Active site residues critical for flavin binding and 5,6-dimethylbenzimidazole biosynthesis in the flavin destructase enzyme BluB

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Abstract: The “flavin destructase” enzyme BluB catalyzes the unprecedented conversion of flavin mononucleotide (FMN) to 5,6-dimethylbenzimidazole (DMB), a component of vitamin B₁₂. Because of its unusual chemistry, the mechanism of this transformation has remained elusive. This study reports the identification of 12 mutant forms of BluB that have severely reduced catalytic function, though most retain the ability to bind flavin. The “flavin destructase” BluB is an unusual enzyme that fragments the flavin cofactor FMNH₂ in the presence of oxygen to produce 5,6-dimethylbenzimidazole (DMB), the lower axial ligand of vitamin B₁₂ (cobalamin). Despite the similarities in sequence and structure between BluB and the nitroreductase and flavin oxidoreductase enzyme families, BluB is the only enzyme known to fragment a flavin isoalloxazine ring. To explore the catalytic residues involved in this unusual reaction, mutants of BluB impaired in DMB biosynthesis were identified in a genetic screen in the bacterium *Sinorhizobium meliloti*. Of the 16 unique point mutations identified in the screen, the majority were located in conserved residues in the active site or in the unique “lid” domain proposed to shield the active site from solvent. Steady-state enzyme assays of 12 purified mutant proteins showed a significant reduction in DMB synthesis in all of the mutants, with eight completely defective in DMB production. Ten of these mutants have weaker binding affinities for both oxidized and reduced FMN, though only two have a significant effect on complex stability. These results implicate several conserved residues in BluB’s unique ability to fragment FMNH₂ and demonstrate the sensitivity of BluB’s active site to

Abbreviations DMB, 5,6-dimethylbenzimidazole; FMN, flavin mononucleotide; FMN_{ox}, oxidized flavin mononucleotide; FMNH₂, reduced flavin mononucleotide; HPLC, high performance liquid chromatography; IYD, iodotyrosinase.

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structural perturbations. This work lays the foundation for mechanistic studies of this enzyme and further advances our understanding of the structure–function relationship of BluB.

Keywords: BluB; flavin destructase enzyme; 5,6-dimethylbenzimidazole; vitamin B₁₂; mutagenesis

Introduction

Vitamin B₁₂ (cobalamin) is an essential cofactor for animals and protists, though its synthesis is restricted to a subset of prokaryotic species.¹ Cobalamin is a tetrapyrrolic cobalt-containing cofactor that participates in isomerization, methyl transfer, and reductive dehalogenation reactions.² The biosynthesis of cobalamin requires approximately 30 enzymatic steps.^{1,3} We previously identified the *bluB* gene in the bacterium *Sinorhizobium meliloti* which is necessary for the biosynthesis of the lower axial ligand of cobalamin, 5,6-dimethylbenzimidazole (DMB).^{4,5} The BluB enzyme catalyzes the oxygen-dependent biosynthesis of DMB from FMNH₂, the reduced form of the cofactor flavin mononucleotide (FMN) (Fig. 1).^{5–7} Flavins are known to carry out one- and two-electron redox reactions, catalyze halogenation and dehalogenation reactions, participate in the sensing and production of light, and in some cases covalently attach to enzyme active sites.^{8–13} The fragmentation of the isoalloxazine ring of flavin by BluB which leads to the formation of DMB is a unique and unprecedented reaction, and thus BluB has been named a “flavin destructase”.⁵

Because of its biochemical novelty, the mechanism that BluB employs to fragment FMNH₂ remains elusive. The X-ray crystal structure of BluB shows overall similarity to the nitroreductase and flavin oxidoreductase enzyme families, suggesting that subtle structural differences in the active site may be responsible for its function as a flavin destructase rather than an oxidoreductase.^{5,14} The active site of BluB contains residues common to oxidoreductases as well as residues found only in BluB orthologs.⁵ An extended “lid” domain is also present in BluB and is proposed to function in shielding the active site from solvent.⁵ Interestingly, the human iodotyrosinase enzyme (IYD), which requires FMNH₂ as a cofactor for reductive deiodination of

iodotyrosine in the thyroid, is structurally similar to BluB, though the significance of this similarity with regard to mechanism is not yet known.¹⁵

The *S. meliloti bluB* gene was originally identified in a genetic screen for mutants with a calcofluor-bright phenotype.⁴ The calcofluor-bright phenotype, visualized as abnormally bright fluorescence on agar plates containing calcofluor, is indicative of an increase or structural alteration in a secreted exopolysaccharide.^{16,17} The calcofluor-bright phenotype of the *S. meliloti bluB* mutant is due to the inability of the *bluB* mutant to synthesize DMB.^{4,5} In this study, we make use of the calcofluor-bright phenotype of an *S. meliloti bluB* null mutant to screen for loss-of-function mutations in *bluB*, with the overall objective of identifying features of the BluB enzyme that account for its unique function as a flavin destructase. We have uncovered a set of previously unidentified mutations that affect catalysis, some of which also affect binding to flavin.

Results

Screen for *BluB* point mutants defective in DMB synthesis

To characterize the residues in BluB that are necessary for catalysis, a random mutagenesis approach was taken to isolate point mutants in BluB that are unable to catalyze DMB biosynthesis. A plasmid containing the *bluB* gene was mutagenized and introduced into a *bluB* null mutant *S. meliloti* strain, and the resulting colonies were screened for the calcofluor-bright phenotype. A total of 156 calcofluor-bright colonies were isolated, of which 16 showed approximately wild-type levels of the BluB protein by western blot and had unique point mutations in the *bluB* open reading frame. The phenotypes of the calcofluor-bright mutants corresponding to proteins that we examined biochemically are shown in Figure

Figure 1. Reaction catalyzed by BluB. Reduced flavin mononucleotide (FMNH₂) is cleaved in an oxygen-dependent reaction to form erythrose 4-phosphate, DMB, and an unknown coproduct(s). The atoms N1, C4a, N5, and N10 in FMNH₂, which are discussed in the text, are labeled.

Figure 2. Calcofluor-bright phenotypes of *S. meliloti* *bluB* point mutants identified in the calcofluor screen. *bluB::gus* strains harboring plasmids expressing *bluB* with the indicated point mutations were streaked on LB plates containing calcofluor and viewed under UV light. The calcofluor-dim wild-type strain containing the parental vector pMS03 (WT) and the calcofluor-bright *bluB::gus* strain harboring pMS03 (vector) are shown for comparison.

2. Three of the mutants, R30C, R30H, and G61D, were also tested for their ability to form a nitrogen-fixing symbiosis with alfalfa, and as expected, their phenotypes were indistinguishable from that of the *bluB* null mutant (not shown).⁴

An alignment of the *S. meliloti* BluB protein sequence and nine putative BluB homologs from distantly related bacteria and archaea shows that 13 of the 16 residues found to be mutated in the calcofluor screen are highly conserved (Fig. 3). Examination of the X-ray crystal structure of BluB shows that three of the mutations identified in the screen, E78K, M94I, and G110S, are located in structural elements unique to BluB and IYD, the active site "lid" and the loop that encloses the active site [Fig. 4(A)].⁴ Seven of the mutations are located in or near the flavin binding pocket [Fig. 4(B, C)]. Two additional active site residues known to be critical for catalysis, D32 and S167, are also highlighted in the alignment and in the crystal structure (Figs. 3 and 4).⁵

Enzymatic activity of *BluB* point mutants

To explore the catalytic properties of the point mutants identified in the calcofluor screen, each mutation was introduced into a BluB expression vector and the corresponding proteins were purified. Of the two double mutants isolated in the calcofluor screen, proteins with the single mutations E78K, M140I, and P202L were purified separately. All of the purified proteins were soluble with the exception of P65L, E78K, A156V, and G193D. The insoluble proteins were not pursued further, as their defect in DMB production is likely due to misfolding of the protein. The mutants D32N and S167G, which were shown previously to be defective in DMB production,⁵ and the double mutant D32N/S167G were also included in this analysis. To determine the extent of the defect in catalysis in each mutant enzyme, DMB production was first measured in a coupled enzyme

assay in which FMNH₂ is generated by the reduction of FMN with NADPH by the SsuE enzyme.^{5,18} HPLC analysis of the reaction shows that wild-type BluB converts 10 nmol of FMN into 8.3 nmol of DMB in 2 h (Table I). In contrast, all of the BluB mutants are severely impaired in DMB biosynthesis, and only four of the 12 mutants tested, M94I, M140I, D32N and S167G, show DMB production above the detection limit (Table I). These four mutants have a reduction in activity between 6- and 58-fold, while those unable to produce detectable DMB have at least 60-fold loss in catalytic function. Because all of the mutants showed a dramatic defect in DMB production after 2 h, detailed kinetic analysis of the mutants was not pursued. No change in DMB production was observed following overnight incubation, indicating that a change in $V_{\max}$ is likely. To test whether the absence of DMB production can be explained by an increase in K_M , mutants for which DMB production was not detected were further examined in reactions containing 50 nmol (0.5 mM) FMN. No DMB was detected in these reactions, indicating either that the enzymes are catalytically inactive or that the K_M for FMNH₂ is drastically increased. Together, these results demonstrate that all of the point mutations identified in the calcofluor screen are either partially or completely defective in DMB production.

We next tested the possibility that some of the BluB mutants are able to catalyze the initial steps in the reaction and accumulate an intermediate that cannot be converted to DMB. HPLC analysis of reactions containing the mutant proteins did not reveal any additional products that could be detected by UV-Vis spectrophotometry (data not shown). We also tested whether any of the BluB mutants were capable of destroying FMN, which would indicate conversion of FMN to a product that could not be detected by HPLC. Depletion of FMN in reactions containing

Figure 3. Sequence alignment of BluB showing point mutations identified in the calcofluor screen. A sequence alignment of BluB homologs from 10 phylogenetically diverse prokaryotic species is shown with identical residues shaded in black and similar residues in gray. Amino acid residues mutated in this study are indicated in the alignment with mutations shown above. Point mutations identified in the calcofluor screen are indicated in bold, and the two additional mutants included in this study are italicized. Mutations analyzed in this study are boxed. * and # indicate the double mutations found in single *bluB* isolates. Black bars under the alignment correspond to residues in or near the FMN binding domain, gray bars represent the alpha-helical "lid" domain, and the white bar represents the loop under FMN that closes the active site. Sm, *Sinorhizobium meliloti*; Ct, *Chlorobium tepidum*; La, *Lentisphaera araneosa*; Cs, *Cenarchaeum symbiosum*; Lf, *Leptospirillum ferrodiazotrophum*; Ss, *Sulfolobus solfataricus*; Sc, *Streptomyces coelicolor*; Pa, *Pseudomonas aeruginosa*; Mt, *Mycobacterium tuberculosis*; and Bc, *Burkholderia cenocepacia*.

a limited concentration of SsuE was examined by monitoring the absorbance at 445 nm (A_{445}) over time. In reactions containing wild-type BluB, nearly all of the FMN was consumed over the 8 min time course (Fig. 5). In contrast, all of the BluB mutants failed to show FMN depletion, suggesting that there

is no significant accumulation of a reaction intermediate derived from FMN (Fig. 5).

Flavin binding by wild-type and mutant BluB

The absence of FMN depletion by the BluB mutants could be explained by an inability of BluB to bind

Figure 4. Point mutations mapped onto the X-ray crystal structure of BluB. (A) The structure of the BluB dimer bound to FMN_{ox} is shown with the lid domain highlighted in red. The active site as viewed from (B) the *si* face and (C) the *re* face is shown with the two monomers in blue and red. The amino acids mutated in this study are rendered as sticks. The figure was made from PDB file 2ISJ.

the substrate. Therefore, we assayed the ability of each protein to bind flavin. Binding to FMN_{ox} was examined based on quenching of the intrinsic fluorescence of FMN. The UV–Vis spectrum of FMN is altered upon binding to wild-type BluB [Fig. 6(A)], suggesting that the quenching of FMN fluorescence by BluB is static through the formation of ground state complexes, and thus the equilibrium dissociation constant (K_d) of the BluB–FMN_{ox} complexes could be obtained from a non-linear best fit to Equation (1), as described in Materials and Methods.¹⁹

Analysis of FMN_{ox} titrated with wild-type BluB shows that the fluorescence of FMN is quenched by

BluB [Fig. 6(B)]. The K_d of wild-type BluB for FMN_{ox} was calculated to be $\sim 7 \mu\text{M}$ based on these fluorescence measurements [Fig. 6(B); Table I]. We obtained a similar K_d value, $8.8 \pm 0.6 \mu\text{M}$, based on the difference in the UV–Vis spectra between free FMN and FMN bound to BluB (Supporting Information Figure 1). These values are higher than our previously reported value of $1.6 \mu\text{M}$ which was obtained by isothermal titration calorimetry;⁵ this difference is likely due to differences in pH, temperature, buffer conditions, or method. The K_d of each catalytically defective BluB mutant for FMN_{ox} was calculated by fluorescence quenching (Table I; Supporting Information

Table I. Biochemical Properties of *BluB* Point Mutants

BluB mutant	DMB produced (nmol) ^{a,b}	K_d for FMN (μ M) ^a	ΔG_b for FMN _{ox} (kcal mol ⁻¹) ^{a,c}	K_d for FMNH ₂ (μ M) ^a	ΔG_b for FMNH ₂ (kcal mol ⁻¹) ^{a,c}
Wild type	8.3 ± 0.1	6.9 ± 0.3	-7.2 ± 0.02	0.62 ± 0.12	-8.6 ± 0.1
<i>Lid domain, active site closure</i>					
M94I	1.5 ± 0.1	29 ± 3	-6.3 ± 0.1	1.6 ± 0.2	-8.0 ± 0.1
G110S	–	38 ± 3	-6.1 ± 0.04	4.5 ± 0.9	-7.4 ± 0.1
<i>Near FMN phosphate group</i>					
R30H	–	31 ± 4	-6.2 ± 0.1	82 ± 21 ^d	-5.7 ± 0.2
A57V	–	24 ± 3	-6.4 ± 0.1	170 ± 44 ^d	-5.2 ± 0.2
P58L	–	24 ± 3	-6.4 ± 0.1	1.7 ± 0.1	-8.0 ± 0.04
P202L	–	28 ± 4	-6.3 ± 0.1	4.1 ± 0.9	-7.5 ± 0.1
<i>Conserved loop near active site</i>					
G61D	–	44 ± 5	-6.0 ± 0.1	5.3 ± 0.8 ^d	-7.3 ± 0.1
<i>Near N5 of FMN</i>					
G133S	–	32 ± 2	-6.2 ± 0.04	4.3 ± 0.8	-7.4 ± 0.1
M140I	0.68 ± 0.06	27 ± 3	-6.3 ± 0.1	4.3 ± 1.1	-7.4 ± 0.1
S167G	0.60 ± 0.07	34 ± 2	-6.2 ± 0.04	2.1 ± 0.2	-7.9 ± 0.1
<i>Near N1 of FMN</i>					
D32N	0.14 ± 0.02	< 0.10 ^e	< -9.7 ^e	< 0.10 ^e	< -9.7 ^e
<i>Double mutant</i>					
D32N/S167G	–	< 0.10 ^e	< -9.7 ^e	< 0.10 ^e	< -9.7 ^e

^a Error represents standard error.

^b Results of three independent experiments. –, none detected; detection limit is 0.12 nmol.

^c ΔG_b , free energy of binding, calculated from K_d .

^d Data were weighted in KaleidaGraph to achieve reasonable curve fits.

^e Binding curves indicate tight binding; exact K_d and ΔG_b values cannot be calculated.

Figure 2). Ten of the *BluB* mutants show a decrease in binding affinity for FMN_{ox} ranging from 3- to 6-fold compared to wild-type *BluB* (Table I). Comparison of the free energy of binding (ΔG_b) of the wild type and these mutant proteins shows that the stability of mutant *BluB*–FMN_{ox} complexes is only modestly lower than that of the wild-type *BluB*–FMN_{ox} complex ($\Delta\Delta G_b$ is 0.8–1.2 kcal mol⁻¹). The D32N mutant and the D32N/S167G double mutant proteins show significantly enhanced affinity for FMN_{ox} (Table I).

Potential differences in the affinity of the *BluB* mutants for FMN_{ox} versus FMNH₂ prompted us to measure the binding affinity of mutant *BluB* enzymes for FMNH₂. This not only provided an analysis of the binding of the true substrate, but also provided insights into the effects of the slight structural differences between FMN_{ox} and FMNH₂ on binding in each mutant. Because FMNH₂ is not fluorescent, binding data were generated instead by a filtration assay under anaerobic conditions (see Methods). A non-linear best fit to Equation (2) resulted in a K_d measurement of ~0.6 μ M for wild-type *BluB* [Fig. 6(C)]. As a control, the filter binding assay was performed with wild-type *BluB* and FMN_{ox}, resulting in a K_d measurement of 2.4 μ M (data not shown). This value is lower than that determined by the methods described above, but higher than our previously reported value,⁵ likely due to differences in the methods. These differences are not relevant when comparing K_d values determined by the same method between wild-type and mutant *BluB* proteins.

Binding measurements of the mutant proteins to FMNH₂ revealed that, similar to FMN_{ox}, the D32N and D32N/S167G mutants show enhanced binding, while the remaining 10 mutants have reduced binding affinity for FMNH₂ (Table I, Supporting Information Figure 3). Eight mutants with slightly decreased binding affinities for FMNH₂ ($\Delta\Delta G_b$ < 1.3 kcal mol⁻¹) also have slightly decreased affinities for FMN_{ox} (Table I). However, two mutants, R30H and A57V, have greater than 100-fold increase in K_d for FMNH₂ despite their relatively modest effect on binding to FMN_{ox}. Thus, the catalytic defect of the R30H and A57V mutants can be explained by their defects in binding FMNH₂, whereas factors other than

Figure 5. FMN depletion assay. The FMN remaining in reactions containing wild-type *BluB* (WT) and each of the *BluB* point mutants was measured as the absorbance at 445 nm (A_{445}).

Figure 6. Determination of the K_d of wild-type BluB for flavin. (A) UV-Vis spectrum of free FMN (solid line) and FMN bound to wild-type BluB (dotted line). (B) Fluorescence quenching of FMN_{ox} upon titration of wild-type BluB protein. (C) Titration of FMNH₂ with wild-type BluB protein. The combined results of three independent experiments are shown in B and C.

substrate binding are responsible for the catalytic defects of the remaining mutants.

Discussion

The reaction catalyzed by BluB is distinct from all other known reactions involving flavins, as FMNH₂ is converted to DMB rather than functioning as a cofactor. Despite this dramatic difference in the reactions catalyzed, BluB has sequence and structural similarity to bacterial nitroreductases and oxidoreductases and the human IYD enzyme, which use FMN as a redox cofactor. Therefore, differences in key amino acid residues and local differences in structure are likely to be responsible for the ability of BluB to fragment FMN. Here, we have utilized an unbiased genetic strategy to generate a collection of BluB point mutants that render the enzyme incapable of catalyzing DMB synthesis. Our biochemical characterization of these proteins demonstrates that the majority have severe defects in DMB production and modest decreases in binding affinity for FMN. Though it is formally possible that the observed defects in binding and catalysis in these point mutants are due to global changes in the protein structure, localized perturbations of the interactions with the substrate or a reaction intermediate are likely to account for these phenotypes in most or all of the mutant proteins. Examination of the locations of these residues in the X-ray crystal structure of BluB has led us to predict their functions, as outlined below.

Function of the unique "lid" domain

Two of the calcofluor-bright mutants identified in this study, M94I and the insoluble E78K, are located in the unique surface-exposed alpha helical "lid" domain that is proposed to shield the active site from solvent.^{5,15} It is surprising that the M94I mutation has any impact on flavin binding and catalysis, given that the side chain of M94 is surface exposed and that mutation to isoleucine is relatively conservative. The long-range influence of this mutation on FMN binding is particularly notable, as its position in the X-ray crystal structure indicates that mutation to isoleucine would not cause steric clashing with any other residue. The fact that the *B*-factors in this area are the highest in the entire protein suggests that BluB may adopt a conformation during the catalytic cycle in which M94 has an important role.

Interactions with the phosphate group and ribityl tail of FMN

Residue R30 is completely conserved in BluB homologs and is predicted to form three hydrogen bonds with the phosphate group of FMN. The contribution of R30 to the stability of the BluB-FMN_{ox} and BluB-FMNH₂ complexes (1 and 3 kcal mol⁻¹, respectively) is similar to the contributions of two threonine residues in a bacterial flavodoxin (2 and 4

kcal mol⁻¹) that form hydrogen bonds to the phosphate group of FMN.²⁰ The presence of an arginine residue at this position is conserved in several proteins with structural similarity to BluB including flavin reductases,^{21,22} NADH oxidase,²³ nitroreductase,²⁴ and the human IYD enzyme.¹⁵ The critical role of an analogously positioned arginine residue in human IYD is underscored by the incidence of severe hypothyroidism in humans carrying a mutation in this residue.²⁵

Surprisingly, the BluB R30H mutant is significantly more impaired in binding FMNH₂ than FMN_{ox}. This difference could be due to alterations in the positions of the side chain of R30 and the ribityl tail of flavin in the structures of BluB-FMN_{ox} and BluB-FMNH₂.⁵ The phenotype of the nearby A57V mutant is similar, suggesting that this mutation may disrupt the interaction of R30 with the FMN phosphate group. In contrast, mutations in residues P58 and P202, which are also located near R30, result in modest effects on binding to both FMN_{ox} and FMNH₂. Our observation that the P58L and P202L mutations completely abolish catalysis suggests that structural changes in the region near the ribityl tail can result in a loss of catalytic activity that is independent of binding. The position of the ribityl tail is thought to be important for both stabilization of O₂ for reaction with C4a of FMNH₂ and the retro-aldol reaction that leads to the formation of erythrose 4-phosphate.^{5,14} The latter reaction is predicted to be the rate-limiting step,¹⁴ and thus mutations that cause a shift in the position of the ribityl tail may further raise the energy barrier and arrest catalysis.

Interactions with the isalloxazine ring of FMN

In previous work, we selected residues D32 and S167 for site-directed mutagenesis based on their positions in the active site, conservation in BluB homologs, and absence of these residues in nitroreductases and flavin oxidoreductases.⁵ In this study, we observed that mutation of S167 to glycine, a residue commonly observed at this position in nitroreductases and flavin oxidoreductases,^{21–23,26} results in a modest decrease in binding affinity for FMN and a significant loss of catalytic function. It is surprising that the S167G mutant retains some catalytic function, given that the hydroxyl group of S167 is proposed to act as a hydrogen bond acceptor throughout the reaction and is considered to be particularly important in facilitating a proton transfer to N5 that allows the retro-aldol reaction to occur.¹⁴ Our observation that a small amount of DMB is produced in some mutants, even in those with mutations in residues thought to be critical for BluB catalysis, could be explained by the non-enzymatic oxidative conversion of the proposed intermediate

dimethylphenylenediimine to DMB and erythrose 4-phosphate.²⁷

Two mutations isolated in the calcofluor screen, M140I and G133S, are located near S167. The phenotype of the M140I mutant is nearly identical to that of the S167G mutant, indicating that its catalytic defect could be due to disruption of the function of S167. Unlike in the S167G mutant, DMB production is completely abolished in the G133S mutant, suggesting that this mutation may affect both the position of S167 and other factors important for catalysis due to the addition of a side chain. A similar phenotype was observed in two other mutants that disrupt glycine residues in conserved loops, G61D and G110S. The G61D mutation may change the position of the backbone amide of G61, which is proposed to participate in the activation of O₂ and stabilization of the proposed C4a-peroxyflavin intermediate.^{5,7,14}

Unlike the other mutants we examined, the D32N mutant has a higher affinity for flavin than wild-type BluB. The β -carboxylate group of residue D32 is located 3.4 and 3.2 Å from N1 in the structures of FMN_{ox} and FMNH₂, respectively.⁵ N1 of reduced flavins is often deprotonated under physiological conditions ($pK_a \sim 6.7$),²⁸ and thus the presence of an acidic residue such as D32 adjacent to N1 of FMNH₂ would reduce the stability of the BluB-FMNH₂ complex. In nitroreductase, flavin oxidoreductase, and IYD enzymes, a serine, threonine, or arginine residue positioned similarly to D32 in BluB serves as a hydrogen bond donor that stabilizes N1 of FMN.^{15,24,26} In contrast, an aspartate residue at this position could fulfill a similar role only if protonated, which is unlikely under our reaction conditions. We have observed qualitatively that mutation of D32 to serine or alanine also results in increased binding affinity for FMN_{ox}, indicating that flavin binding is more stable in the absence of the negatively charged aspartate residue at this position (data not shown). Thus, the β -carboxylate of D32 is likely to be important for stabilization of, or reaction with, an intermediate or transition state in the conversion of FMNH₂ to DMB. Indeed, the β -carboxylate of D32 is proposed to mediate the transfer of a proton from N1 to N10 and N5 to initiate the retro-aldol reaction.¹⁴ An asparagine at this position would function relatively poorly as an acid or base in these reactions, and this likely explains the significant reduction in DMB production in the D32N mutant. D32 is conserved in all BluB homologs, and aspartate has never been observed at this position in nitroreductases, flavin oxidoreductases, or IYD, all of which are structurally similar to BluB and have homology in other active site residues. Thus, residue D32 could be a key factor that drives the fragmentation of the bound flavin rather than utilizing it as a cofactor.

The mutational analysis presented here identifies many of the residues that are important for the “flavin destructase” activity of BluB. With the exception of D32N and D32N/S167G, all of the mutants we analyzed have moderate defects in FMN binding and are severely compromised in catalysis, suggesting that catalysis can be perturbed by relatively small changes in the active site. This analysis may provide additional tools to uncover the reaction mechanism of BluB, as the mutant proteins may be used to analyze steps in the reaction cycle that cannot be discerned in the wild-type enzyme. For example, different mutant enzymes may be capable of catalyzing the initial steps in the reaction to varying degrees, and thus may provide insights into both the reaction mechanism as a whole and the contributions of specific residues to each stage of catalysis. We are currently exploring this possibility by additional biochemical characterization of several of the mutants generated in this study.

Materials and Methods

Bacterial strains and molecular cloning

S. meliloti strains are derivatives of the wild-type strain Rm1021.²⁹ Bacteria were grown in LB or LB containing 2.5 mM magnesium sulfate and 2.5 mM calcium chloride (LBMC)³⁰ and the following supplements when necessary: spectinomycin, 100 mg/L; streptomycin, 500 mg/L; ampicillin, 100 mg/L; and calcofluor, 0.02% (w/v). A derivative of the plasmid pMS03³¹ expressing *S. meliloti bluB* with a C-terminal FLAG tag, pMS03 *bluB-flag*, was constructed by PCR amplification of the *bluB* open reading frame followed by digestion and ligation with the vector pMS03 at the *Xho*I and *Kpn*I restriction sites. For protein expression and purification, the *S. meliloti bluB* open reading frame was cloned into the vector pET11t at the *Nde*I and *Bam*HI sites with a C-terminal octahistidine tag.

Screen for defective *bluB* point mutants

The plasmid pMS03 *bluB-flag* was mutagenized *in vitro* by incubation at 65°C for 4 h in 235 μ L reactions containing 6.6 μ g purified DNA and 1.0M hydroxylamine in 0.1M sodium phosphate buffer, pH 7.4. Reactions were dialyzed against water and introduced into *Escherichia coli* DH5 α by electroporation. Pools of 100–200 colonies were combined in LB and used as the donor in triparental matings to transfer the mutagenized plasmids into *S. meliloti bluB::gus*.^{4,30} Transconjugants were plated on LB with calcofluor at a density of $\sim$ 100 colonies per plate to avoid significant diffusion of DMB to adjacent cells. The fluorescence of candidate calcofluor-bright colonies was compared with that of *bluB::gus* strains containing the parental vector pMS03 or pMS03 *bluB-flag*. To confirm that the calcofluor-

bright phenotype was due to a lack of complementation by the mutagenized plasmid, plasmids were introduced into a *bluB::Tn5* strain by triparental mating and the calcofluor phenotype was screened again.⁴ Calcofluor-bright strains were tested for expression of plasmid-borne BluB-FLAG by immunoblot with a monoclonal antibody against the FLAG epitope (Sigma). The *bluB* alleles encoding non-functional enzymes with normal protein levels were sequenced.

Protein purification and enzyme assays

The point mutations identified in the calcofluor screen were introduced into the *bluB* open reading frame by site-directed mutagenesis, and proteins were purified by nickel affinity chromatography according to the manufacturer's instructions (Bio-Rad). Protein concentrations were calculated based on the estimated molar extinction coefficient at 280 nm, 44,710 M⁻¹ cm⁻¹, as calculated by the ProtParam tool at <http://ca.expasy.org>.³² Prior to performing binding assays, FMN that was present in purified mutant proteins D32N and D32N/S167G was removed by precipitation with trichloroacetic acid (TCA) followed by renaturation of the protein in sodium bicarbonate buffer as described.³³

DMB synthesis was assessed by incubating 100 μ M FMN, 5 mM NADPH, 50 μ M BluB, and 1 μ M SsuE in 20 mM Tris-HCl, pH 7.9 for 2 h and quenching by addition of TCA to 10% (w/v).⁵ The mixtures were centrifuged for 30 s at 16,000 $\times g$ to remove the proteins. 10 μ L of each reaction was loaded onto an Agilent SB-AQ column using an Agilent 1200 series high-performance liquid chromatograph (HPLC) equipped with a diode array detector with two mobile phases, A, 0.1% formic acid in water and B, 0.1% formic acid in methanol. Solvent B was increased from 5 to 37% over 6.5 min with a flow rate of 0.8 mL min⁻¹ at 45°C. DMB concentrations were calculated based on the peak areas at 280 nm as compared with a standard curve.⁵ FMN depletion was measured in reactions containing 50 μ M FMN, 260 μ M NADPH, 50 μ M BluB, and 0.18 μ M SsuE in 20 mM Tris-HCl, pH 7.9 at 30°C. Absorbance at 445 nm was monitored in 96-well plates in a BioTek SynergyTM 2 Microplate Reader every 5–10 s over 8 min. FMN was prepared by digestion of flavin adenine dinucleotide (Sigma, 98% purity) with the venom of *Crotalus adamanteus* to obtain FMN at a level of purity sufficient for the binding experiments.³⁴

Equilibrium binding of flavin to BluB mutants

Binding of proteins to oxidized FMN (FMN_{ox}) was monitored by the change in fluorescence of FMN upon titration of purified protein. 50 μ L of a 1 μ M solution of FMN in 40 μ M Tris-HCl, pH 7.9, was titrated with proteins in a 96-well plate at 30°C. Fifteen additions of 2.5 μ L of 100 μ M protein followed

by five additions of 2.5 μL of 200 μM protein were performed, resulting in a final protein concentration of 61.5 μM . Fluorescence of FMN was measured after each addition of protein with a BioTek Synergy 2 Microplate Reader with excitation and emission wavelengths of 485 and 528 nm, respectively. Titration data were fitted to the single binding site model according to Equation (1)^{19,35} using KaleidaGraph (V 4.03, Synergy Software):

$$\frac{F}{F_0} = 1 + \frac{\Delta F_{\max}}{F_0} \left(\frac{(K_d + [A] + [B]) + \sqrt{(K_d + [A] + [B])^2 - 4[A][B]}}{2[A]} \right) \quad (1)$$

where F represents the fluorescence intensity, F_0 is the initial fluorescence intensity, $\Delta F_{\max}$ is the maximum change in fluorescence intensity, $[A]$ is the FMN concentration, $[B]$ is the protein concentration, and K_d is the dissociation constant.

Binding of proteins to FMNH₂ was monitored by incubating an anaerobic solution of 1 μM FMNH₂ with a range of concentrations of protein in 20 mM Tris-HCl, pH 7.9. All manipulations were performed in an anaerobic chamber containing 98–99% N₂ and 1–2% H₂ (Coy Laboratory Products). Anaerobic protein samples were prepared by gel filtration with P6 resin (Biorad) in anaerobic 20 mM Tris-HCl, pH 7.9. FMNH₂ was prepared by anaerobic titration with sodium dithionite. Following incubation at 30°C for 15 min, reactions were filtered by centrifugation through a 10 kDa MWCO membrane to remove protein and bound FMNH₂. The filtrates were subsequently exposed to oxygen and shielded from light to convert FMNH₂ to FMN_{ox}. Concentrations of free FMN were measured by fluorescence as described above. Data were fitted to the single binding site model according to Equation (2) and plotted using KaleidaGraph:

$$1 - \frac{[A]_{\text{free}}}{[A]_T} = \frac{\Delta F_{\max}[B]}{K_d + [B]} \quad (2)$$

where $[A]_{\text{free}}$ represents the concentration free flavin, $[A]_T$ is the total FMN concentration, $\Delta F_{\max}$ is the maximum change in the fraction of bound FMN, $[B]$ is the protein concentration, and K_d is the dissociation constant. The term $1 - [A]_{\text{free}}/[A]_T$ represents the fraction of FMN bound to protein.

The free energy of binding, ΔG_b , was calculated from K_d measurements according to Equation (3):

$$\Delta G_b = -RT \ln \left(\frac{1}{K_d} \right) \quad (3)$$

where R is the molar gas constant, T is the temperature, and K_d is the dissociation constant.³⁶

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References

- Roth JR, Lawrence JG, Bobik TA (1996) Cobalamin (coenzyme B12): synthesis and biological significance. *Annu Rev Microbiol* 50:137–181.
- Banerjee R, Ragsdale SW (2003) The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes. *Annu Rev Biochem* 72:209–247.
- Warren MJ, Raux E, Schubert HL, Escalante-Semerena JC (2002) The biosynthesis of adenosylcobalamin (vitamin B12). *Nat Prod Rep* 19:390–412.
- Campbell GRO, Taga ME, Mistry K, Lloret J, Anderson PJ, Roth JR, Walker GC (2006) *Sinorhizobium meliloti* *bluB* is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B₁₂. *Proc Natl Acad Sci USA* 103:4634–4639.
- Taga ME, Larsen NA, Howard-Jones AR, Walsh CT, Walker GC (2007) BluB cannibalizes flavin to form the lower ligand of vitamin B12. *Nature* 446:449–453.
- Gray MJ, Escalante-Semerena JC (2007) Single-enzyme conversion of FMNH₂ to 5,6-dimethylbenzimidazole, the lower ligand of B12. *Proc Natl Acad Sci U S A* 104:2921–2926.
- Ealick SE, Begley TP (2007) Biochemistry: molecular cannibalism. *Nature* 446:387–388.
- Mewies M, McIntire WS, Scrutton NS (1998) Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: the current state of affairs. *Protein Sci* 7:7–20.
- Fraaije MW, Mattevi A (2000) Flavoenzymes: diverse catalysts with recurrent features. *Trends Biochem Sci* 25:126–132.
- van Berkel WJ, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *J Biotechnol* 124:670–689.
- Yeh E, Cole LJ, Barr EW, Bollinger JM, Jr., Ballou DP, Walsh CT (2006) Flavin redox chemistry precedes substrate chlorination during the reaction of the flavin-dependent halogenase RebH. *Biochemistry* 45:7904–7912.
- McTamney PM, Rokita SE (2009) A mammalian reductive deiodinase has broad power to dehalogenate chlorinated and brominated substrates. *J Am Chem Soc* 131:14212–14213.
- Crosson S, Moffat K (2002) Photoexcited structure of a plant photoreceptor domain reveals a light-driven molecular switch. *Plant Cell* 14:1067–1075.
- Wang XL, Quan JM (2011) Intermediate-assisted multifunctional catalysis in the conversion of flavin to 5,6-dimethylbenzimidazole by BluB: a density functional theory study. *J Am Chem Soc* 133:4079–4091.
- Thomas SR, McTamney PM, Adler JM, Laronde-Leblanc N, Rokita SE (2009) Crystal structure of iodo-tyrosine deiodinase, a novel flavoprotein responsible for iodide salvage in thyroid glands. *J Biol Chem* 284:19659–19667.
- Doherty D, Leigh JA, Glazebrook J, Walker GC (1988) *Rhizobium meliloti* mutants that overproduce the *R. meliloti* acidic calcofluor-binding exopolysaccharide. *J Bacteriol* 170:4249–4256.

17. Leigh JA, Lee CC (1988) Characterization of polysaccharides of *Rhizobium meliloti* *exo* mutants that form ineffective nodules. *J Bacteriol* 170:3327–3332.
18. Eichhorn E, van der Ploeg JR, Leisinger T (1999) Characterization of a two-component alkanesulfonate monooxygenase from *Escherichia coli*. *J Biol Chem* 274:26639–26646.
19. Liu HY, Xu ZH, Liu XH, Xi PX, Zeng ZZ (2009) Analysis of binding interaction between bovine serum albumin and the cobalt(II) complex with salicylaldehyde-2-phenylquinoline-4-carboylhydrazone. *Chem Pharm Bull* 57:1237–1242.
20. Lostao A, El Harrou M, Daoudi F, Romero A, Parody-Morreale A, Sancho J (2000) Dissecting the energetics of the apoflavodoxin-FMN complex. *J Biol Chem* 275:9518–9526.
21. Koike H, Sasaki H, Kobori T, Zenno S, Saigo K, Murphy ME, Adman ET, Tanokura M (1998) 1.8 Å crystal structure of the major NAD(P)H:FMN oxidoreductase of a bioluminescent bacterium, *Vibrio fischeri*: overall structure, cofactor and substrate-analog binding, and comparison with related flavoproteins. *J Mol Biol* 280:259–273.
22. Tanner JJ, Lei B, Tu SC, Krause KL (1996) Flavin reductase P: structure of a dimeric enzyme that reduces flavin. *Biochemistry* 35:13531–13539.
23. Hecht HJ, Erdmann H, Park HJ, Sprinzl M, Schmid RD (1995) Crystal structure of NADH oxidase from *Thermus thermophilus*. *Nat Struct Biol* 2:1109–1114.
24. Lovering AL, Hyde EI, Searle PF, White SA (2001) The structure of *Escherichia coli* nitroreductase complexed with nicotinic acid: three crystal forms at 1.7 Å, 1.8 Å and 2.4 Å resolution. *J Mol Biol* 309:203–213.
25. Moreno JC, Klootwijk W, van Toor H, Pinto G, D'Alessandro M, Leger A, Goudie D, Polak M, Gruters A, Visser TJ (2008) Mutations in the iodotyrosine deiodinase gene and hypothyroidism. *N Engl J Med* 358:1811–1818.
26. Parkinson GN, Skelly JV, Neidle S (2000) Crystal structure of FMN-dependent nitroreductase from *Escherichia coli* B: a prodrug-activating enzyme. *J Med Chem* 43:3624–3631.
27. Maggio-Hall LA, Dorrestein PC, Escalante-Semerena JC, Begley TP (2003) Formation of the dimethylbenzimidazole ligand of coenzyme B(12) under physiological conditions by a facile oxidative cascade. *Org Lett* 5:2211–2213.
28. Miura R (2001) Versatility and specificity in flavoenzymes: control mechanisms of flavin reactivity. *Chem Rec* 1:183–194.
29. Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J Bacteriol* 149:114–122.
30. Leigh JA, Signer ER, Walker GC (1985) Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc Natl Acad Sci U S A* 82:6231–6235.
31. Keating DH, Willits MG, Long SR (2002) A *Sinorhizobium meliloti* lipopolysaccharide mutant altered in cell surface sulfation. *J Bacteriol* 184:6681–6689.
32. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A, Protein identification and analysis tools on the ExPASy server. In: Walker JM, Ed. (2005) *The proteomics protocols handbook*. Totowa, NJ: Humana Press.
33. Hinkson JW (1968) *Azotobacter* free-radical flavoprotein. Preparation and properties of the apoprotein. *Biochemistry* 7:2666–2672.
34. Sucharitakul J, Chaiyen P, Entsch B, Ballou DP (2005) The reductase of p-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* requires p-hydroxyphenylacetate for effective catalysis. *Biochemistry* 44:10434–10442.
35. Warner JR, Copley SD (2007) Pre-steady-state kinetic studies of the reductive dehalogenation catalyzed by tetrachlorohydroquinone dehalogenase. *Biochemistry* 46:13211–13222.
36. O'Farrell PA, Walsh MA, McCarthy AA, Higgins TM, Voordouw G, Mayhew SG (1998) Modulation of the redox potentials of FMN in *Desulfovibrio vulgaris* flavodoxin: thermodynamic properties and crystal structures of glycine-61 mutants. *Biochemistry* 37:8405–8416.