^{19}F NMR Ligand Perturbation Studies on 6,7-Bis(trifluoromethyl)-8-ribityllumazine-7-hydrates and the Lumazine Synthase Complex of *Bacillus subtilis.* Site-Directed Mutagenesis Changes the Mechanism and the Stereoselectivity of the Catalyzed Haloform-Type Reaction

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The riboflavin synthase/lumazine synthase complex of *Bacillus subtilis* catalyzes the last two steps in riboflavin biosynthesis. The protein comprises a capsid of 60 β subunits with lumazine synthase activity and a core of three α subunits with riboflavin synthase activity. The β subunits catalyze the formation of 6,7-dimethyl-8-ribityllumazine (**3**) from 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**) and 3,4-dihydroxy-2-butanone 4-phosphate (**2**). Complexes of recombinant lumazine synthase (β_{60} capsids) with 6-trifluoromethyl-7-oxo-8-ribityllumazine (**10**) as well as 7*S*- or 7*R*-6,7-bistrifluoromethyl-8-ribityllumazine hydrate (**11**) were studied by ^{19}F NMR spectroscopy. Despite the large molecular weight of approximately 960 kDa of the protein, spectra with separated signals of free and bound ligand could be obtained. An unusually large shift difference of 8 ppm was observed between the 7-trifluoromethyl signals of free and bound ligand for epimer B of **11** and the enzyme. The signal is sensitive to the replacement of amino acid residues F22 and H88. Lumazine synthase catalyzes the elimination of the 7-trifluoromethyl group of R-diastereomer epimer A in a haloform-like reaction. The elimination reaction is also catalyzed by F22 mutants. The H88R mutant displays an opposite stereoselectivity for epimer B and a greatly enhanced reaction rate. From a model of the epimers in the active site of the protein, the main function of the side chain of F22 seems to be to keep the substrate ring in the correct position. H88 is in a position suited to act as proton acceptor in both the physiological as well as the haloform reaction. A different mechanism of the haloform-reaction is proposed in the case of the H88R mutant, initiated by hydrogen bonding of the 7-trifluorormethyl group and the guanidinium group of the arginine residue.

Introduction

Riboflavin (vitamin B_2) is biosynthetically assembled from one molecule of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**) and two molecules of 3,4-dihydroxy-2-butanone 4-phosphate (**2**).[1,2] Initially, the pyrimidine **1** is condensed with the carbohydrate **2** by the catalytic action of 6,7-dimethyl-8-ribityllumazine synthase. The enzyme product, 6,7-dimethyl-8-ribityllumazine (**3**), subsequently undergoes an unusual dismutation catalyzed by riboflavin synthase affording riboflavin (**4**) and the pyrimidine **1**, which is recycled by lumazine synthase (Scheme 1).

In Bacillaceae, riboflavin synthase and lumazine synthase form a high molecular weight complex, which has been studied in considerable detail. Sixty lumazine synthase subunits (β subunits), each with a mass of 16 kDa, were shown to form a capsid with icosahedral 532 symmetry. A trimer of riboflavin synthase subunits (α subunits) is enclosed in the central cavity of that capsid. As a consequence of this topology, the formation of riboflavin from the precursors **1** and **2** is characterized by unusual kinetics indicative of substrate channeling between the two different types of subunits.[3]

The catalytic function of the β subunit, i.e., the formation of 6,7-dimethyl-8-ribityllumazine from 3,4-dihydroxy-2-butanone 4-phosphate (**2**) and 5-amino-6-ribitylaminouracil (**3**), was established relatively recently, since it had to await the isolation and structure elucidation of the carbohydrate substrate **2**.[4,5] The enzyme reaction is presumed to be initiated by formation of a Schiff base between the carbohydrate and the 5-amino group of the

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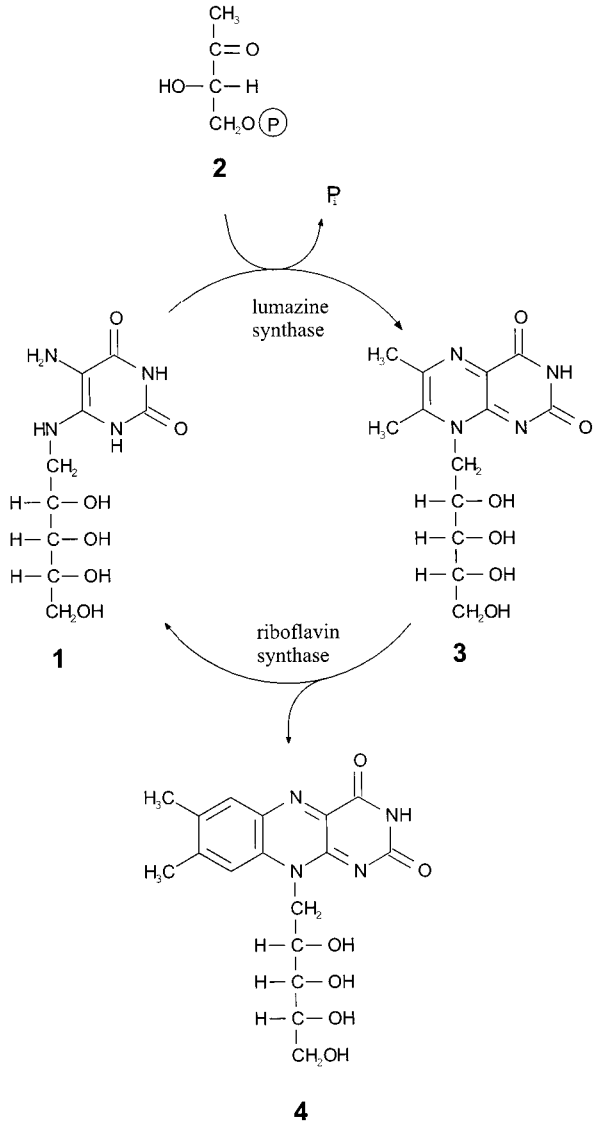
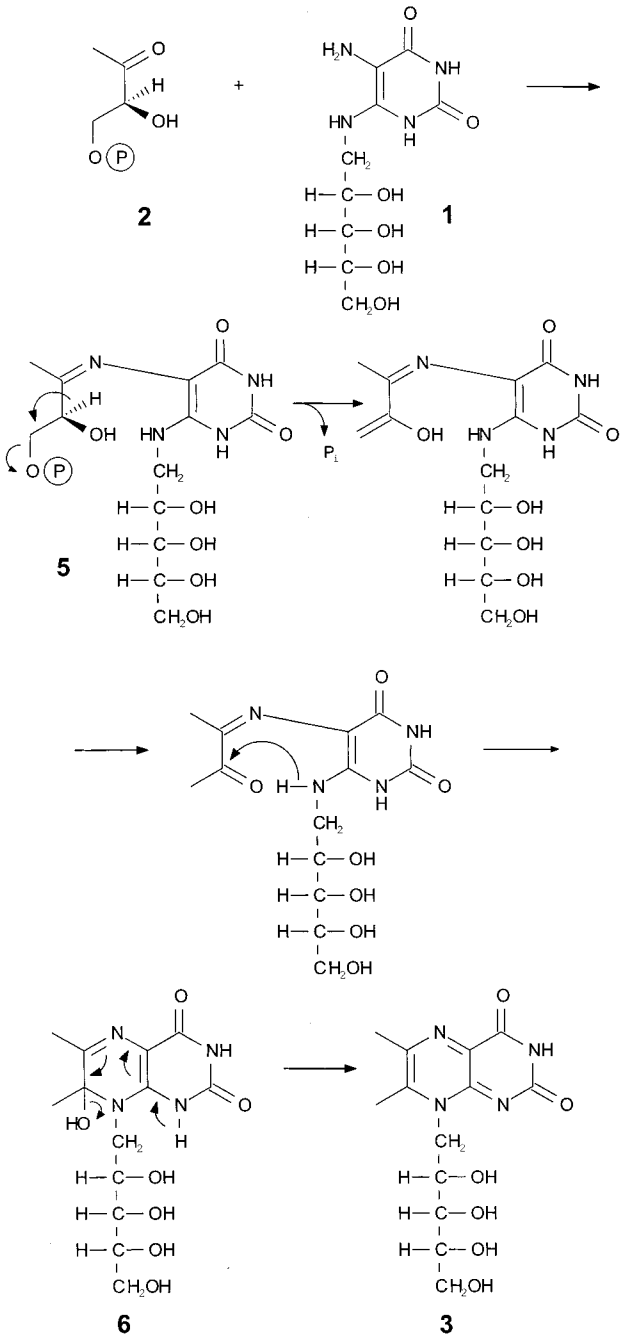
Scheme 1. Biosynthesis of Riboflavin by Lumazine Synthase/Riboflavin Synthase



diaminouracil-type substrate (Scheme 2). Elimination of the phosphate and nucleophilic attack of the secondary amino group at the newly formed carbonyl group yields a 7-hydrate of 6,7-dimethyl-8-ribityllumazine (**6**). The final product **3** is obtained by dehydration.

The three-dimensional structure of the β_{60} capsid has been determined by X-ray crystallography at 2.4 Å resolution.[6] The capsid can be described as an assembly of 12 pentons of β subunits. Crystallization in the presence of the strong binding ligand, 5-nitro-6-ribitylaminouracil (**7**), localized the active site. Each β subunit can bind one molecule of the pyrimidine derivative at its pentamer interface close to the inner capsid wall. In the binding site, the phenyl side chain of residue F22 and the pyrimidindione ring of the ligand are in a stacked position, and they were suggested to form a π-complex. Residue H88 could act as the proton acceptor from a hypothetical intermediate. Both residues are highly conserved in numerous microbial organisms and plants.[7,8]

Scheme 2. Hypothetical Mechanism of Lumazine Synthase



The interaction of lumazine synthase with the pyrimidine derivatives **7** and **8**, the 7-oxolumazine **9**,[9] and a phosphonic acid analogue of the intermediate **5**[10] (Scheme 2) has been studied earlier. The trifluoromethyl-substituted lumazine analogues **10** and **11** have been used in ^{19}F NMR protein perturbation experiments with riboflavin synthase of *B. subtilis* and with the homologous lumazine protein, which participitates in the bioluminescence of certain marine bacteria.[11−13] 6-Trifluoromethyl-7-oxo-8-ribityllumazine **10** is isosteric to 6-methyl-

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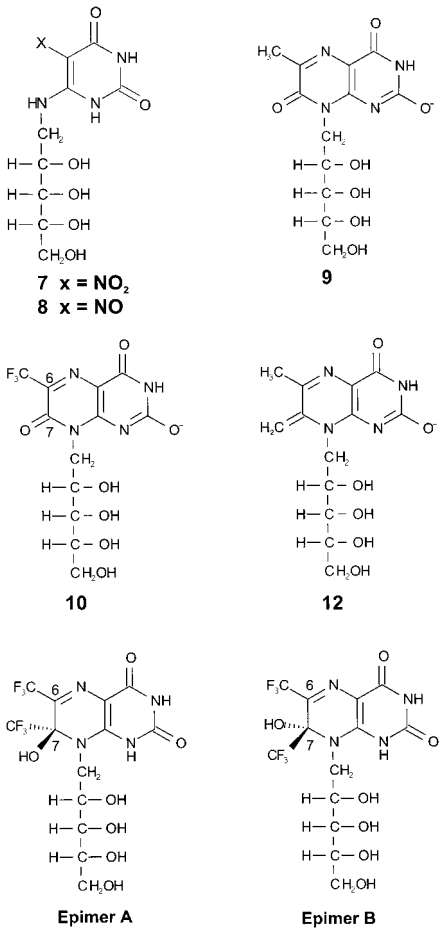
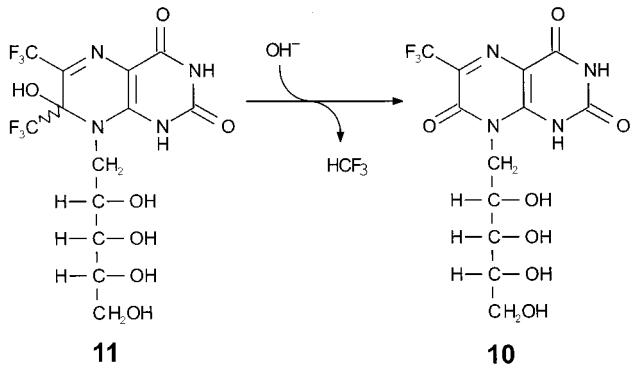


7-oxo-8-ribityllumazine **9**, a high-affinity ligand of the β subunits.[9] It is also an isostere of the exomethylene-type intermediate **12** formed at the acceptor site of riboflavin synthase, which results from deprotonation of the C-7 methyl group. The covalent lumazine hydrate **11** forms two diastereomers, designated epimer A and epimer B, and is structurally similar to the covalent hydrate **6** (Scheme 2) of 6,7-dimethyl-8-ribityllumazine (**3**). Compound **6** has been suggested as an intermediate in the reactions catalyzed by both lumazine synthase and riboflavin synthase. In contrast to the hypothetical reaction intermediate **6**, the covalent hydrates of **11** cannot be dehydrated, nor can any racemization at position 7 of the diastereomers be observed. On the other hand, both epimers of **11** decompose in aqueous solution at increased pH and temperature in a haloform-like reaction yielding the derivative **10** (Scheme 3).[14] Surprisingly, β subunits are able to catalyze the elimination of the 7-CF_3 group stereoselectively for epimer A.

^{19}F nuclei are advantageous for NMR studies on ligand−protein complexes[15] for reasons of (i) their high sensitivity, which is almost comparable to ^{1}H, (ii) absence of signal contribution from protein and solvent, (iii) large chemical shift range, and (iv) high sensitivity of chemical shift to intermolecular perturbation. ^{19}F NMR studies can give insight into the active center of an enzyme and provide information about the direct environment of the ligand.

Scheme 3. Elimination of the 7-Trifluoromethyl Group of 11



In this paper, we describe the extension of our studies using the fluorolumazines as ^{19}F NMR ligand shift perturbation agents on the interaction with *B. subtilis* lumazine synthase wild type and mutant protein.

Results

Lumazine synthase is a complex protein consisting of 60 β subunits with a molecular weight of approximately 960 kDa. Despite the large rotational correlation time expected for such protein−ligand complexes, separate ^{19}F NMR signals for enzyme-bound and free ligand could be observed for the fluorinated lumazine derivatives used in this study (Figure 1). The signals of protein-bound ligands were generally broadened to 250−600 Hz. Signals of free ligands had line widths of 10−60 Hz, which is significantly larger than found in the absence of protein. The line broadening is due to the exchange of free and protein bound ligand. Earlier studies using fluorolumazines as ligands of the 70 kDa enzyme riboflavin synthase and the 23 kDa lumazine binding protein resulted in signals of line widths around 100 and 20 Hz for the bound ligand, respectively.[11−13]

^{19}F NMR Studies on the Wild-Type Enzyme. The lumazine synthase/riboflavin synthase complex from *B. subtilis* as well as recombinant β_{60} subunit assemblies were titrated with the fluorolumazines **10** and both epimers of **11**. Titration was monitored by ^{19}F NMR spectroscopy. There is no observable difference between spectra of recombinant β_{60} assemblies and the native protein $\alpha_3\beta_{60}$ complex. In the complex of **10** with lumazine synthase, a second, broad signal overlapping the signal of the free ligand appeared at 8.3 ppm, in addition to the signal of the free ligand at 7.8 ppm. The signal at 8.3 ppm disappeared after addition of the nitrosopyrimidinedione **8**, an analogue of the pyrimidine substrate **1**. This indicates that the fluoro derivative binds to the active site and is displaced by the stronger binding nitroso ligand (K_D = 1.7 μM).[9]

Binding of the epimers of **11** is highly stereoseselective in the case of riboflavin synthase and lumazine binding protein.[11,13] Only epimer A is bound by these proteins, but there is no evidence for any interaction with epimer B. In contrast, both epimers were bound by the β subunits of the lumazine synthase/riboflavin complex and β_{60} capsids. However, there is a remarkable difference in the

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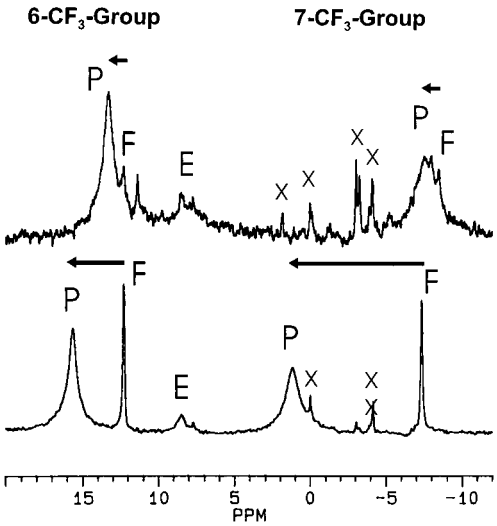

Figure 1. ^{19}F NMR spectra of epimer A (top) and epimer B (bottom) of **11** bound to the wild-type lumazine synthase/riboflavin synthase complex at 24 °C. The signals labeled "E" around 8 ppm correspond to a minor amount of the elimination product **10**, bound to the protein. Shift differences between the signals of the free and the bound ligand are indicated by black arrows for each of the trifluoromethyl groups. The sample contained 10 mg of protein per milliliter and 300 μM of ligand **11,** respectively, in 100 mM phosphate buffer, pH 7.0. The spectrum was processed with 20 Hz line broadening and was calibrated using an external standard of sodium trifluoroacetate at pH 7.0: P, protein bound ligand; F, free ligand; E, elimination product **10**; x, impurities. Note: Because of the broadening of signals of **11** by interaction with the protein, traces of these impurities in the ligand preparation that do not interact with the enzyme appear as large, sharp signals.

^{19}F NMR spectra as shown in Figure 1. The signals of both trifluoromethyl groups of enzyme bound epimer A are only slightly shifted to the low-field side of the ^{19}F NMR spectrum, overlapping the signals of the free ligand (Figure 1, top). In contrast, separate signals of free and enzyme-bound epimer B were found in the spectrum (Figure 1, bottom). There is a shift difference of the 6-CF_3 signals between bound and free ligand of 3.3 ppm, changing to the downfield side of the spectrum from 12.3 ppm in the case of the free to 15.6 ppm of the bound ligand. A remarkable shift difference of 8.8 ppm changing from −7.8 of the free to 1.2 ppm of the bound ligand is observed for the 7-CF_3 group signals. A change of the chemical shift of this order of magnitude could be due to an aromatic amino acid side chain in the direct environment of the 7-CF_3-group. To get an impression about the environment of the trifluoromethyl groups of **11** when bound to the enzyme, the structure of the ligand, ignoring the absolute configuration at C-7, was simply docked into the active site of the crystal structure of lumazine synthase.[6] Two amino acid residues which could influence the trifluoromethyl groups were found in a proximity of about 4−6 Å, the aromatic side chain of F22 and the imidazole ring of H88.

^{19}F NMR Studies on β Subunit Mutants. A series of mutants of active side amino acid residues including the position F22 and H88 was prepared by site-directed mutagenesis. Most of the amino acid replacements in the putative active-site cavity had little or no effect on the catalytic properties of the enzyme, and we used NMR protein perturbation experiments in order to monitor the impact of amino acid replacements on the protein/ligand interaction occurring at the active site of the enzyme.

Mutation of active site amino acid residues other than F22 and H88 did not result in a significant difference in the NMR spectra as compared to those of the wild type. The chemical shifts of the 7-oxo compound **10** bound to F22 or H88 mutant proteins appeared between 9.1 ppm (F22K) and 8.2 ppm (F22Y). For comparison, the bound wild-type ligand has a shift of 8.3 ppm, whereas the shift of the free ligand is 7.8 ppm. The investigations were concentrated on the complexes of the epimers of **11** and mutants at the residues of F22 and H88. The chemical shifts of signals of free and enzyme bound ligand of **11** from spectra with all of the mutants are summarized in Figure 2. The shift of the 7-trifluoromethyl group signal of epimer B bound to mutants of residue F22 was found to be sensitive to the nature of the side chain in this position. In all of the spectra this signal is shifted to the downfield side of the spectrum by at least 5 ppm compared to the signal of the free ligand as observed in the case of F22S. The maximum value of a shift difference of 8.8 ppm as observed in the case of the wild-type protein was not exceeded in any of the mutant proteins. Replacing the phenyl ring by other aromatic side chains or large nonaromatic residues such as arginine resulted in a downfield shift of the signal in comparable magnitude to the wild-type spectrum. On the other hand, short residues such as serine resulted in a smaller downfield shift. The 6-CF_3 group seems to be less sensitive to the changes at F22. Compared to the wild type enzyme there were only minor shift differences observable. Changes in the spectra of epimer A and F22 mutants are insignificant for both trifluoromethyl group signals. None of the fluorolumazines binds to the F22D mutant.

A more dramatic change was found in the pattern of spectra of epimer B and H88 mutants (Figure 2). In all of these cases, the large shift difference between the 7-CF_3 group signals of free and bound ligand disappeared and both signals are nearly overlapping, similar as observed in spectra of epimer A and the wild-type enzyme. The signal of the 6-CF_3 group is also influenced by mutations at residue H88, and the shift difference between free and bound ligand decreased from 3.3 ppm in the wild type enzyme to approximately 2 ppm in mutant protein spectra.

Quantitative evaluation of the NMR spectra and Scatchard analysis gave an estimate for the affinity of the epimers to the lumazine synthase and F22 mutants (Figure 3, Table 1). The K_D value of epimer B and the wild-type protein is 147 μM. To test if integration of NMR signals is suited for the quantitative evaluation of binding data, the dissociation constant of epimer B and the lumazine synthase/riboflavin synthase complex was also determined by equilibrium dialysis. The K_D found is 92 μM, which is in the same order of magnitude as determined from NMR data for the recombinant β_{60} capsid. The K_D of the lumazine synthase/riboflavin synthase complex from *B. subtilis* H94 strain extracted from NMR data is 85 μM. A somewhat increased affinity was found for the F22R, F22Y, and F22V mutants, which showed K_D values below 80 μM. Binding of epimer A was more sensitive to amino acid replacement. Introducing the basic amino acid side chains Lys and Arg decreased the affinity by a factor of 4 with dissociation constants around 200 μM. Epimer A is only weakly bound by F22W (K_D 2

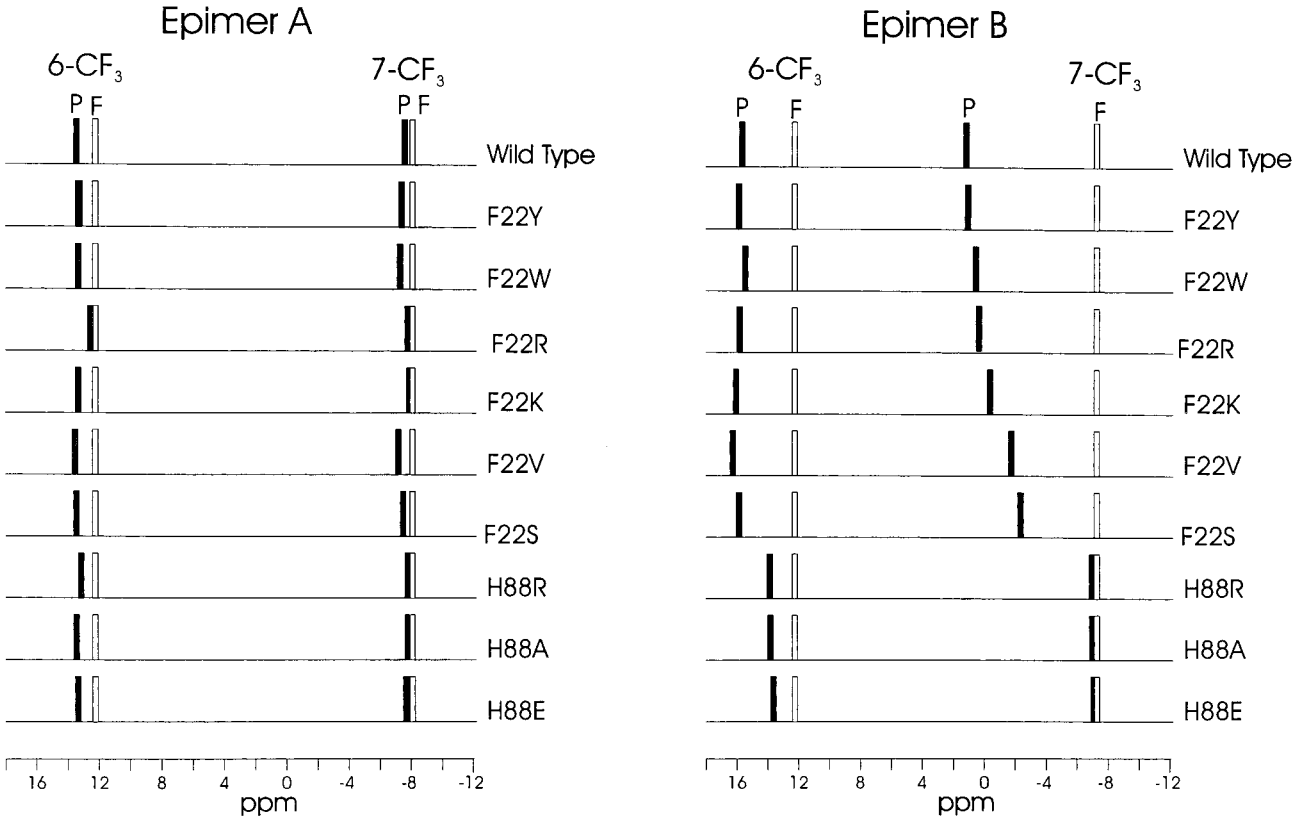

Figure 2. ^{19}F NMR shifts of complexes comprising lumazine synthase β_{60} capsids and epimer A (left panel) or epimer B (right panel) of **11**: P, protein bound ligand; F, free ligand.

mM), whereas epimer B showed a comparable affinity to both the mutant and the wild-type enzyme, respectively.

Haloform Reaction. The 7-trifluoromethyl group of both epimers of **11** is eliminated in a pH-dependent haloform-like reaction in aqueous solution.[14] Moreover, this reaction is catalyzed by lumazine synthase stereoselectively for epimer A. The reaction product is 6-trifluoromethyl-7-oxolumazine **10**. The eliminated trifluoromethyl group is assumed to form HCF_3, which is volatile.

Wild-type enzyme and mutants were assayed for the haloform reaction of both epimers and the formation of the product **10** was monitored by HPLC to determine the kinetic rate constants (Table 1). The ability to catalyze the haloform reaction for epimer A is slightly increased in the cases of the F22W and F22Y mutants compared to the wild-type lumazine synthase. Lower activities between 3 and 7 nmol^{-1} mg^{-1} h^{-1} were found for F22S, F22V, F22R, and F22K. However, these mutants show also a lower activity in the formation of 6,7-dimethyl-8-ribityllumazine **3** (to be published elsewhere). As expected, elimination activities for epimer B as the substrate of all of the F22 mutants are negligible. However, the F22R and F22K showed a slightly enhanced elimination activity.

A surprising situation was found in the case of the H88R mutant. This mutant catalyzes the haloform reaction using epimer B as the substrate, and a rate constant of 228 nmol^{-1} mg^{-1} h^{-1} was determined (Table 1). For comparison, the rate constant of the elimination reaction of epimer A catalyzed by the wild-type enzyme is only 16 nmol^{-1} mg^{-1} h^{-1}. No evidence was found that the elimination reaction using epimer A as the substrate is catalyzed by the H88R mutant in a comparable velocity.

The proposed second product of the haloform reaction, HCF_3, could not be detected up to now, because it is volatile. Mutant H88R made it possible to react a sufficient amount of fluorolumazine in a short time. The haloform reaction was performed in a sealed NMR tube and monitored by ^{19}F NMR. Besides the increasing signal of the 6-trifluoromethyl-7-oxolumazine **10**, an additional ^{19}F signal at −3.2 ppm with a ^{1}H^{19}F coupling constant of 79.4 Hz appeared in the spectrum. Additional NMR experiments, including ^{1}H decoupling and ^{1}H^{19}F correlation spectroscopy, established HCF_3 as the second product formed by the haloform reaction.

Discussion

We analyzed the interaction of the fluorinated derivatives **10** and **11** of 6,7-dimethyl-8-ribityllumazine **3** with lumazine synthase from *B. subtilis* by ^{19}F NMR perturbation studies. Despite the large molecular weight of nearly 1 MDa, it was possible to obtain spectra of complexes showing separate signals for free and enzyme-bound ligands. Mutants at the amino acids F22 and H88 were used to understand the role of these residues in the enzymatic mechanism. The spectra of epimer B of **11** showed an interesting pattern of the shift of the 7-trifluoromethyl group, sensitive to the nature of the residues in these positions. From titration experiments, the dissociation constants K_D could be obtained from quantitative evaluation of the peak areas, and the results are in agreement with data obtained by equilibrium dialysis. Binding of fluorolumazines is relatively weak with dissociation constants in the range of 100 μM. Because of overlapping of the signals in some cases and the instability of the epimers due to the catalyzed haloform reaction,

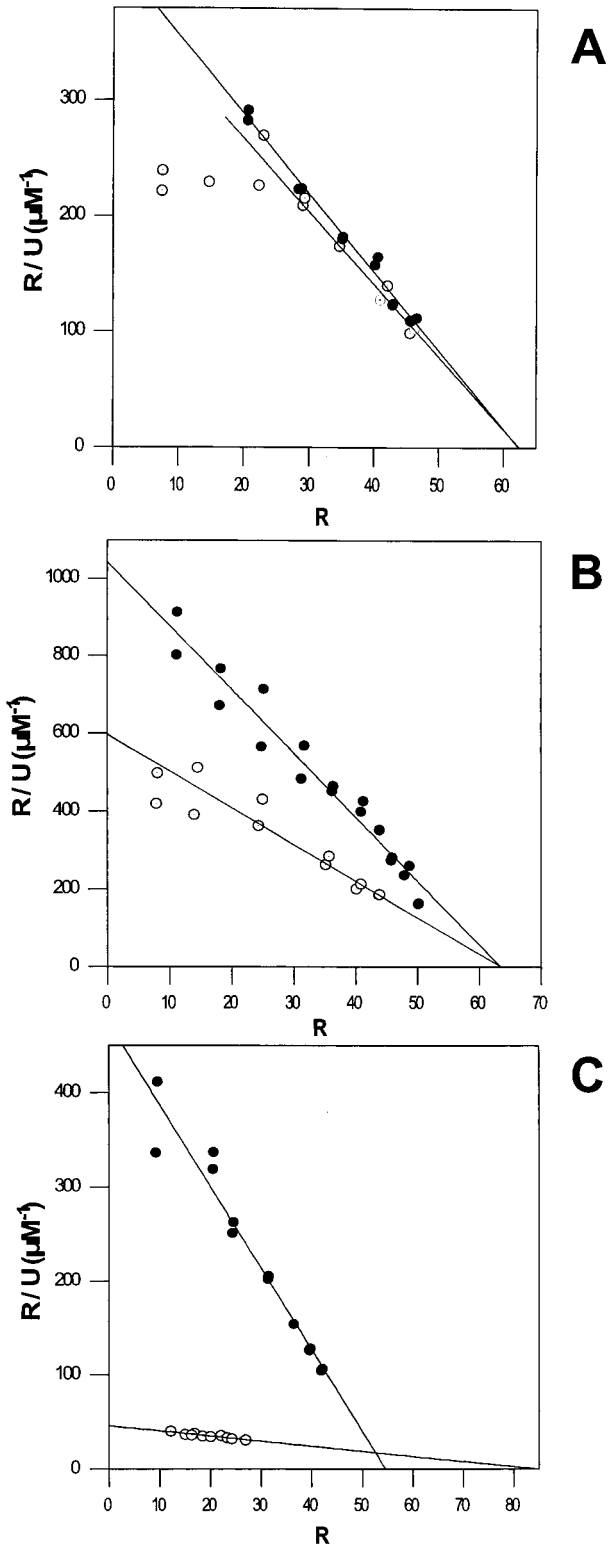

Figure 3. Affinity of wild-type and mutant lumazine synthase to 6,7-bis(trifluormethyl)-7-hydroxyl-8-ribityllumazine. Comparative Scatchard plots of epimer A (hollow circle) and epimer B (solid circle). Recombinant lumazine synthases were titrated with increasing concentrations of epimer ligand (0.1−1.5 mM). Ratios of bound and free ligand were estimated from ^{19}F NMR spectra: (A) wild-type bound to epimer A (k_D = 159 μM), respectively, epimer B (k_D = 142 μM); (B) mutant F22V bound to epimer A (k_D = 106 μM), respectively, epimer B (k_D = 61 μM); (C) mutant F22W bound to epimer A (k_D = 2050 μM), respectively, epimer B (k_D = 115 μM).

Table 1. K_D Values of 11 Binding to Lumazine Synthase Mutants and Kinetic Constants of the Haloform Reaction Catalyzed by Lumazine Synthase Mutants

	epimer A		epimer B	
mutant	v_{elim}[a]	K_D (μM)	v_{elim}[a]	K_D (μM)
wild type	15.9	159	0.4	147
F22W	19.7	2050	0.3	115
F22Y	25.2	92	0.6	79
F22S	3	135	0.6	159
F22V	5.3	106	0.15	61
F22R	7.3	244	2.2	32
F22K	3.4	222	1.1	140
H88R	na[b]	na[b]	228	nd[c]

[a] V_{elim}: nmol^{-1} mg^{-1} h^{-1} at 37 °C and pH 7.0. The velocity constants are corrected by the rates of the uncatalyzed reaction, which is 8.5 nmol^{-1} mg^{-1} h^{-1} under these conditions. [b] No activity observed. [c] Because of the fast conversion of epimer B into **10** it was not possible to determine the K_D value of this complex.

these values may give just an estimate of the binding constants. On the other hand, similar values were found for the interaction with riboflavin synthase α subunits.[11,12]

The X-ray analysis of lumazine synthase of *Bacillus subtilis* is based on the complex of the β_{60} capsid and the inhibitor 5-nitro-6-ribitylaminopyrimidinedione **7**,[6] but there are no data published for lumazine derivatives bound to β_{60}. The distances between the trifluoromethyl groups of **10** and **11** (epimer B) and several side chains, including H88, have been determined by ^{15}N{^{19}F} REDOR NMR.[16] A model of the binding mode of these compounds could be obtained by overlaying ribityl and pyrimidine portions of the structures of these molecules with the nitro ligand **7** in the X-ray structure, followed by REDOR NMR and distance-restrained energy calculations (Figure 4). These studies gave evidence for the *R*-configuration at C-7 of epimer A and the *S*-configuration of epimer B. The ^{19}F NMR spectroscopy data described here and the mechanism of the haloform reaction may be discussed on the basis of these models.

When modeling epimer A (*R*-diastereomer) into the binding site (Figure 4A), the distance of the aromatic side chain of F22 to the trifluoromethyl groups is to far to induce the observed shift of the signals of the bound ligand. This is in agreement with the NMR results where shift differences of only 1.5 ppm between the bound and the free ligand were found. In the model of the epimer B (*S*-diastereomer) complex (Figure 4B), the 7-trifluoromethyl group faces in the opposite direction of the phenyl ring and comes under the influence of the imidazole ring of H88. Therefore, the histidine side chain seems to be responsible for the shift perturbation of this signal by ligand binding to the protein. This is clearly demonstrated in the experiments using H88 mutants. For all H88 mutants used in this study, the shift difference in the spectra between the free and the enzyme bound ligand was less than 1 ppm. On the other hand, there is a shift difference of 4.9 or 5.5 ppm by replacing F22 by a serine or valine residue, respectively. Mutants F22W and F22Y including an aromatic side chain resulted in spectra which were very similar to that of the wild type. Large shift differences of 6.9 and 7.5 ppm were also observed in the spectra of the F22K and F22R mutants, which

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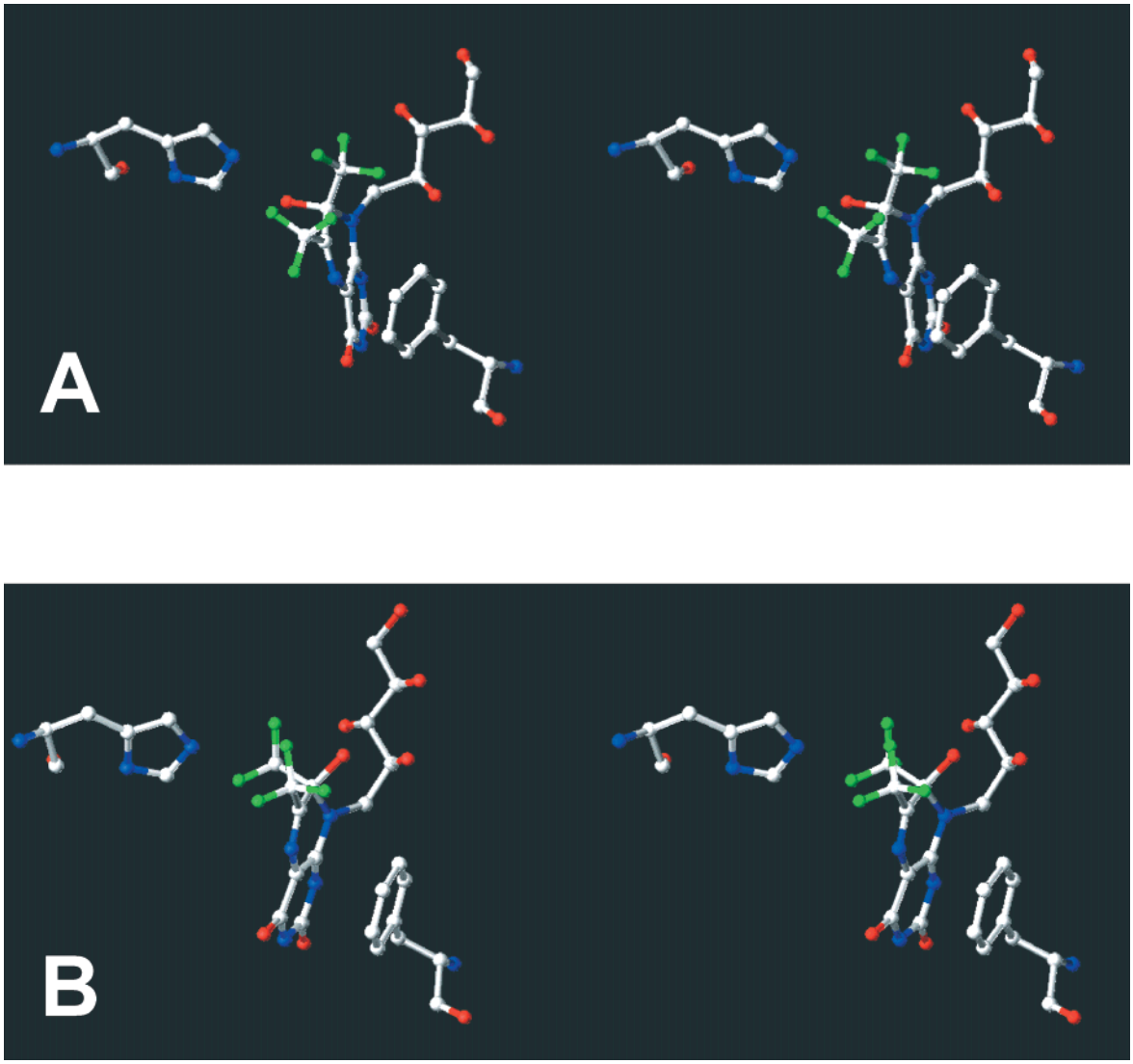


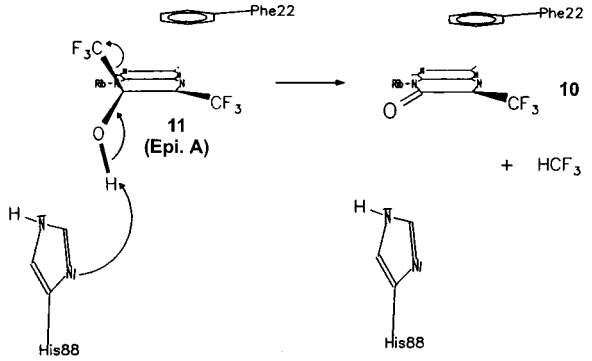
Figure 4. Epimer A (panel A) and epimer B (panel B) of **11** bound to lumazine synthase. Only the ligand and the amino side chains of F22 and H88 are shown. The fluoroligands were modeled into the binding site of lumazine synthase, overlaying the pyrimidindione and ribityl portions of ligand **7** in the X-ray structure[6] and energy minimized using Sculpt and Sybil programs.

have large nonaromatic side chains. The phenyl ring of F22 may act as a panel that keeps the pteridine ring, including the 7-CF_3 group, close to H88. Substitutions of F22 by side chains with a large aromatic ring keep the lumazine derivative in a similar position and do not influence the NMR spectrum in a significant way. Mutants including arginine or lysine with large nonaromatic side chains may have a similar effect. Replacing F22 by amino acids with smaller side chains such as valine or serine allows a somewhat different binding mode increasing the distance to H88 and thus decreasing the imidazole side chain effect on the trifluoromethyl group. Fluorolumazines did not bind to mutant F22D. The introduction of a carboxyl-group seems to be unfavorable for the interaction with the pteridine ring. An aromatic residue at this sequence position is highly conserved in numerous organisms.[7,8] Like *B. subtilis*, most microorganisms produce lumazine synthase with phenylalanine at position 22, but there are also tryptophan residues found as in the case of the yeast enzyme. It should be noted that the *B. subtilis* wild type and an F22W mutant protein showed the same enzymatic activity (manuscript in preparation).

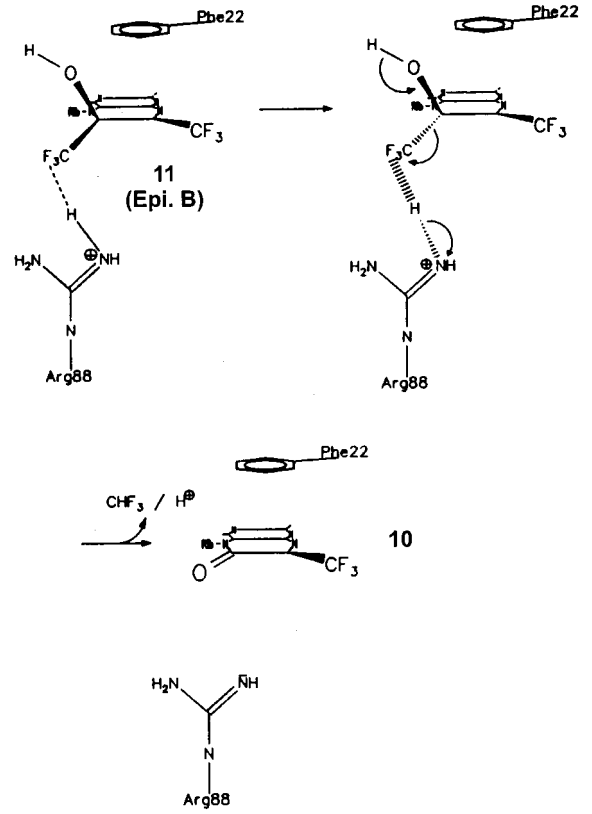
Fitting the fluorolumazines into the binding site at the active center allows an understanding of the mechanism of the haloform reaction. In the suggested mechanism,[14] the reaction is initiated by deprotonation of the 7-OH group, followed by the release of the trifluoromethyl group and formation of the carbonyl oxygen. Deprotonation has been proposed to be the rate limiting step. A basic amino acid side chain in proximity to the hydroxy-group could act as proton acceptor involved in this unusual enzymatic catalysis. In the case of epimer A, H88 is in sufficient proximity to the OH group of approximately 4 Å[16] to act as the proton acceptor. Significant rates of the elimination reaction were only obtained for the wild type and mutants with large aromatic side chains. This is in agreement with a "panel effect" of F22, pushing the 7-OH group close to H88, which positions the imidazole ring as the proton acceptor (Scheme 4). The resulting anionic intermediate stabilizes by formation of a carbonyl oxygen at C-7, cleavage of the C7−C7′ bond and release of the CF_3 group, which is protonated in a subsequent step.

Replacing H88 by an arginine residue resulted in an enhanced reaction rate, and the elimination is catalyzed

Scheme 4. Mechanism of the Elimination of the 7-CF_3 Group of 11, Epimer A, Catalyzed by Lumazine Synthase



Scheme 5. Mechanism of the Elimination of the 7-CF_3 Group of 11, Epimer B, Catalyzed by Lumazine Synthase H88R Mutant



with epimer B. This behavior implies a different mechanism of the elimination reaction (Scheme 5). Here, the 7-CF_3 group of the epimer B is in proximity to the arginine residue in position 88. The guanidine group of this side chain can interact with the CF_3 group by formation of a hydrogen-bridge and polarize and loosen the C7−C7′ bond. The inductive effect facilitates the release of the 7-hydroxyl group proton, followed by elimination of the CF_3 group and formation of the carbonyl oxygen. Initializing the elimination reaction by the interaction of the trifluoromethyl group with the arginine side chain seems to be a novel mechanism. The formation of intramolecular H-bonds between trifluoromethyl groups and hydroxyl groups resulting in conformational stabilization has been reported in the case of 2-trifluoromethylphenol.[17−19] Intramolecular hydrogen bonding between a hydroxyl group and a trifluoromethyl group also influenced the specificity of photocycloadditions in 1-hydroxy-dihydrosemibullvalenes.[20] However, there is no information about hydrogen bonding of an amino acid side chain with a fluorinated ligand available as suggested in the present study.

The configuration at the C-7 of **11** has not been assigned to the epimers in a direct way, because attempts to crystallize these compounds for X-ray analysis failed.[11] Because of their role as analogues of intermediates in the mechanism of both lumazine synthase and riboflavin synthase and the demonstrated stereoselective behavior, elucidation of their absolute configuration is of interest. Based on the results from ^{15}N {^{19}F}REDOR NMR of complexes of ^{15}N labeled lumazine synthase F22W mutant and the lumazines **10** and epimer B of **11**, the R-configuration of epimer A and S-configuration of epimer B was proposed only recently.[16] The results from ^{19}F NMR perturbation and the different haloform reaction mechanism obtained from this study are in agreement with these results and confirm the suggested configuration.

Experimental Section

Chemicals. 6-Trifluoromethyl-7-oxo-8-ribityllumazine (**10**),[12] the epimers A and B of 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrate (**11**),[11] and 5-nitroso-6-(ribitylamino) pyrimidine-2,4(1*H*,3*H*)-dione (**8**)[21] were prepared by published methods.

Synthesis of 6-Trifluoromethyl-7-oxo-8-ribityllumazine (10) by the Haloform Reaction. The epimers of **11** were separated by preparative HPLC (Nucleosil RP-10 C_{18}, 4 × 250 mm; eluent 10% methanol, 0.1% formic acid; flow rate 40 mL/min; retention volume epimer A 480 mL; retention volume epimer B 600 mL).[11] Fractions of the overlapping area of the elution peaks, that contained both epimers, were used to convert **11** to 6-trifluoromethyl-7-oxo-8-ribityllumazine **10** by the haloform reaction.[14] The combined fractions were dried and redissolved in water. The solution containing a 1:1 mixture of both epimers of **11** (100 μM) was adjusted to pH 8 by adding potassium phosphate buffer to a final concentration of 200 mM phosphate, followed by incubation for 4 h at 55 °C until **11** was totally converted into **10**. The reaction was monitored by analytical HPLC (Nucleosil RP-10 C_{18}; eluent 15% methanol, 0.1% trifluororacetic acid). The solution was concentrated, desalted on a Nucleosil RP-10 C_{18} prep-column and lyphylized. The product was identified by ^{19}F NMR (10% D_2O, pH 7.0, 470 MHz), δ 7.8 ppm (s, 6α-CF_3).

Proteins. The lumazine synthase/riboflavin synthase complex of *Bacillus subtilis* strain H94 was purified as described.[22,23] The construction of a plasmid directing the overexpression of wild-type lumazine synthase β_{60} capsids has been published recently.[24]

The construction of the mutant lumazine synthase β_{60} capsids was carried out as described earlier.[25] Proteins were

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overexpressed in *B. subtilis* strain BR 151 [pBL1] and in *E. coli* M15 [pREP4] strains. Cells were grown in LB medium, and overexpression was induced by the addition of 2 mM IPTG for 5 h. Briefly, the protein was partially purified by anion exchange chromatography on Whatman Cellulose DE-52 using a gradient from 100 to 650 mM phosphate, pH 7.0 or gelfiltration on Sephacryl S-300 from Pharmacia. A detailed procedure has been published earlier.[23]

^{19}F NMR Studies. ^{19}F NMR spectra were measured at 338 or 470 MHz in 100 potassium mM phosphate buffer, pH 7.0 containing 10% D_2O at 24 °C. Spectra were calibrated using sodium trifluoroacetate (pH 7.0) as external standard. Samples containing 15−30 mg of protein per milliliter in 100 mM potassium phoshate, pH 7.0, were titrated with the ligand **10** and the epimers of **11** to a final concentration of 0.8 to 1.6 mM. ^{19}F NMR spectra were recorded after every addition step of ligand. The ratio of free and enzyme-bound ligand was determined from the integrals of the signals in the NMR spectrum, respectively. K_D values were obtained from titration series by Scatchard analysis.

Haloform Reaction. Assay mixtures containing identical amounts (200 μM β subunits determined by radial immunodiffusion) of lumazine synthase mutants, and a 5-fold excess of one epimer of 6,7-bis(trifluoromethyl)-8-ribityllumazine **11**, in 200 mM potassium phosphate buffer, pH 7.0 were incubated at 37 °C. Aliquots were retrieved at 30 min intervals, and the formation of 6-trifluoromethyl-7-oxo-8-ribityllumazine **10** was analyzed by HPLC on Nucleosil RP-10 C_{18} columns as described earlier.[14]

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JO001739U

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