Title

Unique Biochemical and Sequence Features Enable BluB To Destroy Flavin and Distinguish BluB from the Flavin Monooxygenase Superfamily

Journal

Biochemistry, 57(11)

ISSN

0006-2960

Authors

Hazra, Amrita B
Ballou, David P
Taga, Michiko E

Publication Date

2018-03-20

DOI

10.1021/acs.biochem.7b01193

Peer reviewed

HHS Public Access

Author manuscript

Biochemistry. Author manuscript; available in PMC 2019 November 01.

Published in final edited form as:

Biochemistry. 2018 March 20; 57(11): 1748–1757. doi:10.1021/acs.biochem.7b01193.

Unique Biochemical and Sequence Features Enable BluB To Destroy Flavin and Distinguish BluB from the Flavin Monooxygenase Superfamily

Amrita B. Hazra[†,‡], **David P. Ballou**[*,§], **Michiko E. Taga**[*,†]

[†]Department of Plant & Microbial Biology, University of California, Berkeley, Berkeley, California 94720, United States

[‡]Department of Chemistry, Indian Institute of Science Education and Research Pune, Pune - 411008, India

[§]Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States

Abstract

Vitamin B_{12} (cobalamin) is an essential micronutrient for humans that is synthesized by only a subset of bacteria and archaea. The aerobic biosynthesis of 5,6-dimethylbenzimidazole, the lower axial ligand of cobalamin, is catalyzed by the "flavin destructase" enzyme BluB, which fragments reduced flavin mononucleotide following its reaction with oxygen to yield this ligand. BluB is similar in sequence and structure to members of the flavin oxidoreductase superfamily, yet the flavin destruction process has remained elusive. Using stopped-flow spectrophotometry, we find that the flavin destructase reaction of BluB from *Sinorhizobium meliloti* is initiated with canonical flavin–O_2 chemistry. A C4a-peroxyflavin intermediate is rapidly formed in BluB upon reaction with O_2, and has properties similar to those of flavin-dependent hydroxylases. Analysis of reaction mixtures containing flavin analogues indicates that both formation of the C4a-peroxyflavin and the subsequent destruction of the flavin to form 5,6-dimethylbenzimidazole are influenced by the electronic properties of the flavin isoalloxazine ring. The flavin destruction phase of the reaction, which results from the decay of the C4a-peroxyflavin intermediate, occurs more efficiently at pH >7.5. Furthermore, the BluB mutants D32N and S167G are specifically impaired in the flavin destruction phase of the reaction; nevertheless, both form the C4a-peroxyflavin nearly quantitatively. Coupled with a phylogenetic analysis of BluB and related flavin-dependent enzymes, these results demonstrate that the BluB flavin destructase family can be identified by the presence of active site residues D32 and S167.

[*]**Corresponding Authors**: dballou@med.umich.edu.; taga@berkeley.edu.

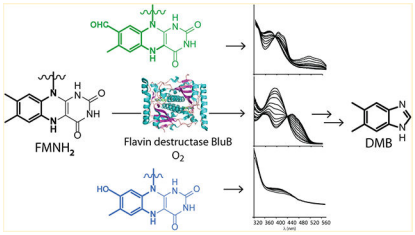
ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/acs.biochem.7b01193. Figures illustrating the stopped-flow reaction of an anaerobic solution of $FMNH_2$ with air-saturated BluB and the rates of C4a-OO(H) formation under various oxygen concentrations in the wild type BluB enzyme and the D32N and S167G mutants with $FMNH_2$ and a table with the full names of the organisms that are shown in the phylogenetic tree in Figure 6A (PDF)

The authors declare no competing financial interest.

Graphical Abstract



B_{12} (cobalamin) is a tetrapyrrolic cobamide cofactor that is synthesized by only <40% of prokaryotes.[1,2] Approximately 30 enzymatic steps are necessary for the complete biosynthesis of cobalamin by either aerobic or anaerobic routes.[2] Of these reactions, the biosynthesis of the lower axial ligand of cobalamin, 5,6-dimethylbenzimidazole (DMB), is among the least understood. The BluB "flavin destructase" enzyme catalyzes the O_2-dependent biosynthesis of DMB in a reaction that requires only flavin mononucleotide (FMN), a reducing agent, and molecular oxygen.[3–5] In this reaction, the reduced form of FMN ($FMNH_2$) is converted to DMB, via the only known enzymatic process that cleaves a flavin isoalloxazine ring[3,4] (Figure 1). The mechanism that BluB employs to catalyze this unusual transformation remains largely unexplored.

Numerous enzymes that utilize a flavin cofactor and O_2 have been characterized by stopped-flow spectroscopy. In these enzymes, the reaction of the reduced flavin cofactor with O_2 commonly results in the formation of a C4a-hydroperoxyflavin when protonated or C4a-peroxyflavin when unprotonated [C4a-OO(H)].[8] In monooxygenases, the distal oxygen atom of the peroxyflavin is transferred to a substrate, resulting in the formation of C4a-hydroxyflavin (C4a-OH), which is subsequently converted to oxidized flavin following the loss of water. In oxidases and other flavoenzymes, such as L-galactono-γ-lactone dehydrogenase, nitroreductase, and vanillyl alcohol oxidase, the reaction of oxygen with the reduced flavin cofactor results in the release of hydrogen peroxide to regenerate oxidized flavin.[9–11] It is often thought that in such enzymes a nascent C4a-OO(H) is formed, but only in the case of pyranose 2-oxidase has such an intermediate actually been documented.[12] In the absence of substrates, most of the flavoprotein monooxygenases stabilize the C4a-OO(H) intermediate but nevertheless will also slowly abortively release hydrogen peroxide. However, in the presence of appropriate substrates, many flavin-dependent monooxygenases are tightly coupled with nearly all of the O_2 and NAD(P)H being used to oxygenate the substrates. Other enzymes, such as ornithine monooxygenases, are not tightly coupled to hydroxylation and release a considerable fraction of the C4a-OO(H) as hydrogen peroxide under all conditions that have been examined.[13,14] There is a large class of two-component flavin-dependent monooxygenases that use reduced FMN or FAD as redox substrates rather than as tightly bound cofactors; these include *p*-hydroxyphenylacetate-3-hydroxylase,[15] dibenzothiophene monooxygenase,[16,17] and bacterial luciferase.[18] These enzymes require a flavin reductase to perform catalysis.

BluB is similar to two-component flavin-dependent enzyme systems in that it also requires a flavin reductase, but it is distinguished from these enzymes because it fragments the

isoalloxazine ring of a reduced flavin substrate rather than simply using it as a redox cofactor. BluB requires O_2 in addition to $FMNH_2$ for catalysis to take place.

The X-ray crystal structure of *Sinorhizobium meliloti* BluB is similar to those of bacterial nitroreductases and flavin oxidoreductases and that of the mammalian enzyme iodotyrosinase (IYD), which is involved in recycling iodine in the thyroid gland.[19,20] Additionally, domains of two human enzymes involved in cobalamin trafficking, CblC and CblD, are structurally similar to BluB, although they are the most divergent members of the superfamily.[21,22] All of these enzymes share a common structural fold for flavin binding and general active site architecture.[3,21] However, BluB and IYD, which catalyze unusual reactions with flavin, contain an extended α-helical "lid" that is proposed to shield the active site from solvent.[3,20,21]

All of the proposed mechanisms of BluB published to date suggest the formation of a C4a-OO(H) intermediate[3,6,7,23] in the first step, and in fact, a C4a-OO(H) intermediate has recently been reported for a BluB homologue from *Rhodobacter capsulatus*.[23] In the work presented here, we report detailed stopped-flow studies of the BluB-catalyzed reaction of oxygen with reduced forms of FMN and FMN analogues with the *S. meliloti* BluB enzyme and its catalytically impaired mutants. We find that both the formation of the C4a-OO(H) intermediate and flavin destruction activity are influenced by the electronic properties of the isoalloxazine ring of flavin and by specific amino acid residues in the active site and lid domains that are unique to BluB.[24] These results, coupled with a bioinformatics analysis of the flavin oxidoreductase superfamily, provide new insights into the mechanism of BluB's unique flavin destructase activity.

EXPERIMENTAL PROCEDURES

Materials.

All reagents and medium components were obtained from Sigma-Aldrich or Fisher Scientific unless otherwise indicated. FMN was prepared from flavin adenine dinucleotide by reaction with *Crotalus adamanteus* venom.[25] The 8-substituted flavin homologues were obtained from Vincent Massey's collection.[26,27] Flavins were reduced anaerobically by titration with sodium dithionite. Overexpression and purification of His-tagged wild type and mutant BluB proteins were performed as previously described.[3,24]

Stopped-Flow Analysis.

Solutions containing 40 μM FMN and 80 μM purified BluB were prepared in a glass tonometer and deoxygenated by repeated cycles of evacuation and purging with purified argon gas.[28] The FMN was then reduced by titration with sodium dithionite added from a syringe attached to the tonometer, and the reduction was monitored spectrophotometrically. Protocatechuate (3,4-dihydroxybenzoate, 200 μM) and purified protocatechuate 3,4-dioxygenase (0.5 unit/mL) were added to remove trace amounts of O_2.[29] Solutions containing 50 mM HEPES buffer (pH 7.9) with 64, 121, 607, and 1210 μM O_2 were prepared by bubbling with 5, 10, 50, and 100% O_2 gas, respectively.

Stopped-flow reactions were performed by mixing 50 μL of each solution in a Hi-Tech Scientific model SF-61DX instrument equipped with either a photomultiplier or a diode array detector. All reactions were performed at 25 °C. Data were analyzed with Kinetasyst 3 (Hi-Tech).

End Point Assays for Single-Turnover Reactions.

End point assays were performed by incubating a 100 μL mixture containing 42 μM $FMNH_2$ and 84 μM BluB in 20 mM Tris-HCl (pH 7.9) in an anaerobic chamber. Aerobic buffer (100 μL) was added to start the reaction. These 100 mM buffers were prepared with mono-, di-, and trisodium phosphate salts and orthophosphoric acid to achieve pH values of 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. Reaction mixtures were then exposed to air for 30 min and reactions quenched with 1.5% (v/v) trichloroacetic acid. The quenched reaction mixtures were centrifuged for 30 s at 14000 rpm to remove the precipitated protein. A 10 μL aliquot of each reaction mixture was loaded onto an Agilent SB-AQ column using an Agilent 1200 series high-performance liquid chromatography (HPLC) system equipped with a diode array detector, and chromatography used two mobile phases: 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Separation of DMB and FMN was achieved by increasing the level of solvent B from 5 to 37% over 6.5 min with a flow rate of 0.8 mL min^{-1} at 45 °C. DMB and FMN concentrations were calculated on the basis of the peak areas at 280 nm as compared with standard curves.

Phylogenetic Analysis.

The sequences were obtained using the Protein Database in NCBI (http://www.ncbi.nlm.nih.gov). Amino acid sequence alignments were performed using MUSCLE,[30] and the sequences were edited with BioEdit.[31] The phylogenetic tree was constructed by using a maximum likelihood/rapid bootstrapping run on RaXML-HPC2 on XSEDE on the Cipres Gateway.[32] The phylogenetic tree was graphed as a Newick Tree using Mega 5.0.

RESULTS AND DISCUSSION

Stopped-Flow Analysis of the BluB-Catalyzed Reaction of $FMNH_2$ and O_2.

Stopped-flow spectrophotometry is commonly used for mechanistic analysis of flavin-containing enzymes because intermediates can be detected on the basis of their distinct spectral properties.[33–35] We conducted stopped-flow studies to examine the mechanism of the reaction of $FMNH_2$ and O_2 with *S. meliloti* BluB. In these experiments, an anaerobic solution of $FMNH_2$ (40 μM) bound to BluB (80 μM) was mixed with an equal volume of aerobic buffer (266 μM O_2) in the stopped-flow apparatus, and the reaction was monitored by either a multiwavelength diode array or single-wavelength detection. We observed the rapid formation of an intermediate with an absorbance peak at 390 nm, characteristic of a C4a-OO(H) flavin species, followed by its decay yielding ~36% of the original FMN (Figure 2A–C). This intermediate, a putative C4a-OO(H), completely formed within 0.02 s under our reaction conditions (25 °C, pH 7.5) with an observed rate constant of 225 s^{-1}, followed by an increase in the absorbance at 457 nm, indicating formation of FMN, with a rate constant of 0.495 s^{-1} (Figure 2D, solid black traces). The C4a-OO(H) intermediate

appears to be similar to that reported recently for *R. capsulatus* BluB.[23] This intermediate was not observed when reactions were conducted anaerobically[3] (data not shown).

In contrast to the results presented above, in reactions initiated by mixing an anaerobic solution of $FMNH_2$ with air-saturated BluB, no C4a-OO(H) intermediate could be clearly discerned, although ~19% of the $FMNH_2$ was consumed in these reactions (Figure S1). This result indicates that, like the $FMNH_2$-dependent alkanesulfonate monooxygenase (SsuD), but unlike the majority of two-component flavin monooxygenases, the binding of BluB to $FMNH_2$ occurs at only 20–30% of the rate that free $FMNH_2$ reacts with oxygen.[25,28,34,36,37] The slow binding of BluB to $FMNH_2$ could be attributed to the buried nature of the active site, the requirement for $FMNH_2$ to bind before O_2 to induce an active protein conformation, or the absence of an associating partner protein, such as a flavin reductase, in our reactions.[3,28,38]

The reaction of $FMNH_2$ bound to BluB with O_2 is expected to be second-order, depending on the concentrations of both $FMNH_2$ and O_2. To characterize the influence of O_2 concentration on the C4a-OO(H) formation step, BluB prebound to $FMNH_2$ was mixed in the stopped-flow apparatus with buffers containing a range of O_2 concentrations. As expected, the observed rate of formation of C4a-OO(H) at 390 nm fit to a single-exponential model and was O_2-dependent. However, at high O_2 concentrations, the rate approached a limiting value of ~500 s^{-1} (Figure 2D, inset). A second-order rate constant was estimated from the linear portion of the curve to be 2.2×10^6 M^{-1} s^{-1} (at 25 °C), a value comparable to rate constants for C4a-OO(H) formation in other two-component flavin monooxygenases such as *p*-hydroxyphenylacetate 3-hydroxylase (1.1×10^6 M^{-1} s^{-1} at 4 °C), tryptophan halogenase (6.3×10^5 M^{-1} s^{-1} at 25 °C), and phenol hydroxylase (1.1×10^5 M^{-1} s^{-1} at approximately 4 °C).[28,39,40] This suggests that the BluB-catalyzed flavin destructase reaction begins with canonical flavin–oxygen chemistry as in flavin-dependent hydroxylases, and the flavin destructase activity of BluB diverges after this step. The hyperbolic nature of the rate of C4a-OO(H) formation with an increasing O_2 concentration (Figure 2D, inset) is likely not due to a technical limitation of the instrument because a rate of >700 s^{-1} with the D32N mutant was observed under the same experimental conditions (Figure S2). Similar to the wild type BluB reaction, the reaction of the D32N mutant with O_2 is also saturated at high O_2 concentrations, but at a somewhat higher rate. Such behavior has also been reported for *p*-hydroxybenzoate hydroxylase and lactate oxidase in studies using modified flavins with different redox potentials. When the potential of the flavin was sufficiently negative, a limiting rate of reaction with oxygen was observed.[27,41] It is possible that some step in the reaction to form the C4a-OO(H) species is limiting.

To examine the possibility that the BluB-catalyzed reaction involves the loss of a hydroxyl group from C4a-OO(H) to form a C4a-OH intermediate, the reaction was monitored by fluorescence, because C4a-OH species are often fluorescent.[39,42] No fluorescent species was observed with excitation at 390 nm, indicating either that a C4a-OH is not formed or that a C4a-OH that is not fluorescent is formed in BluB (data not shown).

In some flavin-dependent enzymes, the effect of pH on different steps in the reaction can provide clues about the reaction mechanism. For example, cyclohexanone monooxygenase

(and other Baeyer-Villiger flavoprotein catalysts) requires a deprotonated C4a-peroxyflavin (C4a-OO^-) intermediate that is favored at high pH, rather than a hydroperoxyflavin (C4a-OOH),42 and in the flavin-containing monooxygenase enzyme SidA, the C4a-OOH hydroxylates the substrate more efficiently at a higher pH.43 In contrast, the hydroxylation efficiency of a modified oxygenase component of *p*-hydroxyphenylacetate-3-hydroxylase is more efficient at a lower pH.44 To examine whether any steps in the BluB reaction are influenced by pH, we conducted stopped-flow analysis on reactions initiated by mixing $FMNH_2$-bound BluB using buffers ranging from 6.0 to 9.5. Examination of reactions at 390 nm across the pH range demonstrated that the rate of formation of the C4a-OO(H) intermediate did not vary with pH (Figure 3A). However, HPLC analysis of the reactions revealed that at higher pH values less FMN remained and more DMB formed (Figure 3A,B). Thus, a step after C4a-OO(H) formation is influenced by pH, indicating that the flavin destruction phase of the reaction, but not the C4a-OO(H) formation step, must occur more efficiently at a higher pH.

The Rate of Formation of C4a-OO(H) Is Influenced by Substitutions at C8 of FMN.

Previous studies have shown that the substituent at the C8 position in the FMN isoalloxazine ring influences the reactivity of the C4a position and can influence the kinetics and absorption spectra of intermediates in the reaction pathway.[27] Hence, the formation of the C4a-OO(H) species or other steps in the reaction may be influenced by inductive effects at the C8 position of FMN and could provide insights into the mechanism.[45] We conducted reactions with one 8-substituted flavin with an electron-donating substituent [8-hydroxyflavin (8-OH-FMN)] and two with electron-withdrawing substituents [8-formyl-FMN (8-CHO-FMN) and 8-chloro-FMN (8-Cl-FMN)]. We observed spectral shifts upon mixing BluB with each of the modified FMNs, indicating that these flavins associated with the protein.[24] Using stopped flow with diode array detection, we examined reactions of O_2 with BluB containing reduced forms of each of the 8-substituted flavins mentioned above. C4a-OO(H) intermediates corresponding to each flavin analogue were observed in all cases, but the fraction of flavin consumed in each reaction was specific for each flavin analogue, ranging from none for 8-Cl FMN to 40% for 8-OH FMN (Figure 4A–I). A comparison of the reactions of O_2 with BluB containing these reduced flavin analogues observed at wavelengths corresponding to the C4a-OO(H) maxima determined in panels B, E, and H of Figure 4 revealed that substituents at C8 influenced the rates of formation of the C4a-OO(H) intermediates (Figure 4J). To quantify the effect of these substituents on the rate of formation of the C4a-OO(H) intermediates, reactions were initiated by mixing buffers containing a range of O_2 concentrations with an anaerobic solution of BluB bound to each reduced substituted flavin. The largest second-order rate constant estimated from these data was with 8-OH-$FMNH_2$, followed by those for unmodified $FMNH_2$ and 8-Cl-$FMNH_2$, and the lowest with 8-CHO-$FMNH_2$ (Figure 4K). An electron-donating group at position 8 of the flavin isoalloxazine ring increases the electron density at the C4a position, while an electron-withdrawing group decreases the electron density at this position; hence, our results are consistent with the expected influence of these position 8 substituents on the rate of C4a-OO(H) formation.[27,46] Furthermore, because we observed that the amount of flavin consumed is smaller with electron-with-drawing substituents than with electron-donating

substituents, steps after C4a-OO(H) formation that lead to DMB formation are apparently favored with more electron density in the isoalloxazine ring.

The Flavin Destructase Reaction Stalls at Different Steps with Catalytically Deficient Mutants of BluB.

We previously reported the isolation and characterization of 12 catalytically deficient point mutants of BluB.[3,24] We selected four of these mutants, D32N (near N1 of the bound FMN), G61D (on a conserved loop in the active site), M94I (in the lid domain), and S167G (near N5 of FMN), for further characterization by stopped-flow spectroscopy, on the basis of their previously determined ability to bind $FMNH_2$, their impaired ability to convert $FMNH_2$ to DMB, and the positions of the residues in the protein structure.[24] An analysis of reaction mixtures containing these mutant enzymes showed that each is blocked at a different point in the reaction. The G61D mutant does not form any detectable C4a-OO(H) intermediate, indicating that the reaction is arrested at an early stage (Figure 5A,B). In this mutant, the bound $FMNH_2$ is completely oxidized to FMN with an observed rate of 14.2 s^{-1}, not much faster than the oxidation of free $FMNH_2$ (Figure 5A,B). The M94I mutant appears to form a small amount of a C4a-OO(H) species, but nearly all of the bound $FMNH_2$ is oxidized to FMN with an observed rate of 11.2 s^{-1}, similar to that of the G61D mutant (Figure 5A,C). In contrast, the S167G mutant forms a C4a-OO(H) intermediate at a rate similar to that for the wild type protein, yet only ~17% of the FMN is consumed in the reaction (Figure 5A,D–F, and Figure S2). This result shows that an interaction between residue S167 and N5 of FMN is not involved in C4a-OO(H) formation but likely facilitates the subsequent flavin destruction phase of the reaction. As in the S167G mutant, the D32N mutant forms a C4a-OO(H) intermediate with kinetics similar to those of wild type BluB, but with only ~11% of the FMN consumed in the reaction (Figure 5A,G–I, Figure S2). A smaller loss of FMN in the reaction compared with that of wild type BluB is consistent with the proposed role of residue D32 in several proton transfer steps in the process of forming the imidazole ring of DMB following C4a-OO(H) formation.[3,6] Note also that the putative C4a-OOH intermediate has an absorbance maximum at 410 nm in the D32N mutant, a wavelength that is considerably longer than that for any of the other C4a-OO(H) intermediates observed.

Phylogenetic Analysis of BluB, Nitroreductases, and Iodotyrosine Deiodinases Reveals Sequence Features Unique to the BluB Flavin Destructase Family.

The results presented above demonstrate that canonical flavin–oxygen chemistry precedes the flavin destructase activity that distinguishes BluB from other flavin-dependent enzymes. We next sought to identify the features of the BluB sequence that endow it with the unique ability to destroy the flavin following the formation of the C4a-OO(H) intermediate. As reported previously, BluB and IYD homologues form two distinct phylogenetic clades within the flavin oxidoreductase superfamily.[3,20,47,48] Analysis of a bootstrapped nearest neighbor phylogenetic tree containing 78 sequences of BluB, IYD, and nitroreductases confirms that all experimentally characterized BluB homologues form a monophyletic group (Figure 6A). Moreover, sequence alignments of the regions surrounding D32 and S167 in BluB reveal that these residues are strictly conserved in the BluB clade and absent in the other enzymes (Figure 6B). In nitroreductases and iodotyrosinases, the residue analogous to D32 is usually replaced with serine, and the residue analogous to S167 with glycine or

threonine.[3,47,48] In summary, our phylogenetic and biochemical analyses indicate that the flavin destructase activity of BluB is defined, in part, by the presence of residues D32 and S167 along with features such as the lid domain that distinguish the BluB family from related enzymes.

CONCLUSION

Herein, we report that the unusual flavin destructase reaction catalyzed by BluB is initiated with a flavin–oxygen reaction with kinetic features typical of other flavin-dependent enzymes, including those of the flavin oxidoreductase superfamily to which BluB belongs. As suggested previously, the reactive C4a-OO(H) intermediate in BluB likely participates in the flavin destructase phase of the reaction by initiating cleavage of the isoalloxazine ring by a mechanism that remains to be elucidated.[3,6,7]

Until now, four mechanisms have been proposed for the formation of DMB by BluB (Figure 1).[3,6,7] Our findings show that substituents at position 8 of FMN affect the rate of formation of the C4a-OO(H) intermediate: electron-donating substituents increase the rate of the reaction with O_2, and electron-withdrawing substituents retard the reaction. In addition, more of the 8-OH-FMN than of 8-Cl-FMN is consumed. Each of the proposed mechanisms includes a ring-closing step to form the five-membered imidazole ring of DMB (Figure 1). All four mechanisms also include an oxidation step of a slightly different nature for the imidazole ring-closing reaction. The first Taga–Larsen mechanism proposes the transfer of the terminal hydroxyl group of C4a-OOH to the ribityl chain that can then act as a leaving group after the ring has closed.[3] The Wang–Quan mechanism suggests that the alloxan that is formed as an intermediate accepts a hydride when the ring is oxidized.[6] The Ealick–Begley mechanism and the second Taga–Larsen mechanism evoke oxidation by an external agent such as O_2 at the time of ring closure.[3,7] Thus, the five-membered benzimidazolyl intermediate that is oxidized in the latter three mechanisms would benefit from the presence of an electron-donating group at position 8 of the isoalloxazine ring.

Our findings also show that the levels of FMN consumption and DMB production increase as the pH increases. This may suggest that a deprotonated C4a-peroxyflavin (C4a-OO^-), which is favored at a higher pH (the pK_a of hydrogen peroxide is 11.6), is involved in breaking the isoalloxazine ring, as indicated by the Wang–Quan mechanism.[6,42] However, if the C4a-OO^- intermediate is indeed favored, then the ring expansion proposed by the Ealick–Begley mechanism would likely be disfavored at a higher pH (Figure 1).

Finally, the D32 residue may also play an important role in ensuring efficient oxidation by forming a hydrogen bond to N10 during formation of the imidazole ring, thereby increasing the efficacy of donation of N10's electrons to enhance hydride formaton,[6] which may also explain the severely inhibited turnover ability of the D32N mutant. Overall, our results are mostly consistent with the Wang–Quan mechanism but do not invalidate any of the other proposed mechanisms.

Our findings will provide information and limitations that can lead to future studies of the mechanism of the flavin destructase phase of the reaction that follows formation of the C4a-

OO(H) intermediate. Furthermore, the key finding that C4a-OO(H) formation is unaffected by the D32N and S167G mutations, despite the fact that these mutants have a weakened ability to fragment the isoalloxazine ring, provides new limits for probing the flavin destruction and DMB formation phases of this remarkable enzymatic transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Sumita Chakraborty for technical assistance and Ta-Yi Yu for some of the purified BluB mutant proteins. The authors thank Pimchai Chaiyen, Graham Walker, and Tadhg Begley for helpful discussions.

Funding

This work was supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund to M.E.T., National Institutes of Health Grants GM083303 to M.E.T. and GM64711 to D.P.B., and the Ramalingaswami Re-entry Fellowship of the Department of Biotechnology, Government of India to A.B.H.

REFERENCES

(1). Zhang Y, Rodionov DA, Gelfand MS, and Gladyshev VN (2009) Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. BMC Genomics 10,78. [PubMed: 19208259]

(2). Roth JR, Lawrence JG, and Bobik TA (1996) Cobalamin (coenzyme B12): synthesis and biological significance. Annu. Rev. Microbiol 50, 137–181. [PubMed: 8905078]

(3). Taga ME, Larsen NA, Howard-Jones AR, Walsh CT, and Walker GC (2007) BluB cannibalizes flavin to form the lower ligand of vitamin B12. Nature 446, 449–453. [PubMed: 17377583]

(4). Gray MJ, and 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. [PubMed: 17301238]

(5). Campbell GRO, Taga ME, Mistry K, Lloret J, Anderson PJ, Roth JR, and Walker GC (2006) *Sinorhizobium meliloti bluB* is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B12. Proc. Natl. Acad. Sci. U. S. A 103, 4634–4639. [PubMed: 16537439]

(6). Wang XL, and 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. [PubMed: 21344938]

(7). Ealick SE, and Begley TP (2007) Molecular cannibalism. Nature 446, 387–389. [PubMed: 17377573]

(8). Massey V (2000) The chemical and biological versatility of riboflavin. Biochem. Soc. Trans 28, 283–296. [PubMed: 10961912]

(9). Cortial S, Chaignon P, Iorga BI, Aymerich S, Truan G, Gueguen-Chaignon V, Meyer P, Morera S, and Ouazzani J (2010) NADH oxidase activity of *Bacillus subtilis* nitroreductase NfrA1: insight into its biological role. FEBS Lett 584, 3916–3922. [PubMed: 20727352]

(10). Leferink NGH, Jose MDF, van den Berg WAM, and van Berkel WJH (2009) Functional assignment of Glu386 and Arg388 in the active site of l-galactono-gamma-lactone dehydrogenase. FEBS Lett 583, 3199–3203. [PubMed: 19737562]

(11). Fraaije MW, and van Berkel WJ (1997) Catalytic mechanism of the oxidative demethylation of 4-(methoxymethyl)-phenol by vanillyl-alcohol oxidase. Evidence for formation of a p-quinone methide intermediate. J. Biol. Chem 272, 18111–18116. [PubMed: 9218444]

(12). Sucharitakul J, Prongjit M, Haltrich D, and Chaiyen P (2008) Detection of a C4a-hydroperoxyflavin intermediate in the reaction of a flavoprotein oxidase. Biochemistry 47, 8485–8490. [PubMed: 18652479]

(13). Franceschini S, Fedkenheuer M, Vogelaar NJ, Robinson HH, Sobrado P, and Mattevi A (2012) Structural insight into the mechanism of oxygen activation and substrate selectivity of flavin-dependent N-hydroxylating monooxygenases. Biochemistry 51, 7043–7045. [PubMed: 22928747]

(14). Mayfield JA, Frederick RE, Streit BR, Wencewicz TA, Ballou DP, and DuBois JL (2010) Comprehensive spectroscopic, steady state, and transient kinetic studies of a representative siderophore-associated flavin monooxygenase. J. Biol. Chem 285, 30375–30388. [PubMed: 20650894]

(15). Chenprakhon P, Trisrivirat D, Thotsaporn K, Sucharitakul J, and Chaiyen P (2014) Control of C4a-hydroperoxyflavin protonation in the oxygenase component of p-hydroxyphenylacetate-3-hydroxylase. Biochemistry 53, 4084–4086. [PubMed: 24878148]

(16). Gonzalez-Osorio L, Luong K, Jirde S, Palfey BA, and Vey JL (2016) Initial investigations of C4a-(hydro)peroxyflavin intermediate formation by dibenzothiophene monooxygenase. Biochem. Biophys. Res. Commun 481, 189–194. [PubMed: 27815073]

(17). Ellis HR (2010) The FMN-dependent two-component monooxygenase systems. Arch. Biochem. Biophys 497, 1–12. [PubMed: 20193654]

(18). Hastings JW, and Nealson KH (1978) Bacterial bioluminescence light emission in the mixed function oxidation of reduced flavin and fatty aldehyde. Crit. Rev. Biochem 5, 163–184. [PubMed: 363350]

(19). Gnidehou S, Caillou B, Talbot M, Ohayon R, Kaniewski J, Noël-Hudson M-S, Morand S, Agnangji D, Sezan A, Courtin F, Virion A, and Dupuy C (2004) Iodotyrosine dehalogenase 1 (DEHAL1) is a transmembrane protein involved in the recycling of iodide close to the thyroglobulin iodination site. FASEB J 18, 1574–576. [PubMed: 15289438]

(20). Thomas SR, McTamney PM, Adler JM, Laronde-Leblanc N, and Rokita SE (2009) Crystal structure of iodotyrosine deiodinase, a novel flavoprotein responsible for iodide salvage in thyroid glands. J. Biol. Chem 284, 19659–19667. [PubMed: 19436071]

(21). Koutmos M, Gherasim C, Smith JL, and Banerjee R (2011) Structural basis of multifunctionality in a vitamin B12-processing enzyme. J. Biol. Chem 286, 29780–29787. [PubMed: 21697092]

(22). Yamada K, Gherasim C, Banerjee R, and Koutmos M (2015) Structure of Human B12 Trafficking Protein CblD Reveals Molecular Mimicry and Identifies a New Subfamily of Nitro-FMN Reductases. J. Biol. Chem 290, 29155–29166. [PubMed: 26364851]

(23). Collins HF, Biedendieck R, Leech HK, Gray M, Escalante-Semerena JC, McLean KJ, Munro AW, Rigby SEJ, Warren MJ, and Lawrence AD (2013) *Bacillus megaterium* has both a functional BluB protein required for DMB synthesis and a related flavoprotein that forms a stable radical species. PLoS One 8, e55708. [PubMed: 23457476]

(24). Yu T-Y, Mok KC, Kennedy KJ, Valton J, Anderson KS, Walker GC, and Taga ME (2012) Active site residues critical for flavin binding and 5,6-dimethylbenzimidazole biosynthesis in the flavin destructase enzyme BluB. Protein Sci 21, 839–849. [PubMed: 22528544]

(25). Sucharitakul J, Chaiyen P, Entsch B, and Ballou DP (2005) The reductase of p-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* requires p-hydroxyphenylacetate for effective catalysis. Biochemistry 44, 10434–10442. [PubMed: 16042421]

(26). Chaiyen P, Brissette P, Ballou DP, and Massey V (1997) Thermodynamics and reduction kinetics properties of 2-methyl- 3-hydroxpyridine-5-carboxylic acid oxygenase. Biochemistry 36, 2612–2621. [PubMed: 9054568]

(27). Ortiz-Maldonado M, Ballou DP, and Massey V (1999) Use of free energy relationships to probe the individual steps of hydroxylation of p-hydroxybenzoate hydroxylase: studies with a series of 8-substituted flavins. Biochemistry 38, 8124–8137. [PubMed: 10387058]

(28). Yeh E, Cole LJ, Barr EW, Bollinger JM, Ballou DP, and Walsh CT (2006) Flavin redox chemistry precedes substrate chlorination during the reaction of the flavin-dependent halogenase RebH. Biochemistry 45, 7904–7912. [PubMed: 16784243]

(29). Patil PV, and Ballou DP (2000) The use of protocatechuate dioxygenase for maintaining anaerobic conditions in biochemical experiments. Anal. Biochem 286, 187–192. [PubMed: 11067739]

(30). Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acid Res 32, 1792–1797. [PubMed: 15034147]

(31). Hall TA (1999) BioEdit: A user-friendly biological sequence alignment program for Windows 95/98/NT. Nucleic Acids Symp. Ser 41, 95–98.

(32). Miller MA, Pfeiffer W, and Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees In Proceedings of the Gateway Computing Environments Workshop (GCE), pp 1–8, New Orleans, LA.

(33). Chaiyen P, Sucharitakul J, Svasti J, Entsch B, Massey V, and Ballou DP (2004) Use of 8-substituted-FAD analogues to investigate the hydroxylation mechanism of the flavoprotein 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase. Biochemistry 43, 3933–3943. [PubMed: 15049701]

(34). Massey V, Palmer G, and Ballou DP (1971) On the reaction of reduced flavins and flavoproteins with oxygen In Flavins and Flavoproteins (Kamin H, Ed.) pp 349–361, University Park Press, Baltimore.

(35). Ballou DP, and Entsch B (2013) The reaction mechanisms of groups A and B flavoprotein monooxygenases In Handbook of Flavoproteins (Hille R, Miller SM, and Palfey B, Ed.) pp 1–28, Walter de Gruyter.

(36). Zhan X, Carpenter RA, and Ellis HR (2008) Catalytic importance of the substrate binding order for the FMNH2-dependent alkanesulfonate monooxygenase enzyme. Biochemistry 47, 2221–2230. [PubMed: 18198899]

(37). Valton J, Mathevon C, Fontecave M, Nivière V, and Ballou DP (2008) Mechanism and regulation of the Two-component FMN-dependent monooxygenase ActVA-ActVB from *Streptomyces coelicolor*. J. Biol. Chem 283, 10287–10296. [PubMed: 18245777]

(38). Fagan RL, and Palfey BA (2010) Flavin-dependent enzymes In Comprehensive Natural Products II: Chemistry and Biology, pp 37–113. Elsevier, Amsterdam.

(39). Sucharitakul J, Chaiyen P, Entsch B, and Ballou DP (2006) Kinetic mechanisms of the oxygenase from a two-component enzyme, p-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii*. J. Biol. Chem 281, 17044–17053. [PubMed: 16627482]

(40). Maeda-Yorita K, and Massey V (1993) On the reaction mechanism of phenol hydroxylase. New information obtained by correlation of fluorescence and absorbance stopped flow studies. J. Biol. Chem 268, 4134–4144. [PubMed: 8440702]

(41). Yorita K, Misaki H, Palfey BA, and Massey V (2000) On the interpretation of quantitative structure-function activity relationship data for lactate oxidase. Proc. Natl. Acad. Sci. U. S. A 97, 2480–2485. [PubMed: 10706608]

(42). Sheng D, Ballou DP, and Massey V (2001) Mechanistic studies of cyclohexanone monooxygenase: chemical properties of intermediates involved in catalysis. Biochemistry 40, 11156–11167. [PubMed: 11551214]

(43). Frederick RE, Ojha S, Lamb A, and Dubois JL (2014) How pH modulates the reactivity and selectivity of a siderophore-associated flavin monooxygenase. Biochemistry 53, 2007–2016. [PubMed: 24490904]

(44). Dhammaraj T, Pinthong C, Visitsatthawong S, Tongsook C, Surawatanawong P, and Chaiyen P (2016) A Single-Site Mutation at Ser146 Expands the Reactivity of the Oxygenase Component of p-Hydroxyphenylacetate 3-Hydroxylase. ACS Chem. Biol 11, 2889–2896. [PubMed: 27541707]

(45). Moore EG, Ghisla S, and Massey V (1979) Properties of flavins where the 8-methyl group is replaced by mercapto- residues. J. Biol. Chem 254, 8173–8178. [PubMed: 475888]

(46). Schopfer LM, Wessiak A, and Massey V (1991) Interpretation of the spectra observed during oxidation of p-hydroxybenzoate hydroxylase reconstituted with modified flavins. J. Biol. Chem 266, 13080–13085. [PubMed: 1906463]

(47). Fraaije MW, and Mattevi A (2000) Flavoenzymes: diverse catalysts with recurrent features. Trends Biochem. Sci 25, 126–132. [PubMed: 10694883]

(48). Phatarphekar A, Buss JM, and Rokita SE (2014) Iodotyrosine deiodinase: a unique flavoprotein present in organisms of diverse phyla. Mol. BioSyst 10, 86–92. [PubMed: 24153409]

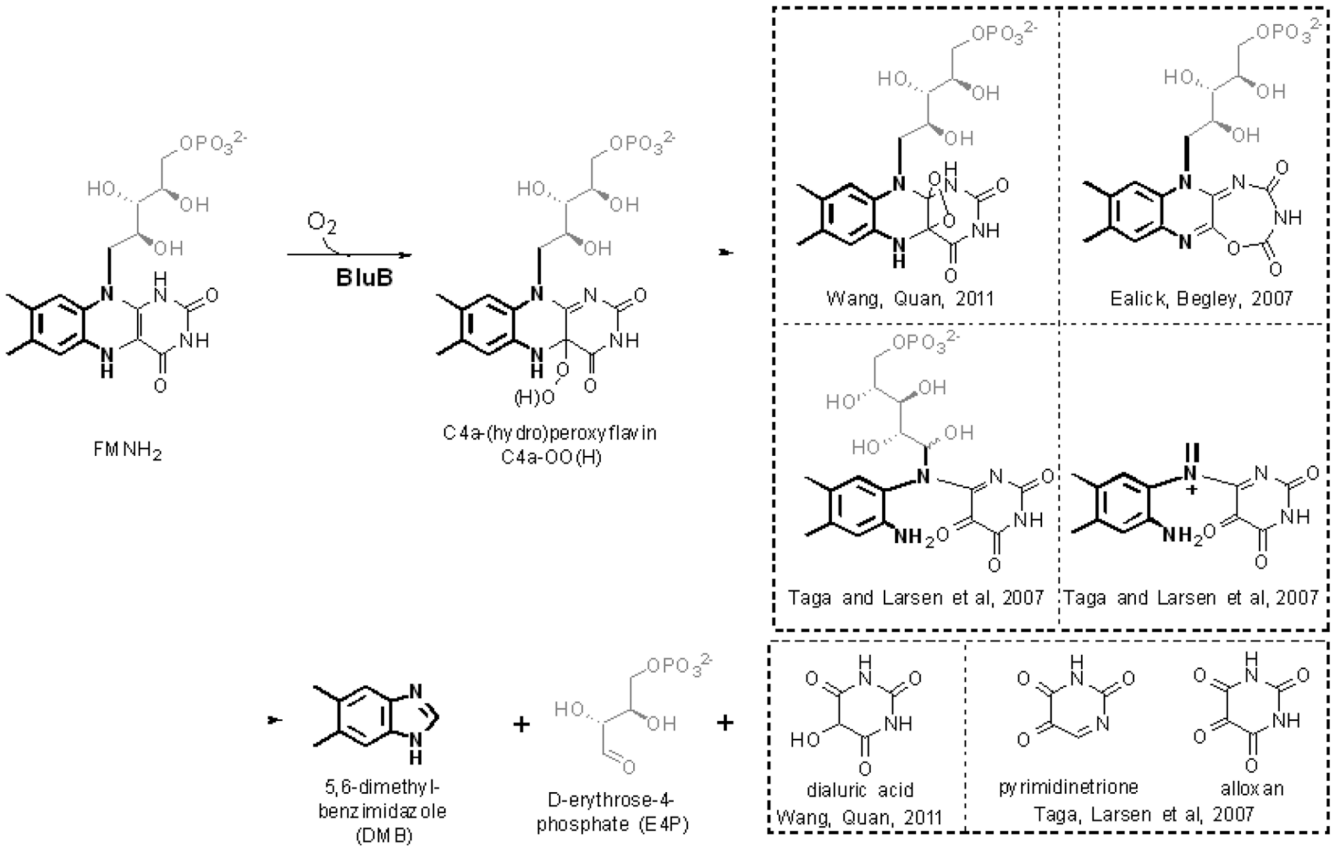


Figure 1.

Reaction catalyzed by BluB. Four reaction mechanisms have been proposed for the BluB-catalyzed conversion of $FMNH_2$ to DMB. Proposed intermediates and co-products in the four mechanisms are shown.[3,6,7] The portion of FMN that is converted to DMB is shown in bold.

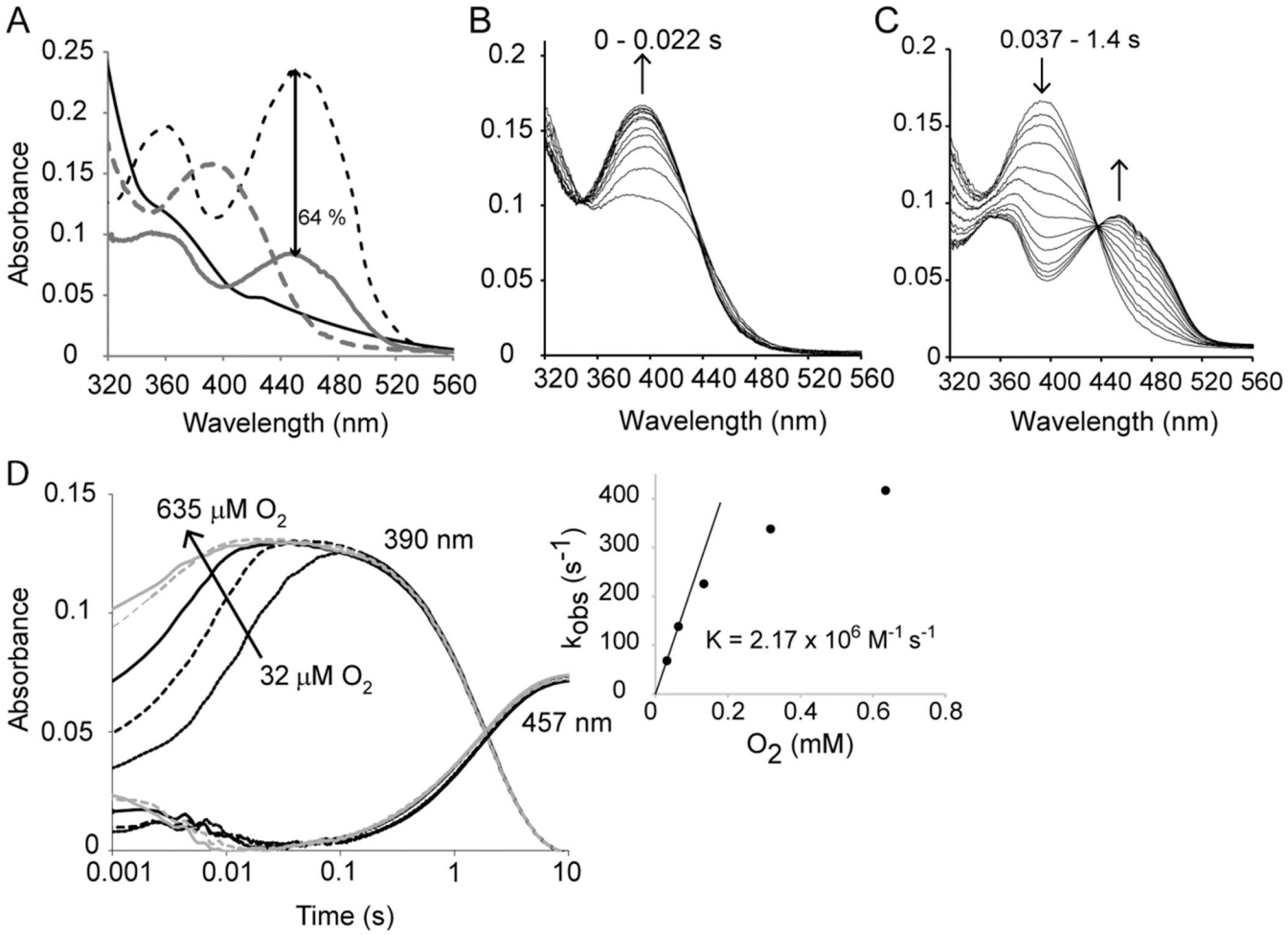


Figure 2.

Stopped-flow analysis of wild type BluB with $FMNH_2$ and O_2. (A) Ultraviolet–visible spectra of different points in the reaction: dashed black trace, FMN bound to BluB prior to reduction; solid black trace, $FMNH_2$ bound to BluB; dashed gray trace, C4a-OO(H) intermediate 0.022 s after mixing with O_2; solid gray trace, reoxidized FMN remaining after completion of the reaction at 14 s. The amount of flavin consumed (64%) in the reaction is indicated. (B) Formation of C4a-OO(H) occurs within the first 0.022 s. (C) Decay of C4a-OO(H) leading to formation of DMB and FMN between 0.037 and 1.4 s. (D) C4a-OO(H) formation shows a dependence on the concentration of oxygen. Concentrations of O_2 in these reactions were 32, 63, 133, 317, and 635 μM. The inset shows the rate of C4a-OO(H) formation as a function of O_2 concentration. The second-order rate constant calculated from the linear portion of the graph is shown.

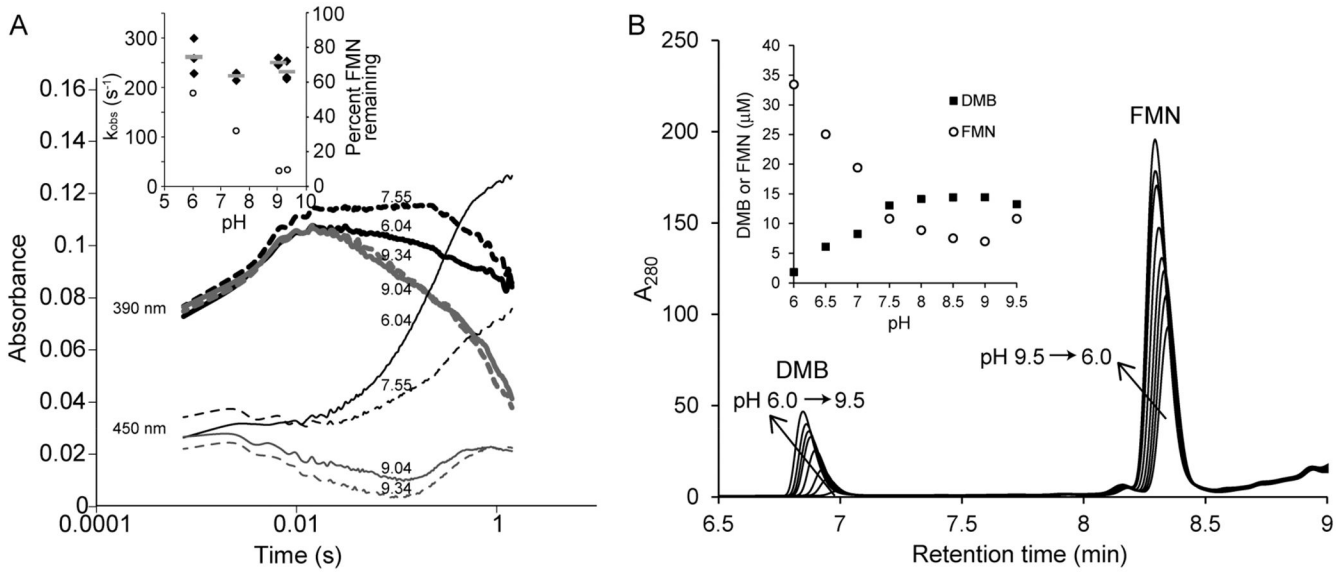


Figure 3.

Flavin destructase activity of BluB is pH-dependent. (A) A_{390} and A_{450} traces for reaction mixtures containing 40 μM $FMNH_2$ and 80 μM BluB mixed with aerobic buffer (266 μM O_2) performed at pH 6.04–9.34 showing the formation of the C4a-OO(H) and the reappearance of the oxidized flavin. The inset shows the rate of C4a-OO(H) formation (♦) with the average indicated by gray lines, and the FMN remaining as a percentage of the initial amount (○). (B) HPLC analysis of reactions at varying pHs. The inset shows the concentrations of DMB and FMN in these reactions as calculated from the average peak areas in two independent experiments.

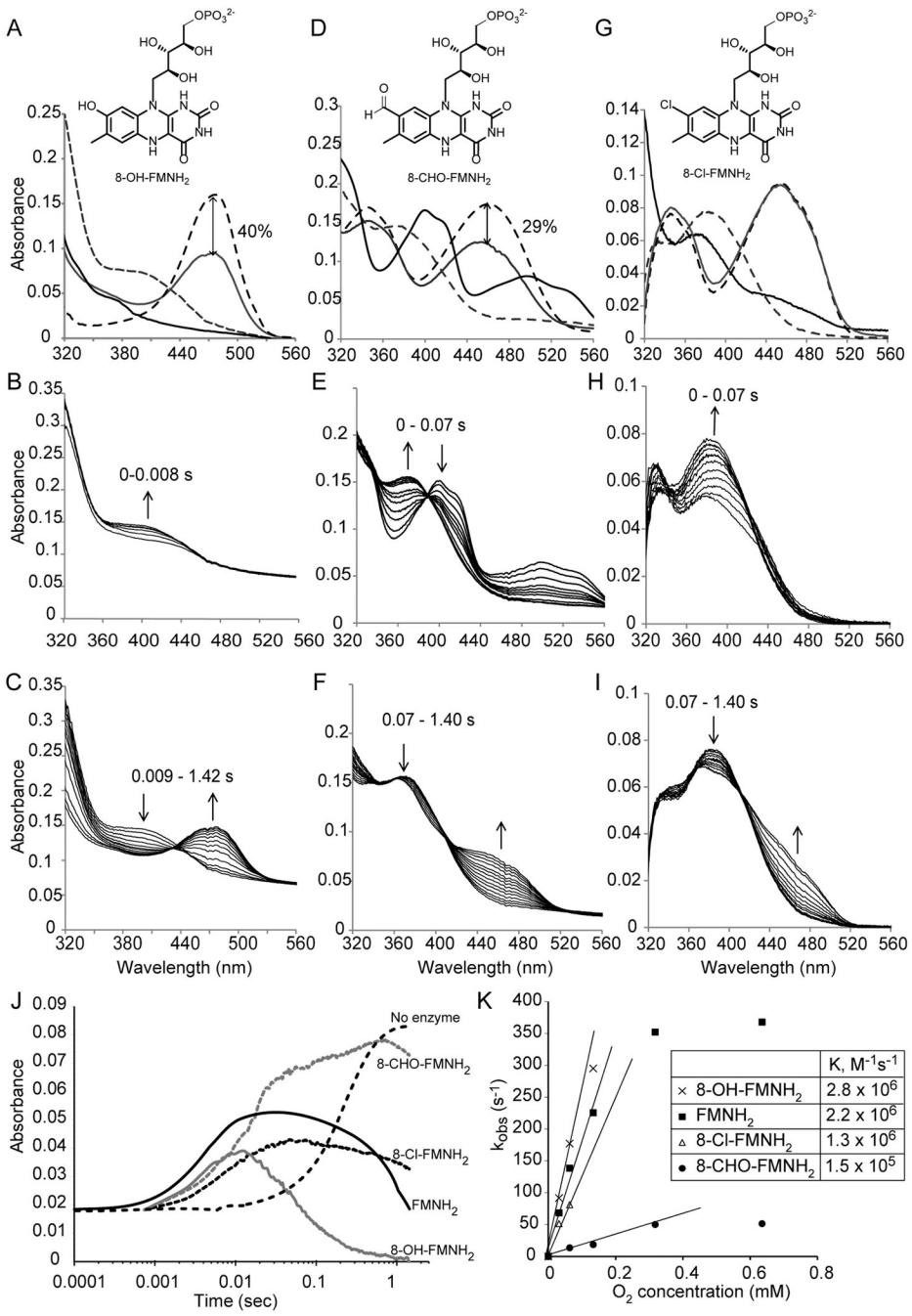


Figure 4.

Stopped-flow analysis of wild type BluB with various 8-substituted FMN analogues. (A, D, and G) Ultraviolet–visible spectra of different points in the reaction of 8-OH-FMN, 8-CHO-FMN, and 8-Cl-FMN: dashed black trace, oxidized flavin bound to BluB; solid black trace, reduced flavin bound to BluB; dashed gray trace, C4a-OO(H) intermediate; solid gray trace, reoxidized flavin after completion of the reaction. (B, E, and H) Formation of C4a-OO(H) intermediates. (C, F, and I) Decay of C4a-OO(H) and formation of the oxidized flavin analogues. (J) Formation of the C4a-OO(H) intermediate for the substituted flavins occurs

during the first 1.42 s of the reaction of O_2 with the reduced flavin analogues as compared to that with $FMNH_2$ (solid black line) and with a control using $FMNH_2$ and no enzyme (black dashed line). The reaction with 8-CHO-$FMNH_2$ is shown at 370 nm, and the other reactions are shown at 390 nm. (K) C4a-OO(H) formation shows a dependence on the concentration of oxygen for the substituted flavin analogues. The table shows calculated second-order rate constants for C4a-OO(H) formation for each analogue.

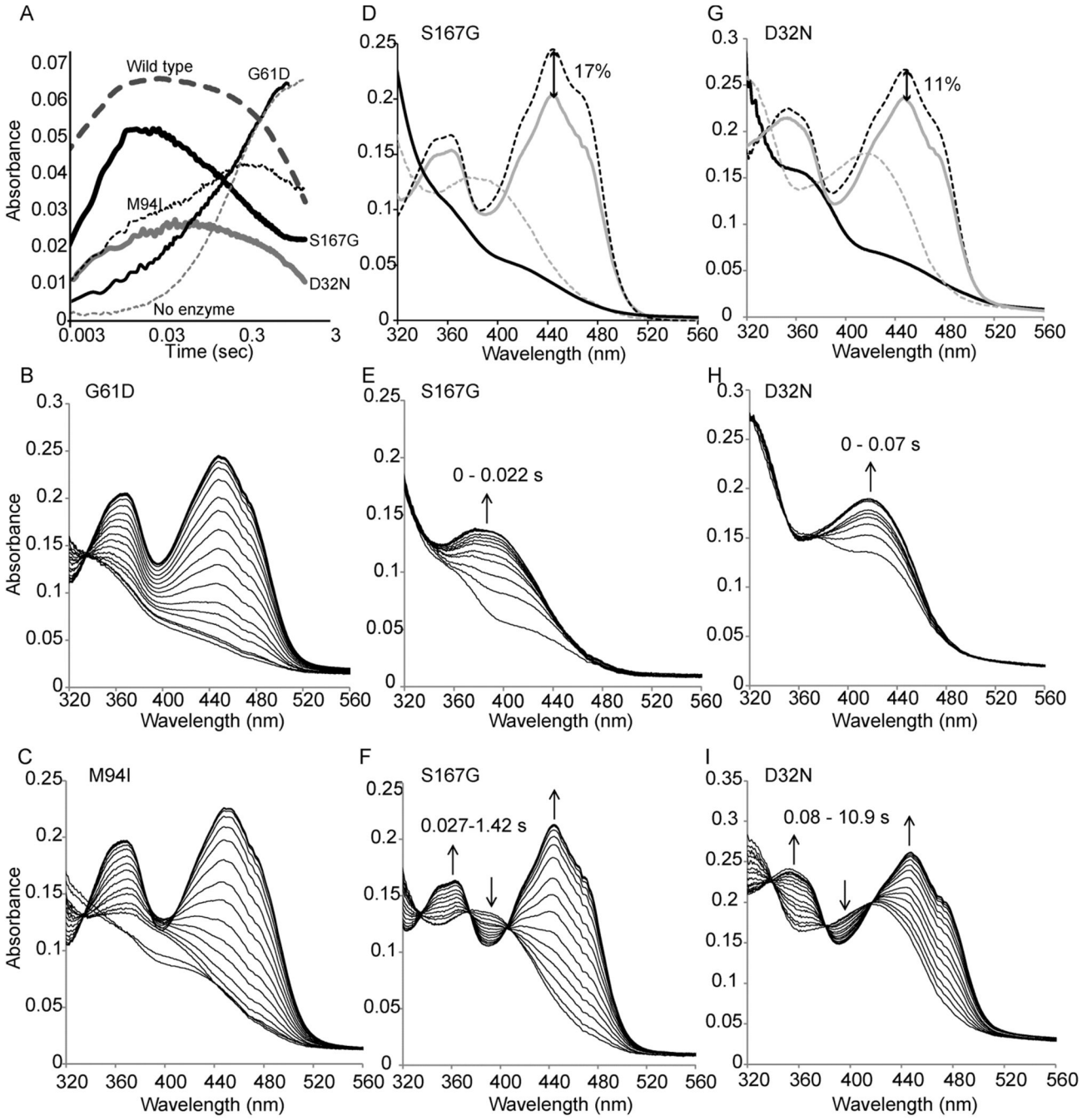
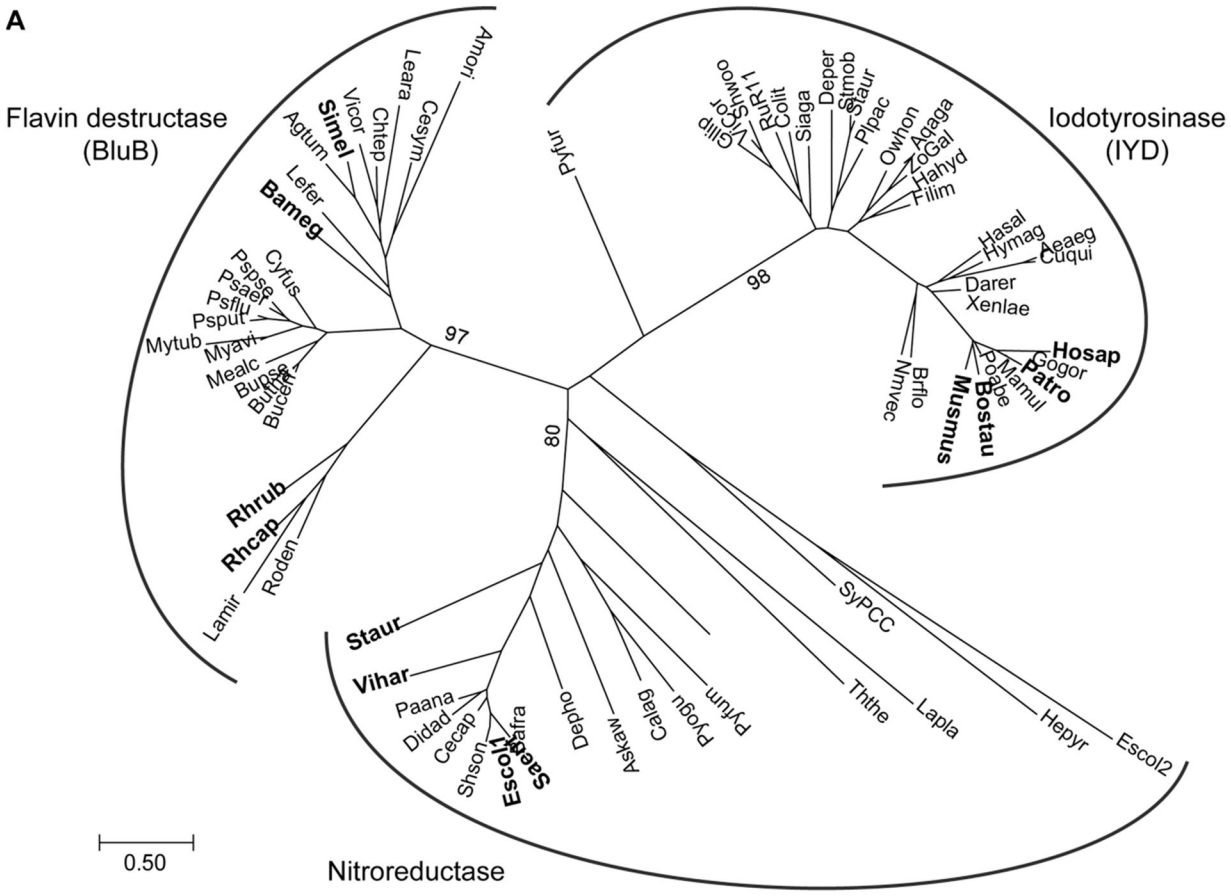


Figure 5.

Stopped-flow analysis of the reaction of O_2 (265 μM) with $FMNH_2$ bound to catalytically deficient BluB mutants. (A) Comparison of G61D (450 nm), M94I (450 nm), D32N (410 nm), and S167G (390 nm) with wild type BluB (390 nm) and a control with no enzyme (390 nm). (B) Reaction of the G61D mutant with $FMNH_2$ and O_2 results in the formation of only oxidized flavin. (C) Reaction of M94I with $FMNH_2$ and O_2 appears to result in the formation of a small amount of C4a-OO(H), but most of the $FMNH_2$ is converted directly to oxidized flavin. (D and G) Reaction of S167G and D32N with $FMNH_2$ results in the

formation of C4a-OO(H) in each case, followed by loss of only 17 and 11% of the flavin, respectively, with the remaining flavin forming FMN: dashed black trace, oxidized flavin bound to the mutant enzyme; solid black trace, reduced flavin bound to the mutant enzyme; dashed gray trace, C4a-OO(H) intermediate; solid gray trace, reoxidized flavin after completion of the reaction. (E and H) C4a-OO(H) forms in S167G at 0.022 s and in D32N at 0.07 s. Note that nearly complete formation of the C4a-OO(H) species occurs in each case. (F and I) Continuation of the reactions of S167G and D32N to 1.42 and 10.9 s, respectively. The majority of flavin in each case is subsequently reoxidized to FMN.



B

	D32	S167
S. meliloti BluB	RAIETRR**D**VRDEFLPEP	GVGWV**S**IFHESEIK
R. capsulatus BluB	EVLRWRR**D**VRH-FRPDP	GLGMV**S**VLDPKAVE
M. tuberculosis BluB	RVIAERR**D**MRR-FVPGG	GMGWV**S**LFDPQRLA
R. rubrum BluB	ALFQWRR**D**VRH-FRKDP	GVGWV**S**VLDPQTVT
H. sapiens IYD	ELLNKRR**S**VRFISNEQV	VNNGI**T**MRHQTARH
M. musculus IYD	ELLNKRR**S**VRFISSEHV	NAGLV**T**VTTTPLNC
B. taurus IYD	ELLNKRR**S**VRFISNEQV	NAGLV**T**VTTTPLNC
P. troglodytes IYD	ELLNKRR**S**VRFIRNEQV	NAGLV**T**VTTTPLNC
S. aureus NfrA	YLVKKHH**S**VRKFKNKPL	GIVFL**G**SLRNDVER
V. harveyi NfsA	ETILAHR**S**IRKFTAVPI	GGVYI**G**GLRNSAAQ
E. coli NfsA	ELICGHR**S**IRHFTDEPI	GGVYI**G**GLRNNIEA
S. enterica NfsA	ELLCGHR**S**IRHFTDEPV	GGVYI**G**GIRNNIES

Figure 6.

Phylogenetic tree with enzymes from the BluB, IYD, and nitroreductase families. (A) The BluB and IYD homologues each form a single clade distinct from the nitroreductases. Table S1 provides the complete names of the organisms that have been used to generate the tree. (B) Alignment of BluB, IYD, and nitroreductase (NfsA) sequences for which biochemical data are available (indicated in bold in panel A). The D32 and S167 residues are conserved

in BluB enzymes and differ in the corresponding regions of IYD and nitroreductase homologues.