(Trifluoromethyl)lumazine Derivatives as ^{19}F NMR Probes for Lumazine Protein†

Johannes Scheuring,‡ John Lee,§ Mark Cushman,⊥ Hemantkumar Patel,⊥ Donald A. Patrick,⊥ and Adelbert Bacher*,‡

Institut für Organische Chemie und Biochemie, Lehrstuhl III, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Federal Republic of Germany, Department of Biochemistry, University of Georgia, Athens, Georgia 30602, and Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907

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ABSTRACT: Lumazine protein acts as an electronic excited state transducer in bioluminescence of *Photobacterium* species. The protein binds 6,7-dimethyl-8-(D-ribityl)lumazine (**1**) which serves as the fluorophore. This compound also serves as a biosynthetic precursor of riboflavin and is the substrate of the enzyme riboflavin synthase. This enzyme and lumazine protein show considerable sequence homology. The interaction of lumazine apoprotein with several trifluoromethyl analogs of 6,7-dimethyl-8-ribityllumazine was investigated by ^{19}F NMR spectroscopy. Upon binding to the protein, the ^{19}F NMR resonances of the ligand shift to lower field with broadened line widths to around 30 Hz. By comparison, all ligands studied show more complex NMR spectra when bound to riboflavin synthase. Only one position 7 epimer (designated epimer A) of 6,7-bis(trifluoromethyl)-7-hydroxy-8-(D-ribityl)lumazine binds to lumazine apoprotein and riboflavin synthase. The apoprotein can also bind lumazine derivatives with a quarternary C-7. It is suggested that the binding site of lumazine protein corresponds to the donor binding site of riboflavin synthase.

In the brightly bioluminescent bacteria belonging to the genus *Photobacterium*, the origin of the emission is the fluorescent state of lumazine protein, a 21-kDa protein overproduced by these cells [for reviews, see Lee et al. (1991) and Lee (1993)]. In the *in vitro* reaction involving purified luciferase (77 kDa) together with its substrates $FMNH_2$, O_2, and tetradecanal, the fluorescent species producing the bioluminescence is a flavin intermediate bound to the luciferase. The involvement of the phototransducer, lumazine protein, explains why the *in vivo* bioluminescence maximum, 475 nm, differs from that of the luciferase reaction, 495 nm.

Lumazine proteins have been obtained from several strains of *P. phosphoreum* and *P. leiognathi* and have been studied in considerable detail by Lee and co-workers (Gast & Lee, 1978; Lee, 1982; Vervoort et al., 1983; O'Kane et al, 1985). The protein is monomeric and binds one molecule of 6,7-dimethyl-8-(1′-D-ribityl)lumazine (**1**), hence its name (Koka & Lee, 1979; O'Kane & Lee, 1985). The binding is noncovalent and reversible, with a K_D in the range 10–50 nM at 2 °C, depending on the type (Lee et al., 1985).

Besides this fluorophore function, this lumazine derivative also serves as the precursor to riboflavin in its biosynthetic pathway [for reviews, see Plaut (1971), Plaut et al. (1974), and Bacher (1990)]. Dismutation of the lumazine **1** yields riboflavin (**4**) and the pyrimidine **5** which is recycled in the biosynthetic pathway. The reaction is catalyzed by the enzyme riboflavin synthase (Scheme 1).

Reaction mechanisms for riboflavin synthase have been proposed by Plaut, Wood, and their co-workers (Plaut & Harvey, 1971; Plaut & Beach, 1976; Paterson & Wood, 1972). The enzyme reaction involves the transfer of a four-carbon moiety from one lumazine molecule serving as donor to a second lumazine molecule serving as acceptor. It is assumed that the donor molecule is attacked in the initial reaction step by a nucleophile (Nu), e.g., water, at position 7 of the pteridine ring yielding the adduct **3** (Nu = OH). This species then undergoes nucleophilic attack at C-6 by the anion **2** which is formed by deprotonation of the methyl group at C-7 of the acceptor molecule. Further transformations yield the products **4** and **5** (Scheme 1). This mechanism requires binding of the donor and acceptor substrate molecules in proximity.

Riboflavin synthase from *Bacillus subtilis* is a trimer of identical 24-kDa subunits (Bacher et al., 1980). Earlier studies showed a remarkable correspondence in the ligand binding stereospecificity of riboflavin synthase and lumazine protein (Plaut & Beach, 1976; Otto & Bacher, 1981; O'Kane et al., 1990; Lee et al., 1992). This implied a similarity in the structure of the binding site between the two proteins. This hypothesis is supported by the evolutionary relationship of the two proteins, based on the comparison of their primary sequences (Schott et al., 1990; O'Kane et al., 1991; O'Kane & Prasher, 1992).

Riboflavin synthase of *B. subtilis* and lumazine protein show extensive sequence similarity. Of the lumazine protein's 189 amino acid residues, 27% are identical to riboflavin synthase and 17% are structurally conservative replacements (O'Kane et al., 1991; O'Kane & Prasher, 1992).

The sequences of both proteins show extensive internal similarity between their respective N-terminal and C-terminal portions. Whereas three-dimensional structures are not available, the close internal homology suggests that each peptide folds into two similar domains. It has been proposed that riboflavin synthase binds the two substrate molecules at the interface of the two domains and that each of the domains provides the environment for one of the adjacent substrate molecules at the active site (Schott et al., 1990).

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‡ Technische Universität München.
§ University of Georgia.
⊥ Purdue University.

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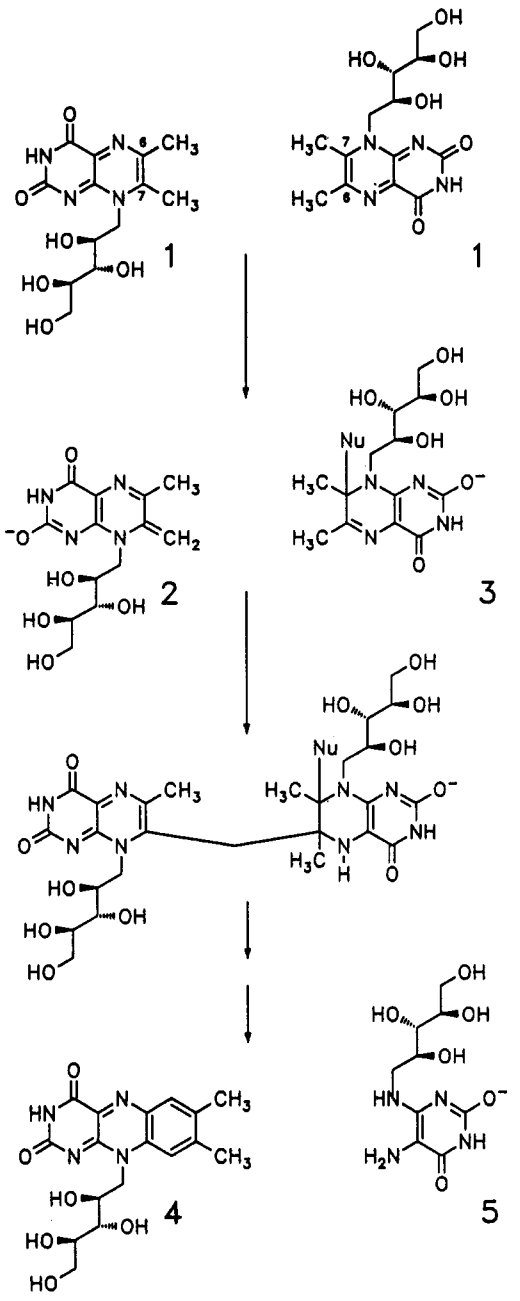
Scheme 1



In an attempt to find inhibitors of riboflavin synthase which could be useful as antimicrobial agents, we have recently prepared a series of trifluoromethyl derivatives of 8-ribityllumazine. Whereas these compounds had only inhibition constants in the range of 20–80 μM, they were found to be highly suitable ^{19}F NMR probes in order to study the enzyme/ligand interaction. A hypothetical reaction mechanism involving conformational changes in the protein was proposed on the basis of these data (Cushman et al., 1991, 1992, 1993).

Lumazine protein, which has no known enzyme activity, can be considered as a simplified model of riboflavin synthase. Whereas riboflavin synthase is a trimer and can bind two substrate molecules per subunit, lumazine protein is a monomer and binds only one ligand molecule. It should therefore be of interest to compare the two proteins with regard to their interaction with (trifluoromethyl)lumazine derivatives. This paper reports ^{19}F NMR studies using lumazine protein from *P. phosphoreum*.

MATERIALS AND METHODS

Chemicals. 6,7-Dimethyl-8-(D-ribityl)lumazine was prepared as described by Plaut and Harvey (Plaut & Harvey, 1971). The preparation of the following compounds has been described previously: 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (**6**) (Cushman et al., 1992), 6-(trifluoromethyl)-8-(D-ribityl)lumazine (**7**) (Cushman et al., 1993), 6,7-bis(trifluoromethyl)-8-(D-ribityl)lumazine hydrate (**9**) (Cushman et al., 1991), and 6-(trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine (**10**) (Cushman et al., 1992).

Protein. Lumazine protein was purified from *P. phosphoreum* strain P by published procedures (O'Kane et al., 1985). Apoprotein was prepared by ultrafiltration in the presence of urea as described earlier (O'Kane & Lee, 1985; Lee et al., 1992).

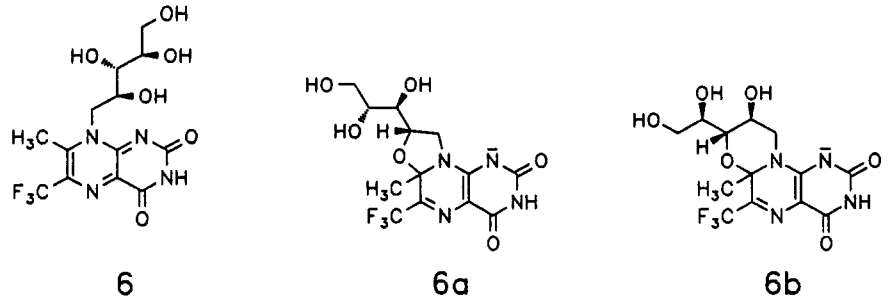
NMR Spectroscopy. ^{19}F NMR spectra were recorded at 338 and 235 MHz using AM 360 and AC 250 instruments from Bruker, Karlsruhe, Federal Republic of Germany. Measurements were performed at 4 °C and at 24 °C using a variable-temperature probe. The experimental conditions were as follows: pulse angle, 30° (2 μs); repetition time, 1.0 s; 8K data set; line broadening as indicated.

Determination of Binding Isotherms. Aliquots of ligand solution were added to samples of lumazine apoprotein in 0.5 mL of 50 mM phosphate buffer, pH 7.0. After each addition, a ^{19}F NMR spectrum was recorded. The ratio of bound and free ligand was obtained from ^{19}F NMR signal integrals. The concentration of functionally competent apoprotein (Lee et al., 1992) was determined from the integral of the bound ligand's resonance at ligand saturation.

RESULTS

6-(Trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (**6**, Chart 1) is a close structural analog of 6,7-dimethyl-8-(D-ribityl)lumazine (**1**, Scheme 1), the natural ligand of lumazine protein. However, the pK of the fluoroanalog is 6.5 (Cushman et al., 1992) as compared to the pK of 8.4 of the natural ligand (Bown et al., 1986). Thus, the fluoroanalog at neutral pH comprises a mixture of the neutral molecule form and several anionic species giving rise to separate ^{19}F NMR signals (Figure

Chart 1



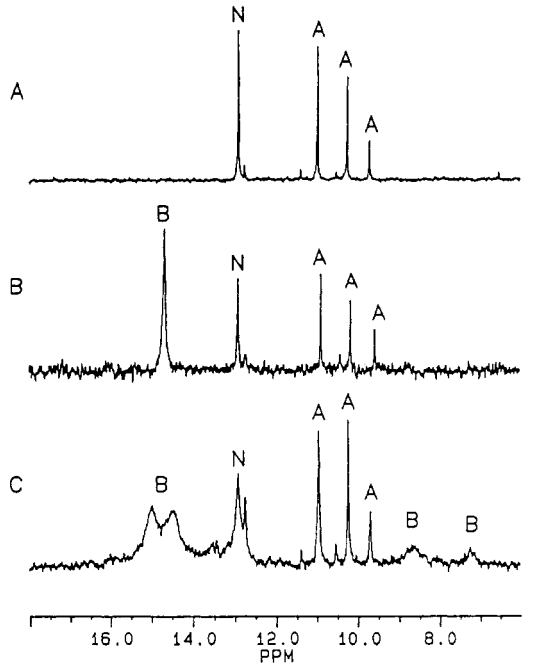


FIGURE 1: ^{19}F NMR spectra of 6-(trifluoromethyl)-7-methyl-8-(D-ribityl)lumazine (**6**). Spectra of (A) the free compound, (B) bound to lumazine protein, and (C) bound to riboflavin synthase. Sample B contained 115 μM ligand and 100 μM lumazine protein. Measurements were performed in 50 mM phosphate buffer, pH 7.0, at 4 °C. Sample C contained 930 μM ligand and 300 μM protein. The measurements were performed in 170 mM phosphate buffer, pH 6.8 at 24 °C. B: bound ligand. N: free neutral form of **6**. A: free anionic species. The line broadening was 2 Hz.

1A). The structures of the anions have not been studied in detail but they are likely to involve a reaction of the chromophore with one of the hydroxyl groups of the side chain to form 5- or 6-membered rings (diastereomeric forms of **6a** and **6b**, Chart 1) in analogy to earlier observations made with 6,7-dimethyl-8-(D-ribityl)lumazine (**1**) (Pfleiderer et al., 1971; Beach & Plaut, 1971; Bown et al., 1986).

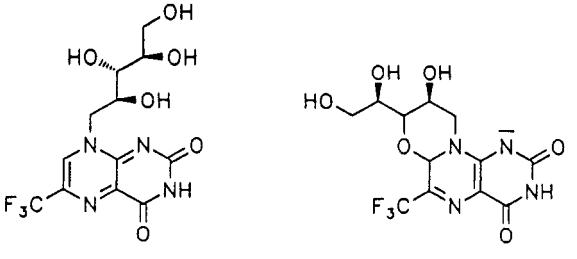
The ^{19}F NMR spectrum of **6** in the presence of lumazine apoprotein at pH 7.0 is shown in Figure 1B. The NMR signal at 12.9 ppm represents the free neutral molecule (designated N), and the signals at 10.9, 10.2, and 9.6 ppm represent free monoanionic species (designated A). The signal at 14.6 ppm corresponds to the bound ligand (designated B). This signal has a line width of about 25 Hz. The signal of the free neutral molecule is broadened to about 12 Hz in the presence of the protein. In contrast, the signals corresponding to the various free anions in Figure 1B have line widths around 8 Hz. This may signify that the signal of the free neutral molecule, but not those of the free anion forms of the ligand, are broadened by chemical exchange with the protein bound form, thus suggesting that the protein binds only the neutral molecule and not the anions. This may explain the presence of only a single signal corresponding to the bound form. In contrast, the spectrum of **6** in the presence of light riboflavin synthase shows, besides two minor signals at 7.2 and 8.2 ppm, two broadened signals at 14.5 and 15.0 ppm (Figure 1C).

Titration of lumazine apoprotein with **6** was monitored by ^{19}F NMR and yielded a linear Scatchard plot (data not shown) consistent with a binding constant of 30 μM. Thus, the affinity of the protein for **6** is smaller by about 3 orders of magnitude as compared to the natural ligand (**1**) at the same pH (Lee et al., 1985). This observation parallels the reduced affinity of the fluoro derivative for riboflavin synthase by comparison with the natural substrate. The ^{19}F NMR signal of the bound ligand is completely quenched by the addition of the natural ligand **1**, thus indicating that the fluorinated ligand is bound reversibly at the chromophore-binding site of lumazine protein (data not shown).

6-(Trifluoromethyl)-8-(D-ribityl)lumazine (**7**, Chart 2) is also closely related to **1**. However, the compound forms predominantly one anion corresponding to one diastereomer of structure **7a** (Cushman et al., 1993) (Chart 2). Perturbation of the ^{19}F NMR spectrum of **7** by lumazine apoprotein is shown in Figure 2a. Signals at 8.7 ppm (designated N) and at 7.9 ppm (designated A) represent the free neutral molecule and the free anion, respectively. The signal of the free neutral molecule is broadened to 20 Hz in the presence of the protein, whereas the free anion signal is not broadened appreciably. A signal pertaining to the enzyme-bound species is seen at 10.0 ppm. This signal has a line width of about 30 Hz. It appears likely that this signal represents the neutral molecule bound to the lumazine protein. A minor, relatively broad signal is also observed at 11.1 ppm.

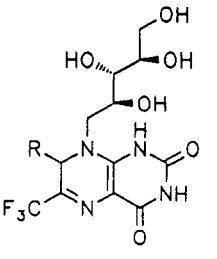
The lumazine **7** can react with a variety of sulfur compounds such as sulfite, sulfide, and organic thiols (Cushman et al., 1993). These compounds can add to C-7 of the lumazine ring with formation of a C–S bond (Chart 2). Formation of diastereomers is expected in each case and has been demonstrated by ^{19}F NMR in the case of the sulfite adduct, although the ^{19}F chemical shifts of the two diastereomers differ only by 0.05 ppm. The formation of the adducts is reversible, and the equilibrium constants have been reported. The derivatization with various sulfur compounds yields

Chart 2



7

7a



	R
8a	SO_3^-
8b	S^-
8c	SCH_2CH_2OH

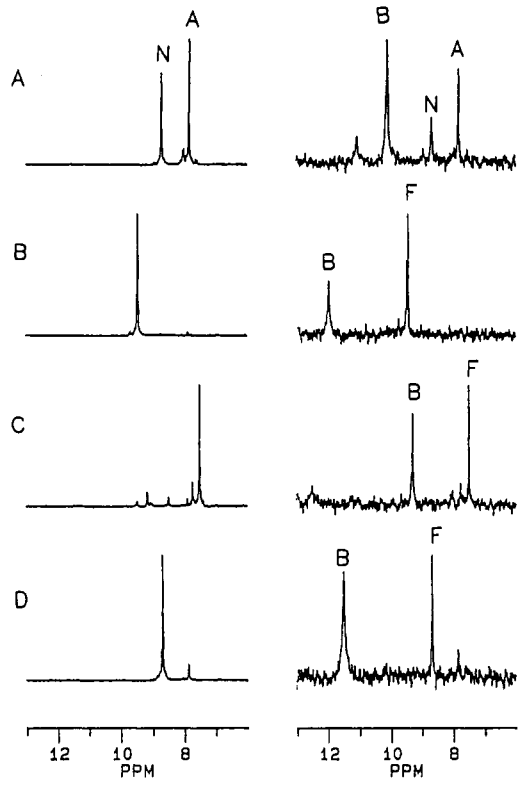


FIGURE 2: ^{19}F NMR spectra of **7** and the products obtained by the addition of sulfur compounds to 6-(trifluoromethyl)-8-(D-ribityl)-lumazine. Left: spectra of the free compounds. Right: spectra in the presence of lumazine protein. Key: A, 120 μM **7**; B, 60 μM **8a** and 20 mM sulfite; C, 100 μM **8b** and 20 mM sulfide; D, 85 μM **8c** and 50 mM mercaptoethanol; 100 μM lumazine protein. The measurements were performed in 50 mM phosphate buffer, pH 6.8 at 4 °C. B: bound ligand. F: free ligand (**8a-c**). N: free neutral **1**. A: free anion **7a**. The line broadening was 10 Hz.

derivatives of **3** which differ by their bulkiness in the environment of C-7. All of these derivatives are characterized by quaternary C-7.

All adducts shown in Chart 2 can be bound by riboflavin synthase of *B. subtilis*. The bound species show complex multiplets as described earlier (Cushman et al., 1993). As shown in Figure 2B–D, these compounds can also bind to lumazine apoprotein. In each case, the bound species contributes a singlet with a downfield shift of 1.5–3 ppm, and the signal of the bound ligand is broadened to about 30 Hz. Moreover, the signals of the free ligand are broadened to about 10–20 Hz. The signals of the bound ligands are quenched by the addition of **1**. These data give additional support to the close similarity between riboflavin synthase and lumazine protein. Apparently, both proteins can tolerate relatively bulky substrates at position seven of the Michael-type adducts.

Binding isotherms for **7**, **8a**, and **8c** were determined by ligand titration of lumazine apoprotein monitored by ^{19}F NMR. Scatchard plots are shown in Figure 3. All compounds are characterized by straight lines in the Scatchard plots. The binding of the adduct **8c** is considerably tighter than **7** whereas the adduct **8a** shows a reduced affinity (Table 1).

The presence of two trifluoromethyl groups at positions 6 and 7 of the lumazine chromophore stabilizes the two covalently hydrated diastereomers **9a** and **9b** (Chart 3), which can be separated by HPLC (Cushman et al., 1986a,b, 1991). Since the absolute configurations of the diastereomers are as yet unknown, they are designated epimer A and B on the basis of their HPLC retention times.

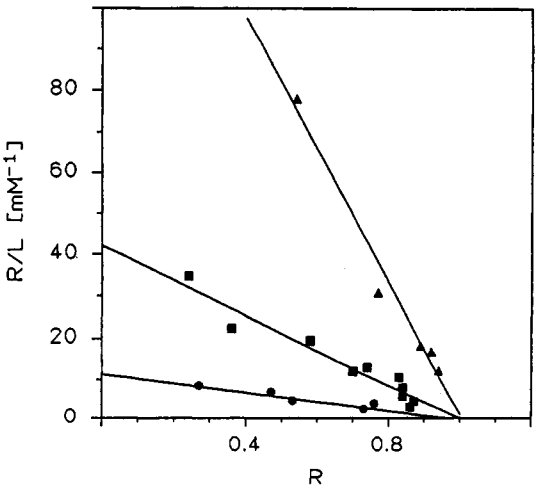


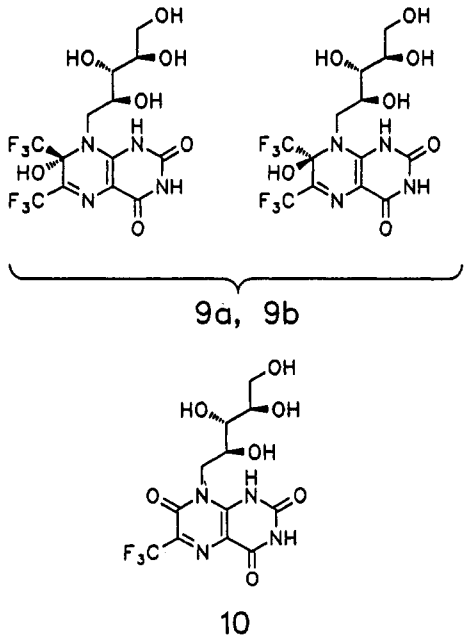
FIGURE 3: Binding of 6-(trifluoromethyl)-8-(D-ribityl)lumazine and sulfur adducts to lumazine protein of *P. phosphorum*. Scatchard plot data from titration experiments monitored by ^{19}F NMR spectroscopy at 4 °C, pH 7.0: ■, **7**; ●, **8a**; ▲, **8c**.

Table 1: Chemical Shifts and Binding Constants of the Complexes between Lumazine Protein and (Trifluoromethyl)lumazines

	^{19}F chemical shift (ppm[a])		
compd	free ligand	bound ligand	K_D (μM) at 4 °C
6	12.9, 10.9, 10.2, 9.6	14.6	30
7	8.7, 7.9	10.2	24
8a	9.45, 9.50	11.9	88
8b	7.5, 7.7	9.0	about 50
8c	8.68	11.6	6
9 (epimer A)	12.3, −8.0	16.1, −6.5	5
9 (epimer B)	12.3, −7.5	nb[b]	nb[b]
10	7.8	10.0	3[c]

[a] Referenced to trifluoroacetate, pH 7.0, in D_2O. [b] No binding detected. [c] At 24 °C.

Chart 3



9a, 9b

10

We have shown earlier that epimer A but not epimer B binds to riboflavin synthase of *Bacillus subtilis*. Thus, binding is highly diastereospecific with respect to C-7. Bound epimer A displays a complex NMR pattern in which the trifluoromethyl groups correspond to two or three broad signals, respectively (Figure 4B).

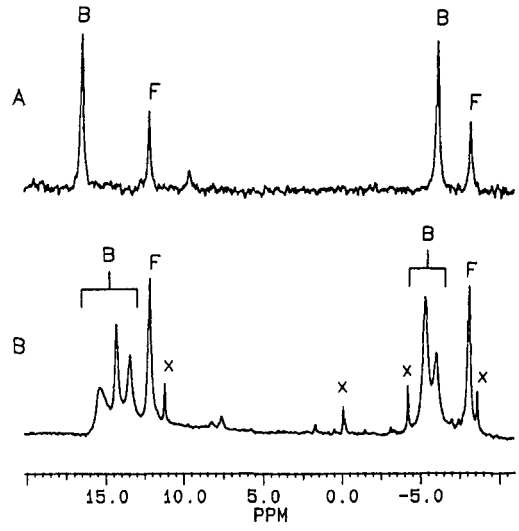


FIGURE 4: ^{19}F NMR spectra of 6,7-bis(trifluoromethyl)-7-hydroxy-8-(D-ribityl)lumazine (**9**) (epimer A) bound to (A) lumazine protein of *P. phosphoreum* and (B) light riboflavin synthase of *B. subtilis*. Sample A contained 50 μM protein and 50 μM of ligand; sample B contained 225 μM protein and 950 μM ligand, pH 6.8: A, 4 °C; B, 25 °C. B: bound ligand. F: free ligand. x: impurities. The line broadening was 10 Hz.

We have shown earlier that riboflavin synthase and lumazine protein have similar stereospecificities with respect to the 8-alditiyl side chain of **1** (O'Kane et al., 1990; Lee et al., 1992). It was therefore of interest to analyze whether the similar diastereoselectivity of the two proteins extends to the heterocyclic ring system.

Figure 4A shows the ^{19}F NMR spectrum of epimer A bound to lumazine apoprotein. The trifluoromethyl groups at C-6 and C-7 of the free ligand (F) yield the signals at 12.3 and −8.0 ppm. Signals representing the protein-bound ligand appear at 16.5 and −6.0 ppm. The enzyme-bound signals have a line width of about 40 Hz. The signals of the free ligand are appreciably broadened to about 30 Hz in the presence of the protein. Again, the signals of the bound fluorolumazine disappeared by the addition of the natural ligand **1** (data not shown). The perturbation of epimer A by riboflavin synthase is shown for comparison (Figure 4B). Both trifluoromethyl groups of the bound ligand yield multiple signals with line widths in the range of 100 Hz.

A series of ^{19}F NMR spectra was acquired during a titration of lumazine apoprotein with epimer A. The integrals of the free and bound signals afforded a linear Scatchard plot resulting in a binding constant of about 5 μM (Table 1).

Both the chemical shifts and the line widths of the ^{19}F NMR signals of epimer B are completely unperturbed by lumazine protein. It follows that the protein displays absolute diastereospecificity, precisely as riboflavin synthase. Upon the addition of epimer A to a mixture of epimer B and lumazine apoprotein, the characteristic signals of bound epimer A appeared, and it was thus obvious that the apoprotein was intact and capable of binding appropriate ligands.

6-(Trifluoromethyl)-7-oxo-8-(D-ribityl)lumazine (**10**, Chart 3) is isosteric with 6-methyl-7-oxo-8-(D-ribityl)lumazine which is a tight-binding inhibitor of riboflavin synthase (Winestock et al., 1963; Al-Hassan et al., 1980). Compound **10** binds to lumazine apoprotein, and Scatchard analysis results in a K_D of about 3 μM at 24 °C (Table 1). Binding of **10** to lumazine apoprotein is accompanied by a downfield shift of about 2 ppm. The signal of the bound ligand has a line width of about 20 Hz, whereas the linewidth of the free ligand was about 10 Hz (Figure 5A).

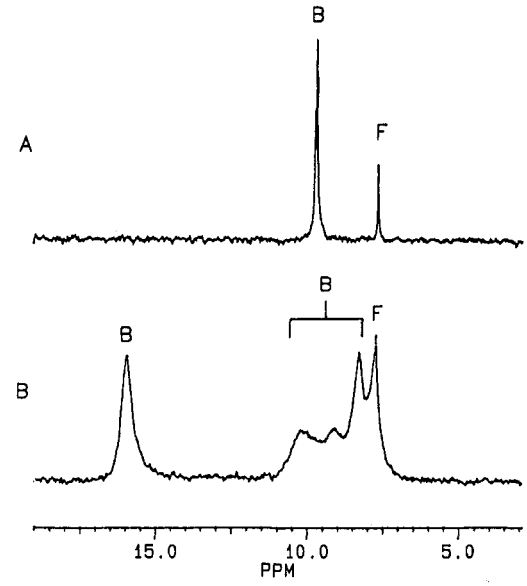


FIGURE 5: ^{19}F NMR spectra of 6-trifluoro-7-oxo-8-(D-ribityl)-lumazine (**10**) bound to (A) lumazine protein of *P. phosphorum* and (B) light riboflavin synthase of *B. subtilis*. Sample A contained 150 μM protein and 200 μM ligand, sample B contained 250 μM protein and 1140 μM ligand. B: bound ligand. F: free ligand. The line broadening was 10 Hz.

The ^{19}F NMR pattern of **10** bound to riboflavin synthase is shown for comparison (Figure 5B). The ^{19}F NMR signature of the bound ligand is very complex and consists of three signals around 8–11 ppm and an additional signal with a large downfield shift at 16 ppm. The enzyme has been shown to bind two molecules of this ligand per subunit, presumably at the donor and acceptor site of the catalytic center (Cushman et al., 1992). In contrast, no evidence for binding of more than one ligand molecule was obtained with lumazine apoprotein.

All fluorolumazines studied could be displaced from lumazine protein by the natural ligand, 6,7-dimethyl-8-ribityllumazine, as demonstrated by NMR spectroscopy (data not shown). It follows that all fluorolumazines bind reversibly at the ligand binding site of lumazine apoprotein.

DISCUSSION

The primary structures of riboflavin synthase and lumazine protein indicate that the two proteins are related by a divergent evolutionary process. The riboflavin derivatives, FMN and FAD, serve as cofactors for a wide variety of redox enzymes. It has therefore been argued that flavocoenzymes originated at an early period of biochemical evolution. Moreover, it appears likely that the phototransducer, lumazine protein, is of more recent origin. This protein could have evolved by gene duplication followed by loss of enzymatic function (O'Kane et al., 1991; O'Kane & Prasher, 1992).

Apparently, the highly fluorescent riboflavin precursor **1** has found a second function as the chromophore of an electronic excitation transducer, and riboflavin synthase, the enzyme catalyzing its conversion to the vitamin, has been remodeled to serve as the apoprotein for the fluorescence function. Whereas riboflavin synthase is a trimer and binds two ligand molecules per subunit (Bacher et al., 1980; Otto & Bacher, 1981), lumazine protein is a monomer and binds only one ligand molecule (O'Kane & Lee, 1985).

A comparison between the two proteins must address the following features: complexity of the ^{19}F NMR signal pattern,

chemical shift perturbation, line width, and stereoselectivity of the protein–ligand interaction.

The major difference observed between the two proteins is the multiplicity of protein-bound signals. Rather narrow singlets are observed for trifluoromethyl groups of ligands bound to the monomeric lumazine apoprotein even in cases where multiple anionic forms or two diastereomeres are present in equilibrium. In contrast, two to four maxima extending over a chemical shift range of 1–8 ppm are observed with riboflavin synthase. The multiplicity of signals seen when the fluorolumazines are bound to riboflavin synthase provides evidence that the three binding sites of the trimer may be structurally nonequivalent. The multiple ^{19}F NMR signals are not the result of enzyme heterogeneity, since identical spectra are observed with recombinant, homogeneous enzyme preparations (to be published elsewhere).

In both proteins under study, the ^{19}F NMR signals of all bound ligands show a moderate low-field shift of 1–5 ppm. Only the 7-oxo compound **10** shows one signal component with an exceptionally large low-field shift of 8 ppm when bound to riboflavin synthase (Cushman et al., 1992).

Fluorolumazines bound to lumazine apoprotein are characterized by a line width of about 30 Hz. Riboflavin synthase gives considerably broader lines in the range of 150 Hz. This should be due, at least partially, to the larger molecular weight and, consequently, the larger rotational correlation time of light riboflavin synthase. Both proteins also have a tendency to broaden the signals of the free ligands. In cases where the free ligand is present as a mixture of species in slow equilibrium on the NMR time scale, the individual nonbound ligand species are broadened to different extents. The broadening of the free ligand signals is probably due to exchange of the free and bound ligand.

Both riboflavin synthase and lumazine protein tolerate sp^3-hybridized as well as sp^2-hybridized carbon atoms at position 7 of the pteridine chromophore. Moreover, relatively bulky substituents at position 7 (SO_3^-, $S(CH_2)_2OH$, CF_3 and OH) are acceptable to both proteins. Indeed, the affinity for both proteins is increased by bulky substituents at position 7. Both proteins have strong and identical diastereoselectivity as shown with the diastereomers of **9** (epimers A and B).

All fluorolumazine derivatives studied are displaced completely from lumazine protein by the addition of the natural ligand, 6,7-dimethyl-8-(D-ribityl)lumazine. Thus, their binding is reversible and appears to occur at the normal ligand binding site.

The obvious similarities of ligand interaction with lumazine protein and riboflavin synthase appear to result from the extensive sequence homology between the two proteins which may imply a similar folding pattern (O'Kane et al., 1991). As a consequence, the bound ligands would have a similar environment at the binding sites of the two proteins. It appears likely that the binding sites correspond to highly conserved sequence motifs.

With the exception of **10**, the ligands used in this study bind to riboflavin synthase at a stoichiometry of one ligand molecule per protein subunit, but **10** binds at a stoichiometry of two ligand molecules per subunit, and its ^{19}F NMR signature is characterized by an unusual signal shifted far downfield (Cushman et al., 1992). For the mechanism of riboflavin synthase, the formation of an intermediate at the donor site with a quarternary C-7 carbon has been proposed. It has been shown earlier that the enzyme can simultaneously bind two lumazine type derivatives per subunit or one molecule of riboflavin and one molecule of a pyrimidine product analog (Otto & Bacher, 1981; Cushman et al., 1992). We suggest tentatively that the signals with the relatively small downfield shift represent the contribution of ligand bound at the donor site, whereas the signal with the large downfield shift represents ligand bound at the acceptor site. This would be consistent with the small downfield ^{19}F NMR shifts of bound **9** (epimer A) relative to free **9** (epimer A), which is assumed on the basis of its structure to bind to the donor site.

The evolution of lumazine protein has involved two major changes: (1) the formation of stable monomer from a trimer and (2) the apparent loss of one of the two binding sites. In parallel with riboflavin synthase, lumazine apoprotein can still bind compounds with a quaternary carbon at C-7. This appears to be a remnant property as a result of the evolution of this protein and is not useful for its purpose because reaction intermediate **3** is nonfluorescent and this would not be useful for the bioluminescence activity of lumazine protein. However, the fact that lumazine protein can bind ligands with quaternary carbon at C-7, in conjunction with the small downfield shift of bound **10** relative to free **10**, suggests tentatively that the binding site of lumazine protein corresponds to the donor site of riboflavin synthase.

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