant *E. coli* strain BL21(DE3) [pPHL36] and were solubilized as described earlier [37]. A solution (25 ml) containing 50 mM Tris hydrochloride, pH 7.2, 0.5 mM DTT and solubilized protein was passed through a column of Q Sepharose Fast Flow (2 × 18 cm, Amersham Pharmacia Biotech, Freiburg, Germany) (flow rate, 1 ml min^{-1}). The column was washed with 100 ml of 50 mM Tris hydrochloride, pH 7.2, containing 0.5 mM DTT and was then developed with a linear gradient of 0 – 0.5 M NaCl in the same buffer (total volume, 280 ml). Lumazine protein was eluted from 220 to 260 ml. The enzyme fraction was dialyzed against 50 mM sodium/potassium phosphate, pH 7.0, containing 5 mM 2-mercaptoethanol. The solution was centrifuged for 30 min at 26,000× g, and the supernatant was passed through a column of Superdex 75 (2.6 × 60 cm, Amersham Pharmacia Biotech, Freiburg, Germany) (flow rate, 3 ml min^{-1}). The column was developed with 360 ml of 50 mM phosphate, pH 7.0, containing 5 mM DTT. The enzyme was eluted from 180 to 200 ml. Fractions were combined and concentrated by ultrafiltration.

Estimation of protein concentration

Protein concentration was estimated by a modified Bradford procedure [38].

SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with 16 % polyacryl-amide gels by published procedures [39]. Molecular weight standards were supplied by Sigma (Munich, Germany).

Protein sequencing

Sequence determination was performed by the automated Edman method using a 471 A Protein Sequencer (Perkin Elmer).

Steady state kinetics

Assay mixtures containing 100 mM phosphate, pH 7.2, 10 mM EDTA, 10 mM sodium sulfite, 0.6 mM 6,7-dimethyl-

8-ribityllumazine, and protein were incubated at 37°C. Formation of riboflavin was monitored photometrically at 470 nm. The absorbance coefficient for riboflavin is 9,100 M^{-1} cm^{-1} at 470 nm [7,40].

Analytical ultracentrifugation

Boundary sedimentation and sedimentation equilibrium experiments were performed using an analytical ultracentrifuge Optima XL-I from Beckman Instruments (Palo Alto, CA) equipped with absorbance and interference optics. Aluminum double sector cells equipped with sapphire windows were used throughout. Protein solutions were dialyzed against 20 mM potassium phosphate, pH 7.0, containing 100 mM potassium chloride. The partial specific volume was estimated from the amino acid composition yielding a value of 0.73 ml g^{-1} [41]. Sedimentation equilibrium experiments were performed with a solution containing 20 mM potassium phosphate, pH 7.0, 100 mM potassium chloride, and protein (0.7 mg ml^{-1}). Samples were centrifuged at 11,000 rpm and 4°C for 72 h. Protein concentration was monitored photometrically at 280 nm. For boundary sedimentation experiments the protein (1.6 mg ml^{-1}) was centrifuged at 55,000 rpm and 20°C for 2 h. Protein concentration was monitored interferometrically at intervals of 10 min.

Electrospray mass spectrometry

Experiments were performed with a triple quadrupol ion spray mass spectrometer API365 (SciEx, Thornhill, Ontario, Canada) [42].

NMR spectroscopy

^{19}F NMR spectra were recorded at 470 MHz using an AVANCE 500 MHz spectrometer from Bruker Instruments (Karlsruhe, Germany) equipped with a ^{19}F probehead. Experimental parameters were as follows: pulse angle, 70° (10 μsec); repetition rate, 3 sec; 64 K data set; 500 to 20,000 scans. Chemical shifts were referenced to an external standard containing 50 mM sodium trifluoroacetate, pH 7.0. Measurements were performed at 11 °C. Samples contained 20 mM phosphate, pH 7.0, and 100 mM potassium chloride in 10 % D_2O.

Authors contributions

The study was designed and coordinated by MF and AB. The study was performed as follows: AKS, BI, KK, MF, WE contributed to the NMR part of the manuscript. MF cloned and overexpressed the protein. RF, AKS purified the protein. MC synthezised the ligands used in this study. BI constructed the mutant proteins. SS, SG, RH, GR participated in the discussion of the study.

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