Riboflavin Synthase of Escherichia coli

EFFECT OF SINGLE AMINO ACID SUBSTITUTIONS ON REACTION RATE AND LIGAND BINDING PROPERTIES*

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Conserved amino acid residues of riboflavin synthase from Escherichia coli were modified by site-directed mutagenesis. Replacement or deletion of phenylalanine 2 afforded catalytically inactive proteins. S41A and H102Q mutants had substantially reduced reaction velocities. Replacements of various other conserved polar residues had little impact on catalytic activity. 13C/15N NMR protein perturbation experiments using a fluorinated intermediate analog suggest that the N-terminal sequence motif MFTG is part of one of the substrate-bindng sites of the protein.

Riboflavin synthase catalyzes a mechanistically complex dismutation of 6,7-dimethyl-8-ribityllumazine (Compound 1) affording riboflavin (Compound 3) and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (Compound 4) (Fig. 1) (1–3). The enzyme is a potential target for the chemotherapy of infections by Gram-negative bacteria which are unable to absorb riboflavin from the environment due to the absence of a transport system and are therefore absolutely dependent on its endogenous synthesis. Specifically, it has been shown that riboflavin-deficient mutants of Escherichia coli and Salmonella typhimurium require riboflavin concentrations well above 10 mg/liter of culture medium (7, 8). Similar levels of the vitamin are required for the growth of riboflavin-deficient mutants of the yeasts Saccharomyces cerevisiae and Candida guilliermondii (9–11). Inhibitors of riboflavin biosynthesis may therefore also qualify as potential agents for the therapy of yeast infections, which are becoming progressively more important in immuno-compromised patients.

The riboflavin synthases of eubacteria and yeasts are homotrimeric proteins with an approximate subunit mass of 24 kDa. The sequence similarity between the N-terminal and C-terminal part of the enzyme suggested that each subunit folds into two domains with similar folding topology (12). In line with this hypothesis, preliminary crystallographic analysis of riboflavin synthase from E. coli provided evidence for pseudo d3 symmetry of the homotrimeric molecule (13).

The reaction mechanism of the enzyme is incompletely understood despite the efforts of several research groups extending over a period of 4 decades (for review, see Refs. 6, and 14). The dismutation reaction involves the transfer of a 4-carbon unit between the two identical substrate molecules and requires the simultaneous presence of two 6,7-dimethyl-8-ribityllumazine molecules at the active site of the enzyme (15–17). This is well in line with the hypothesis of two topologically similar folding domains and with the experimentally observed binding of two substrate molecules per enzyme subunit.

Recently, the two-domain hypothesis received considerable support by the recombinant expression of N-terminal and C-terminal segments of riboflavin synthase from E. coli. Both recombinant proteins were shown to bind the intermediate analog, Compound 5, but not its diastereomer, Compound 6 (Fig. 2) (19). Moreover, the N-terminal domain could bind riboflavin and 6,7-dimethyl-8-ribityllumazine with high affinity. Surprisingly, the N-terminal domain was found to form a homodimer in contrast to the heterotrimeric riboflavin synthase.

The three-dimensional structure of riboflavin synthase is still unknown despite considerable efforts. Therefore, we decided to use systematic mutation analysis to identify catalytically relevant amino acid residues.

EXPERIMENTAL PROCEDURES

Materials—6,7-Bis(trifluoromethyl)-8-ribityllumazine hydrazide (Compound 5) and 6,7-dimethyl-8-ribityllumazine were prepared by published procedures (20, 21).

Restriction enzymes were from New England Biolabs (Schwalbach, Germany), Amersham Pharmacia Biotech (Freiburg, Germany), or from Life Technologies, Inc. (Karlsruhe, Germany). T4 DNA ligase was from Life Technologies, Inc. (Karlsruhe, Germany). Oligonucleotides were custom-synthesized by Life Technologies, Inc. (Karlsruhe, Germany) or MWG Biotech (Ebersberg, Germany). Nucleobond AX20 columns for plasmid DNA purification were from Macherey and Nagel (Daren, Germany). DNA fragments were purified with the QiAquick Gel Extraction Kit or QiAquick PCR Purification Kit from Qiagen (Hilden, Germany). Casein hydrolysate and yeast extract were from Life Technologies, Inc. (Karlsruhe, Germany), isopropyl β-D-thiogalactopyranoside was from Biovitrum (Hamburg, Germany), Q-Sepharose Fast Flow and phenyl-Sepharose CL-4B were from Amersham Pharmacia Biotech (Freiburg, Germany).

Construction of a ribC Hyperexpression Strain—The ribC gene coding for riboflavin synthase of E. coli was amplified by PCR using the plasmid pERS (22) as template and oligonucleotides 1 and 2 as primers (Table I). The amplificate was purified, digested with XbaI and PstI,
and ligated into plasmid pT7-7 (23), which had been digested with the same restriction enzymes. The resulting plasmid was designated pT7-RS.

**Bacterial Culture—**

Recombinant *E. coli* strains were grown in LB medium at 37 °C in shaking flasks to an optical density of 0.7. Isoproplyl-D-thiogalactopyranoside was added to a concentration of 0.5 mM, and incubation was continued at 37 °C for 4 h. The cells were harvested by centrifugation and stored at −78 °C.

**Site-directed Mutagenesis—** A procedure modified after Marini et al. (24) was used for site-directed mutagenesis. PCR was performed with Vent DNA Polymerase (New England Biolabs, Schwalbach, Germany) to minimize the error rate. Plasmid pERS was used as template. The internal mismatch primers are shown in Table I. The general scheme of mutagenic PCR involved two rounds of amplification cycles using one mismatch and two flanking primers (primers 1 and 2, Table I). During the first round, 5 amplification cycles were carried out with the respective mismatch primer and with one of the flanking primers. Then the second flanking primer was added and the reaction was continued for an additional 10 cycles. The amplificate was subjected to agarose gel electrophoresis, digested with XbaI and PstI, purified using the QIAquick PCR Purification Kit, and ligated into plasmid pT7-7 that had been digested with the same restriction enzymes. The ligation mixture was transformed into *E. coli* XL1-blue cells (Stratagene, Heidelberg, Germany). Mutant clones were identified by the presence of the respective new restriction site. All plasmid constructs were sequenced by the automated dideoxynucleotide method (Sanger) using a 377 Prism DNA sequencer from Applied Biosystems (Weiterstadt, Germany).

**Protein Purification—**

Purification procedures were performed at 4 °C, unless otherwise stated. Frozen cell mass (5 g) was thawed in 25 ml of 50 mM Tris hydrochloride, pH 7.2, containing 0.5 mM EDTA and 0.5 mM dithiothreitol (buffer A). The suspension was subjected to ultrasonic treatment and then centrifuged. The supernatant was dialyzed against 10 volumes of buffer A and centrifuged. The supernatant was passed through a column of Q-Sepharose Fast Flow (2 × 18 cm) pre-equilibrated with buffer A (flow rate, 1 ml min⁻¹). The column was washed with 100 ml of buffer A and developed with a linear gradient of 0–0.5 M NaCl in buffer A (total volume, 280 ml). Riboflavin synthase was eluted from 200 to 240 ml. The enzyme fraction was brought to a concentration of 1 M (NH₄)₂SO₄ by the slow addition of an equal volume of 2 M (NH₄)₂SO₄ in buffer A. The solution was passed through a column of phenyl-Sepharose CL-4B (1.5 × 12 cm) pre-equilibrated with 1 M (NH₄)₂SO₄ in buffer A (flow rate, 1 ml min⁻¹). The column was washed with 40 ml of 1 M (NH₄)₂SO₄ in buffer A and developed with a linear gradient of 1.0–0 M (NH₄)₂SO₄ in buffer A (total volume, 120 ml). The enzyme was eluted from 80 to 95 ml. Fractions were combined, concentrated by ultrafiltration, and dialyzed against 20 mM sodium/potassium phosphate, pH 6.9, containing 0.2 mM EDTA and 0.5 mM dithiothreitol (buffer B). The dialyzed solution was stored at −78 °C. According to SDS-polyacrylamide gel electrophoresis (25), the protein samples contained less than 5% impurities. The oligomeric structure of mutant proteins was determined using a 6% polyacrylamide nondenaturing gel electrophoresis system as previously described (26). The protein concentration was determined photometrically ($\varepsilon_{280} = 47,700$ M⁻¹ cm⁻¹).

**Monitoring of Riboflavin Formation—**

Reaction rates were measured using a stopped-flow module SFM-4 and Diode Array Spectrometer (Bio-Logic, Claix, France) at 20 °C. All mixing solutions were prepared in buffer B. The reaction was initiated by rapid mixing of equal volumes of 8 μM enzyme solution and 25–160 μM 6,7-dimethyl-8-ribityllumazine solution. The reference absorbance spectrum of 4 μM protein solution in reaction buffer was automatically subtracted from the experimental data. Absorbance spectra were collected and analyzed using Spectralys software from J&M Analytische Meß- und Regeltechnik GmbH (Aalen, Germany).

FIG. 1. Hypothetical reaction mechanism of riboflavin synthase based on the data by Plaut, Wood, and co-workers (3–6).

FIG. 2. Diastereomeric 6,7-bis(trifluoromethyl)-8-ribityllumazine hydrates. R = β-ribityl.

FIG. 3. Amino acid sequence of *E. coli* riboflavin synthase. Amino acid residues selected for mutagenesis are boxed. Absolutely conserved amino acid residues are shown in bold type. Genetic variation of certain boxed residues are shown below the *E. coli* sequence.
Germany). Riboflavin concentration was determined using an extinction coefficient \(e_{470} = 10,300 \text{ M}^{-1} \text{ cm}^{-1}\).

Equilibrium Dialysis—Equilibrium dialysis experiments were performed at 24°C in buffer B using Dianorm microdialysis cells (Bachofen, Beutlingen, Germany) and Visking dialysis tubes (Medicell, London, United Kingdom). The protein concentration was 17 \(\mu\)M. The total ligand concentration was between 7.5 \(\mu\)M and 1.1 mM. The dialysis cells were allowed to equilibrate for 4 h under slow rotation. Control experiments had shown that the concentrations of compound 5 in the two parts of a microdialysis cell had reached equilibrium after 2 h of incubation. Ligand concentration was determined photometrically (\(e_{440} = 7,943 \text{ M}^{-1} \text{ cm}^{-1}\)).

Protein Perturbation Experiments—Samples contained 20 mM sodium/potassium phosphate, pH 6.9, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% D_2O and protein (10.5–35.0 mg ml^{-1}). Aliquots (5–30 \(\mu\)l) of 6,7-dimethyl-1,3,8-triﬂuoromethyl)-8-ribityllumazine hydrate (epimer A) were added. After each addition, \(^{19}F\) NMR spectra were recorded at 338 MHz using an AM 360 NMR spectrometer from Bruker Instruments (Karlsruhe, Germany) at 24 °C. Experimental parameters were as follows: pulse angle, 30° (2 \(\mu\)s); repetition rate, 1.6 s; 32 K data set; 800 to 3000 scans.

Chemical shifts were referenced to an external standard containing sodium trifluoroacetate, pH 7.0. The concentrations of bound and free ligands were calculated from \(^{19}F\) NMR signal integrals.

### RESULTS

Construction of Riboflavin Synthase Mutants—Plaut, Wood, and co-workers (4, 15, 27) had proposed a hypothetical mechanism for riboflavin synthase involving the initial addition of a nucleophile to the lumazine molecule serving as donor of the 4-C unit which is transferred between the two substrate molecules in the enzyme-catalyzed reaction (Fig. 1). More specifically, this hypothetical nucleophile could be either a water molecule or a polar amino acid residue. To check this hypothesis, we decided to mutate polar amino acid residues in search of the hypothetical nucleophile thought to be involved in catalysis.

Sequence comparison of all known or presumed riboflavin synthases of the fungal/eubacterial type showed that the similarity between the proteins from different species was relatively low (data not shown). More specifically, the enzymes from E. coli and the closely related Hemophilus influenzae share 63% identical amino acid residues. Otherwise, the fraction of identical amino acids was in the range of 29 to 38%.

The sequence comparison revealed 11 polar amino acid residues that were absolutely conserved (Fig. 3). Moreover, several other amino acids (Ser126, Asn127, Thr128, Thr129, Thr130, Tyr131, Tyr133, and Asp134) were rarely replaced. All these residues were selected for mutagenesis.

In light of the relatively low overall conservation of the riboflavin synthase sequence, it was most notable that all enzymes invariably had the N-terminal sequence MXGK. A functional role of this sequence segment appeared likely, and it was therefore included in the mutagenesis study.

Site-specific mutagenesis was performed by PCR and generated mutant genes were checked for sequence accuracy by restriction analysis of novel restriction sites and by full-length sequence analysis. Five mutated genes (C48S, T50R, T67R, T145R, and T165R) could not be expresses in recombinant E. coli strains. It was tentatively assumed that the mutated amino acid residues affected the folding and overall conformation rather than the active site of the enzyme, and the respect-
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A substantial reduction in catalytic capacity. \(V_{\text{max}}\) (Table II).

\textit{Riboflavin Formation}—The catalytic activity of the mutant proteins was studied by single turnover experiments using a stopped-flow photometer. The respective mutant protein and the substrate, 6,7-dimethyl-8-ribityllumazine, were rapidly mixed at a molar ratio of 6:1 to 20:1 and the absorbency at 470 nm was recorded. At this wavelength, the absorbency contribution of 6,7-dimethyl-8-ribityllumazine is low, and the recorded signal is dominated by the concentration of riboflavin formed. The maximal rate of riboflavin formation \(V_{\text{max}}\) in the case of the wild type enzyme was \(21\) nmol \(mg^{-1} min^{-1}\) (Table II), which is equivalent to a turnover number of 0.5 per min and subunit.

A substantial number of absolutely or highly conserved, polar amino acid residues could be exchanged without significant impact on the catalytic rate. Thus, the replacement of residues Thr\(^{4}\), Asn\(^{67}\), Thr\(^{71}\), Asn\(^{83}\), Glu\(^{85}\), His\(^{97}\), Lys\(^{137}\), Ser\(^{146}\), and Glu\(^{183}\) decreased the reaction velocity by less than 10-fold (Table II).

The replacement of serine 41 by alanine was accompanied by a substantial reduction in catalytic capacity. \(V_{\text{max}}\) decreased to \(3\) pmol \(mg^{-1} min^{-1}\). Similarly, the replacement of histidine 102 by glutamine afforded a mutant protein with a decreased \(V_{\text{max}}\) of \(83\) pmol \(mg^{-1} min^{-1}\).

The replacement of the absolutely conserved phenylalanine 2 by alanine afforded soluble protein that had no detectable catalytic activity. No riboflavin whatsoever could be observed even after long-term incubation with substrate. Similarly, the deletion of phenylalanine 2 resulted in the complete loss of catalytic activity. The more conservative replacement of phenylalanine by tyrosine decreased \(V_{\text{max}}\) of the enzyme 50-fold, to 0.42 nmol \(mg^{-1} min^{-1}\).

\textit{Protein Perturbation Experiments}—In the absence of protein structural data, we decided to perform protein perturbation studies to characterize the interaction of the mutant proteins with a ligand that binds to the catalytic site of riboflavin synthase. The fluorinated Compounds 5 and 6 have been used extensively in ligand binding studies with riboflavin synthase and lumazine synthase of \textit{Bacillus subtilis} (17, 20, 28, 29). Fluorine substitution favors the covalent hydration of the pteridine ring system to such an extent that the diasteromeric covalent hydrate structure mimics the hypothetical intermediate (protein, 0.08 mM; ligand, 0.18 mM), respectively, C-terminal (protein, 0.08 mM; ligand, 0.18 mM) domain are shown for comparison (Footnote 1).

FIG. 4. \(^{19}\)F NMR titration of \textit{E. coli} riboflavin synthase with Compound 5. Initial protein concentration, 0.4 mM. Ligand concentrations: \(a, 0.33 \text{ mM}; b, 0.92 \text{ mM}; c, 1.3 \text{ mM}; d, 1.8 \text{ mM}\). Spectra of recombinant N-terminal (protein, 1.5 mM; ligand, 0.56 mM), respectively, C-terminal (protein, 0.08 mM; ligand, 0.18 mM) domain are shown for comparison (Footnote 1). \(F\), free ligand; \(B\), bound ligand; \(x\), impurity.

enzyme-bound ligand. With progressive saturation of the enzyme’s binding sites, the signals of the free ligand increase. The position 7 trifluoromethyl group of the enzyme-bound ligand appears as a relatively broad signal with a line width of 180 Hz which is 1.4 ppm downfield shifted by comparison with the signal of the free ligand. The position 6 trifluoromethyl group of the enzyme-bound ligand affords a more complex signal pattern. At low ligand concentrations (Fig. 4a), a relatively intense signal with a line width of 160 Hz is observed at 14.2 ppm and a signal of lower intensity with a line width of 90 Hz at 15.7 ppm. The latter signal increases with progressive addition of ligand, thus suggesting that the enzyme has two different types of binding sites whose affinities to Compound 5 differ significantly (Table III).

The quantitative evaluation of the signal integrals afforded the nonlinear Scatchard plot shown in Fig. 5A. Besides the experimental values obtained from the NMR titration, the plot shows experimental values obtained by equilibrium dialysis. The data obtained by the two different experimental methods fit a model with \(\sim 3.2\) high affinity sites \((K_{D} = 2.0 \mu \text{M})\) and 2.5 low affinity sites \((K_{D} = 41 \mu \text{M})\) per enzyme molecule. Incidentally, it should be noted that the separate evaluation of the
NMR signal integrals affords a $K_D$ value of 30 $\mu$M for the low affinity site (Fig. 5B).

These data are well in line with our recent finding that the recombinant N-terminal and C-terminal domains of *E. coli* riboflavin synthase can both bind Compound 5 (Fig. 4, Table III). The comparison between the spectra of the wild type enzyme and the domains suggests tentatively that the ligand signal at 15.7 ppm is associated with the N-terminal domain of the wild type enzyme, whereas the signal at 14.2 ppm is associated with the C-terminal domain.

Titration experiments with Compound 5 were performed with all mutants listed in Table II. In most cases, the spectra were qualitatively similar to that obtained with the wild type enzyme. $K_D$ values obtained from $^{19}$F NMR titration differ from that of the wild type enzyme by less than a factor of 10 (Table III) and the number of ligand molecules bound per protein molecule (trimer) varied from 5.2 to 6.3 (relative standard error, 6–15%). The few exceptional cases are discussed in detail below.

An NMR titration experiment with the catalytically inactive F2A mutant with a deletion of phenylalanine 2 is shown in Fig. 6. The position 7 trifluoromethyl group of the bound ligand affords two signals with a distance of 0.9 ppm, respectively (Table III). On the other hand, the signals of the position 6 trifluoromethyl group are located very close to each other with a distance of 0.4 ppm (Fig. 6). The signal pattern suggests that the mutant retains the capacity to bind one ligand molecule each to the N-terminal and C-terminal domain. The signals of

### Table III

| Proteins            | Chemical shift (ppm) | Chemical shift (ppm) |
|---------------------|----------------------|----------------------|
|                      | 6-CF$_3$ group       | 7-CF$_3$ group       |
| Wild type           | 14.2                 | 15.6                 | -5.9                  | -5.7                  |
| F2A                 | 13.5                 | 13.9                 | -6.2                  | -7.1                  |
| F2Y                 | 13.8                 | 13.8                 | -6.1                  | -7.2                  |
| K137A               | 14.3                 | 15.2                 | -6.3                  | -7.0                  |
| S146G               | 14.2, 13.6           | 15.7                 | -6.2                  | -5.8                  |
| E183G               | 14.1                 | 15.1, 15.6           | -5.9                  | -6.4                  |
| N-terminal domain   | 14.1                 | 15.6                 | -5.5                  | -6.4                  |
| C-terminal domain   |                      |                      |                      |                      |

**Fig. 5.** Scatchard plots based on data from equilibrium dialysis (triangles) and NMR titration experiments (circles) with wild type riboflavin synthase. A, integral ligand binding; B, ligand binding to the low affinity site.

**Fig. 6.** $^{19}$F NMR titration of F2A mutant with Compound 5. Initial protein concentration, 0.29 mM. Ligand concentrations: a, 0.22 mM; b, 1.1 mM; c, 1.4 mM; d, 1.7 mM. The chemical shifts of Compound 5 bound to recombinant N-terminal, respectively, C-terminal domain are indicated on top. See legend of Fig. 4 for other details.
The dissociation constant of 57 mM ligand (wild type); 0.22 mM protein, 1.7 mM ligand (F2Δ); 0.39 mM protein, 2.0 mM ligand (K137A); 0.18 mM protein, 1.0 mM ligand (S146G); 0.28 mM protein, 1.6 mM ligand (E183G). See legend of Fig. 5 for other details.

The low affinity site is characterized by a dissociation constant less than 1 mM ligand (wild type); 0.22 mM protein, 1.7 mM ligand (F2Δ); 0.39 mM protein, 2.0 mM ligand (K137A); 0.18 mM protein, 1.0 mM ligand (S146G); 0.28 mM protein, 1.6 mM ligand (E183G). See legend of Fig. 5 for other details.

The replacement of serine 41 or histidine 102 reduced the catalytic activity of the enzyme by several orders of magnitude. The mutations did not decrease substantially the affinity of the ligand molecule assumed to represent the ligand bound to the N terminus. By comparison with the wild type enzyme (Table III), the low affinity site cannot be measured accurately for experimental reasons (the NMR signals of the free ligand are too small to be integrated in the early titration steps; the value of the dissociation constant for the high affinity site is estimated to be less than 1 mM).

The F2Δ mutant is very similar to the F2Δ mutant. It is catalytically inactive, and it modulates the NMR spectrum of Compound 5 in the same way as the deletion mutant (Table III). The low affinity site is characterized by a dissociation constant of 57 μM. The dissociation constant for the high affinity site cannot be measured accurately for experimental reasons (the NMR signals of the free ligand are too small to be integrated in the early titration steps; the value of the dissociation constant is estimated to be less than 1 μM).

The F2Δ mutant is very similar to the F2Δ mutant. It is catalytically inactive, and it modulates the NMR spectrum of Compound 5 in the same way as the deletion mutant (Table III). However, the conservative replacement of phenylalanine 2 by tyrosine affords a catalytically active protein (Table II) whose interactions with Compound 5 are similar to that of the wild type protein.

A comparison of the NMR chemical shift values shown in Table III indicates that, except of Glu183, replacements of other amino acids in N- or C-terminal halves of riboflavin synthase affected exclusively (Lys137 and Ser146) or largely (Phe2) the NMR pattern afforded by the ligand molecule bound to the same half of the protein. The fact that all mutations listed in Table III have a visible effect on the appearance of the 6- and 7-trifluoromethyl groups of the ligand, relative to the wild type enzyme, suggests that residues 2, 137, 146, and 183 may be in close proximity to the ligand and to each other.

The hypothetical reaction mechanisms suggested by Wood, Plaut, and co-workers (3–6) implicate the addition of a nucleophile to one of the substrate molecules as an early reaction step. In the uncatalyzed reaction, a water molecule could serve this purpose. Either a water molecule or a polar amino acid side chain could serve as a nucleophile in the enzyme-catalyzed reaction.

The protein perturbation data provide evidence for the existence of two different types of binding sites whose affinities for Compound 5 differ by approximately 1 order of magnitude. Evidence for two different types of binding sites had been obtained earlier by studies with riboflavin synthase from B. subtilis and 6-trifluoromethyl-7-oxo-8-ribityllumazine (16, 28), but the 19F NMR spectra produced by Compound 5 in this case not only differed substantially from those shown in Fig. 4, but also gave the molar ratio of 1:1 of bound ligand per subunit, indicating that only one type of binding site could bind the ligand. E. coli and B. subtilis proteins share only 33% identical amino acids (data not shown), so the structural difference between the ligand-binding sites of these two enzymes that leads to the different NMR signal pattern seems plausible.

We have shown recently that the N-terminal and C-terminal parts of the riboflavin synthase of E. coli can be expressed as recombinant proteins.1 Specifically, the N-terminal domain was shown to form a relatively stable, soluble homodimer whereas the full-length riboflavin synthase forms a homotrimer. The recombinant C-terminal domain has comparatively low stability and could not be obtained in pure form. Both recombinant domains were shown to bind Compound 5 by protein perturbation experiments similar to those reported in the present study. A comparison of the chemical shift values indicates that binding of Compound 5 to the N-terminal domain is associated with a relatively large downfield shift of the position 6 trifluoromethyl signal whereas the binding to the C-terminal domain has a more pronounced effect on the signals from the position 7 trifluoromethyl group.

The replacement of serine 41 or histidine 102 reduced the catalytic activity of the enzyme by several orders of magnitude. The mutations did not decrease substantially the affinity of the enzyme for the putative intermediate analog, Compound 5. Moreover, the mutations appeared to have little impact on the
environment of enzyme-bound Compound 5 as gleaned by $^{19}$F NMR spectrometry. If either of these amino acids were involved in covalent catalysis, the residual activity of the mutant proteins could be due to a water molecule replacing the amino acid nucleophile. Whereas the data are insufficient to prove a direct catalytic role of serine 41 or histidine 102, they rule out covalent catalysis by any of the other polar amino acid residues addressed in the present mutagenesis study.

Whereas this study was primarily focused on conserved polar amino acid residues, the inclusion of the lipophilic N terminus appeared mandatory in light of the absolute conservation of the terminal MF$^X$G motif. Deletion or nonconservative replacement of phenylalanine 2 afforded completely inactive proteins. Although these mutants could still bind two lumazine-type molecules, the chemical shifts of the ligand bound at the putative N-terminal-binding site were modulated significantly. This finding suggests that the N terminus of the polypeptide forms an essential part of the active site.

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