Published in final edited form as:

Biochemistry. 2012 September 18; 51(37): 7342–7347. doi:10.1021/bi300887m.

Isotope effects suggest a stepwise mechanism for Berberine Bridge Enzyme†

Helena M. Gaweska, Kenneth M. Roberts, and Paul F. Fitzpatrick*

Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229

Abstract

The flavoprotein Berberine Bridge Enzyme (BBE) catalyzes the regioselective oxidative cyclization of (*S*)-reticuline to (*S*)-scoulerine in an alkaloid biosynthetic pathway. A series of solvent and substrate deuterium kinetic isotope effect studies were conducted in order to discriminate between a concerted mechanism in which deprotonation of the substrate phenol occurs before or during hydride transfer from the substrate to the flavin cofactor and substrate cyclization, and a stepwise mechanism, in which hydride transfer results in the formation of a methylene iminium ion intermediate that is subsequently cyclized. The substrate deuterium isotope effect of 3.5 on k_{red} , the rate constant for flavin reduction, is pH-independent, indicating that C-H bond cleavage is rate-limiting during flavin reduction. Solvent isotope effects on k_{red} are one for both wild-type BBE and the E417Q mutant, indicating that solvent exchangeable protons are not in flight during or before flavin reduction, thus eliminating a fully concerted mechanism as a possibility for catalysis by BBE. An intermediate was not detected by rapid chemical quench or continuous-flow mass spectrometry experiments, indicating that it must be short-lived.

Berberine Bridge Enzyme (BBE) is a plant enzyme that participates in an alkaloid biosynthetic pathway that produces a variety of pharmacologically active alkaloids, including berberine, sanguinarine, and palmatine (1). The enzyme, an FAD-containing amine oxidase, catalyzes a unique oxidative cyclization reaction, converting (*S*)-reticuline to (*S*)-scoulerine (Scheme 1) (2). This regioselective reaction has not yet been reproduced by synthetic organic methods. Furthermore, only a couple of other enzymes have been identified that catalyze similar reactions: cannabidiolic acid synthase and Δ^1 -tetrahydrocannabinolic acid synthase from *Cannabis sativa* (3, 4). Neither of these enzymes has been characterized enzymatically.

Stopped-flow analyses of the BBE reductive half-reaction have failed to identify a flavin semiquinone intermediate, suggesting that substrate oxidation does not proceed via single electron transfer from the substrate to the cofactor (5) and instead likely occurs via hydride transfer, as observed with other flavoprotein oxidases (6, 7). Both concerted and stepwise mechanisms have been proposed for BBE (Scheme 2) (8, 9). In the stepwise mechanism, a hydride equivalent is transferred from the substrate to the flavin, forming a methylene iminium ion intermediate; subsequently, an active site base, Glu417, deprotonates a substrate phenol, making the adjacent carbon more nucleophilic and enabling its attack on the *N*-methylene group, yielding the cyclized product. In the concerted mechanism,

†This work was supported by NIH grants R01 GM058698 to PFF and F32 GM097762 to HMG.

*To whom correspondence should be addressed: Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229. Phone: (210) 567-8264. Fax: (210) 567-8778. fitzpatrick@biochem.uthscsa.edu.

Supporting Information. Figure S1 is provided in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

deprotonation of the phenol occurs during concomitant attack on the *N*-methyl group by the C2' and transfer of a hydride to the flavin.

Several studies support each mechanistic proposal. Oxidation by BBE of (*S*)-*N*-methylcoclaaurine yields a demethylated product, suggesting that the reaction proceeds via formation of a methylene iminium ion intermediate (Scheme 3) (8). BBE oxidizes the tetrahydropyprotoberberine alkaloids (*S*)-coreximine, (*S*)-scoulerine and (*S*)-norsteponine, forming a double bond between the nitrogen and C-8 of each (Scheme 3) (8, 10); these observations also suggest a two-step mechanism for the oxidative cyclization of (*S*)-reticuline (8). However, analyses of the products resulting from a different set of substrate analogs have been taken as support for a concerted mechanism (9). Oxidation of laudanosine is associated with an absorbance increase around 360 nm, suggesting formation of a double bond within the isoquinoline ring system (Scheme 3). BBE oxidizes the C1-N bond of 6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline, as opposed to the *N*-CH₃ bond expected in a two-step mechanism (Scheme 3) (9). Mutation of the proposed active site base Glu417 to glutamine leads to a 1500-fold decrease in the rate constant for flavin reduction, resulting in substrate oxidation becoming the rate-determining step during turnover (9). This has been interpreted as additional evidence for a concerted mechanism in which deprotonation of the substrate phenol is necessary for the concerted attack on the incipient methyl aminium and the transfer of a hydride to the flavin cofactor. In the present study, we performed a series of substrate deuterium and solvent kinetic isotope effect studies to discriminate between these two mechanistic proposals.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO) or Fisher Scientific (Waltham, MA) unless otherwise stated. Superdex 200 prep grade and Sephacryl S-200 FPLC columns were purchased from GE Life Sciences (Pittsburgh, PA). (*R,S*)-Norreticuline was purchased from Hang Zhou Sage Chemical Co. (Hang Zhou, China).

Syntheses

(*R,S*)-Reticuline was synthesized from (*R,S*)-norreticuline in two steps, using the method of Rice and Brossi (11). Deuterated (*R,S*)-reticuline (*-N*-CD₃) was synthesized with the same protocol using ethyl formate-d₁ (CDN Isotopes, Quebec, Canada) and BD₃ in THF. ¹H NMR (Bruker Avance 500 MHz) analysis of the deuterated substrate yielded essentially the same NMR spectrum as that reported for protiated (*R,S*)-reticuline (12), but with the singlet of the *-N*-CH₃ protons at 2.45 ppm missing.

Protein Expression and Purification

Wild-type BBE and the E417Q mutant were expressed, purified, and quantified as described previously (13, 14); protein was expressed with *Pichia pastoris* strains provided by Professor Peter Macheroux (University of Graz, Austria). Volumes were adjusted for a 2.5-l fermentation in a BioFlo/Celligen 115 Fermentor (Edison, NJ). A Sephacryl S-200 column was sometimes used instead of a Superdex 200 prep grade column for purification of BBE. An additional chromatographic purification step was added during the purification of the E417Q mutant: fractions eluted from the Superdex 200 column that contained BBE were concentrated, dialyzed against buffer containing no salt (100 mM Tris, pH 8.0), applied onto a Mono Q column equilibrated with the same buffer, and eluted with a gradient of increasing salt concentration (0–250 mM NaCl in 100 mM Tris buffer, pH 8.0).

Enzyme Assays

HPLC-based steady-state assays with BBE were based on those described previously (14), using 0.5 nM enzyme and varying concentrations of (*R,S*)-reticuline (0.19–6.0 μ M) in 50 mM of the appropriate buffer at 25 °C. The assays were initiated by the addition of enzyme. The following buffers were used: MOPS, pH 6.75–7.75; Bicine, pH 7.75–8.75; and CHES, pH 8.75–9.75. Instead of the continuous assay described previously, reactions were quenched with an equal volume of 1 N NaOH at varying time intervals (40 s – 20 min). Subsequently, the pH was neutralized with the same volume of either 1 N HCl or 1 N acetic acid before quantifying product formation by HPLC analysis. The HPLC analysis was performed as described previously (14), but using a Phenomenex (Torrance, CA) C18 column (250 $\times$ 4.6 mm) and a Waters (Milford, MA) HPLC system. Substrate and product were separated using an isocratic elution of 65% methanol and 35% 50 mM potassium phosphate, pH 7.0.

Wild-type enzyme k_{cat} values were determined in 50 mM CHES, pD 9.5, in 98–99% D₂O, and wild-type and mutant flavin reduction rate constants were measured in 100 mM of the appropriate buffer listed above, pD 7–10, in 94–95% D₂O. All experiments were performed at saturating substrate concentrations (100–400 μ M (*R,S*)-reticuline or 100–400 μ M deuterated-(*R,S*)-reticuline). Solvent isotope effects were calculated by directly comparing the kinetic parameters obtained in H₂O and D₂O. The solvent isotope effect on k_{cat} was determined by comparing rates measured at pH 9 and pD 9.5. The effects on k_{red} with the wild-type enzyme were calculated using the pL-independent values obtained by fitting the entire k_{red} -pL curves to eq 1, and the effects on k_{red} with the E417Q mutant were determined by comparing the values in the pH-independent region, pH 8.5–9.5 and pD 9–10.

The dependence of BBE activity on oxygen concentration was determined using an oxygen electrode (Model 5300A, Yellow Springs Instruments, Yellow Springs, OH) to measure oxygen consumption at 25 °C. A 1-ml reaction contained 50 mM CHES, pH 9, and 200 μ M (*R,S*)-reticuline, and the reaction was initiated by the addition of BBE (0.25 or 0.125 μ M final). The oxygen concentration was varied from 20–1250 μ M by bubbling the reaction solution with the appropriate concentration of oxygen for 15 minutes before addition of enzyme.

Flavin reduction during substrate oxidation was monitored at 445 nm using an Applied Photophysics (United Kingdom) SX-20 MV stopped-flow spectrophotometer under anaerobic conditions. The system was made anaerobic by overnight incubation of the flow cell and syringes with 100 mM appropriate buffer containing 5 mM glucose. The buffer was deoxygenated in a tonometer by exposing it to 16 cycles of vacuum and oxygen-scrubbed argon; subsequently, glucose oxidase (30 nM final) was added to maintain anaerobic conditions and remove any remaining oxygen. The following buffers were used: MES, pH 6.0; MOPS, pH 6.75–7.75; Bicine, pH 8.0–8.55; and CHES, pH 9–9.75. Substrate solutions were made anaerobic by bubbling them with oxygen-scrubbed argon for 5 minutes. Oxygen was removed from enzyme solutions by exposing them to 16 cycles of vacuum and oxygen-scrubbed argon. Both substrate and enzyme solutions included 5 mM glucose, and 30 nM glucose oxidase was added after either bubbling with argon or the vacuum/argon cycles. Experiments were performed using final concentrations of 100 mM buffer, 17 μ M BBE, and 75–400 μ M (*R,S*)-reticuline. Substrate deuterium kinetic isotope effects on the rate constant for reduction were measured in 100 mM buffer under anaerobic conditions with saturating deuterated substrate concentrations (100–400 μ M (*R,S*)-reticuline) at 25 °C.

Rapid-Quench Analyses

Rapid-quench experiments were performed in air-saturated buffers using an SFM-400 Bio-Logic (France) instrument. Reactions contained final concentrations of 10 μ M BBE, 200 μ M (*R,S*)-reticuline, and 100 mM CHES, pH 9.0, at 25 °C. Reactions were quenched with 0.67 M NaOH. The solution was neutralized with 1 N acetic acid before HPLC analysis to determine the amount of product formed.

Continuous Mass Spectrometry (MS) Experiments

BBE reaction progress was analyzed using an LTQ Orbitrap Discovery Mass Spectrometer (Thermo Scientific) equipped with a custom nano-electrospray ionization source containing a small volume continuous-flow mixer driven by a NanoFlow Metering System (Eksigent, Dublin, CA). Wild-type and E417Q BBE were exchanged into 50 mM ammonium acetate, pH 9.0, using a series of concentration and dilution steps. Reactions were initiated by mixing substrate and enzyme solutions in a 1:1 ratio at varying flow rates of 1–10 μ l/min at 10 and 25 °C; the delay volume was approximately 0.04 μ l, yielding reactions times of 0.25 – 2.5 s. The reactions consisted of final concentrations of 10 μ M wild-type or E417Q BBE and 40 or 400 μ M (*R,S*)-reticuline in 50 mM ammonium acetate, pH 9.0. The following were monitored by high-resolution MS: reticuline (m/z 330.1705), products scoulerine and coreximine, (m/z 328.1549), the imine intermediate (m/z 328.1549) and the demethylated intermediate norreticuline (m/z 316.1549).

Data Analysis

Kinetic data were analyzed using the KaleidaGraph program (Synergy Software, Reading, PA). Steady-state data were fit to the Michaelis-Menten equation to determine k_{cat} and K_m values. pH-Dependence data from steady-state and stopped-flow experiments were analyzed using equation 1, which accounts for a single pK_a value with rates increasing to a constant value at high pH. Here Y is the parameter being measured, C is the pH-independent value of the parameter, H is the hydrogen ion concentration, and K is the dissociation constant of the ionizable group. Stopped-flow experiments measured the rate of flavin absorbance decrease during reduction of the flavin cofactor by substrate. For wild-type BBE, the flavin absorbance decrease over time was fit to equation 2; here A is the absorbance at time t , A_{∞} is the final absorbance, and A_1 and A_2 are the absorbance changes associated with each of the first-order rate constants, k_1 and k_2 , respectively. One of the first-order rate constants, attributed to k_{red} , corresponds to the majority of the flavin absorbance change, while the other constant, likely due to product release, is approximately 10-fold slower and corresponds to only 10–20% of the absorbance change. Rate constants were measured at three saturating substrate concentrations in triplicate and averaged. The E417Q mutant stopped-flow data were fit to Equation 3, which describes a single exponential decay.

$$\log Y = \frac{C}{1 + \frac{H}{K}} \quad (1)$$

$$A = A_{\infty} + A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (2)$$

$$A = A_{\infty} + A e^{-k t} \quad (3)$$

RESULTS AND DISCUSSION

Steady-state kinetics

BBE activity under steady-conditions was determined using an HPLC-based assay to quantify product formation and an oxygen electrode to measure oxygen consumption. The resulting steady-state kinetic parameters are listed in Table 1. The HPLC-based assay performed in air-saturated buffers at 25 °C yielded a $k_{\text{cat(app)}}$ value consistent with the value previously reported at 37 °C (14). The $K_{\text{m(app)}}$ value of (*S*)-reticuline for BBE has been most recently reported to be 3 μM (15), but has been previously reported to be as low as 0.14 μM (16), and this latter lower value is more consistent with our observations. The k_{cat} and oxygen K_{m} values were measured at varying oxygen concentrations (0.02 – 1.25 mM) at 25 °C. The oxygen K_{m} value of $280 \pm 70 \mu\text{M}$ (Table 1) indicates that assays performed in air-saturated buffers ($[\text{O}_2] \approx 250 \mu\text{M}$) only yield apparent k_{cat} values. The $k_{\text{cat}}/K_{\text{O}_2}$ value is consistent with the k_{ox} rate constant for flavin reoxidation that has been reported previously (5).

Measurement of substrate deuterium and solvent kinetic isotope effects necessitates identification of a pH-independent region of activity for accurate analyses of results. The $k_{\text{cat(app)}}$ -pH profile with wild-type BBE at 25 °C fit to a curve with a $\text{p}K_{\text{a}}$ of $\text{pH } 7.8 \pm 0.1$ for a single ionizable group that must be deprotonated for maximal activity (Figure 1). It was previously reported that BBE activity is maximal at pH 8.9, but decreases below and above this pH value (2). The decrease in enzymatic activity observed above pH 9 could be due to enzyme instability at high pH over the prolonged, hour-long incubation times utilized in that study, compared to the initial rate assay utilized here. The solvent kinetic isotope effect on k_{cat} was measured in the pH-independent region, at pH 9 and pD 9.5, in buffers containing 1.25 mM O_2 , yielding a value of 1.4 ± 0.1 (Table 2). A solvent isotope effect on $k_{\text{cat}}/K_{\text{m}}$ could not be determined accurately due to the low (*S*)-reticuline K_{m} value and the sensitivity limits of the assay.

Rapid-reaction kinetics

The modest solvent isotope effect suggested that amine oxidation may not be rate-limiting for turnover by BBE (5). Consequently, we measured isotope effects on k_{red} , the rate constant for flavin reduction at saturating substrate concentrations, using stopped-flow spectroscopy (Figure 2). The overall shape of the k_{red} -pH profile was similar to that for k_{cat} ; the data fit to the same equation yielded a $\text{p}K_{\text{a}}$ value of 7.3 ± 0.1 (Figure 3). This $\text{p}K_{\text{a}}$ value is likely due to the necessity for the substrate nitrogen to be deprotonated for oxidation; this has been observed with other flavin amine oxidases (17–22). The $\text{p}K_{\text{a}}$ value of the tertiary amine in reticuline does not appear to have been reported, but it is likely close to that of trimethylamine of approximately 9.8. While we cannot rule out the possibility that the $\text{p}K_{\text{a}}$ is due to an amino acid residue in the protein, the most likely candidates are Glu417, His459 and Tyr106; mutagenesis of His459 or Tyr106 does not have a drastic effect on k_{red} (9), while the data below rule out Glu417.

Substrate deuterium kinetic isotope effects on k_{red} were measured over pH 6–10; Figure 2 compares flavin reduction at pH 9 with protiated and deuterated reticuline. The $^{\text{D}}k_{\text{red}}$ values are pH-independent with the wild-type enzyme (Figure 3, Table 2), demonstrating that reduction is not limited by substrate binding (23). The k_{red} -pH profile with the deuterated substrate yielded a similar $\text{p}K_{\text{a}}$ value of 7.4 ± 0.1 (Figure 3).

The k_{red} was measured at varying pD values; the $\text{p}K_{\text{a}}$ value increased to 7.9 ± 0.1 in D_2O (Figure 3). In contrast to the solvent isotope effect on k_{cat} , the pH-independent value for the solvent isotope effect on the rate constant for flavin reduction is 1.00 ± 0.02 , within error of one. The value is similar with the deuterated substrate (Table 2). Solvent isotope effects near

one on the rate of flavin reduction indicate that the substrate phenolic proton is not in flight during C-H bond cleavage. The $^Dk_{\text{red}}$ did not change significantly in D₂O, consistent with CH bond cleavage remaining rate-limiting for flavin reduction.

Kinetics of E417Q BBE

The possibility of rapid and equilibrium deprotonation before slower C-H bond cleavage remained; hence, isotope effects were measured with a variant lacking the proposed glutamate active-site base. In the E417Q mutant, the k_{red} value decreases approximately 1500-fold and becomes equal to k_{cat} , which itself decreases, indicating that reduction is rate-limiting for turnover with this enzyme (9). If the glutamate residue functions to deprotonate the phenolic proton, a significant solvent isotope effect should be observed in the mutant protein as the deprotonation becomes more rate-limiting (24).

The k_{red} with the E417Q mutant was measured over pH 7–9.5; the $\text{p}K_{\text{a}}$ value decreased to 6.5 ± 0.1 with this variant (Figure 4). The deuterium kinetic isotope effect is 6.7 ± 0.3 at pH 9 with the E417Q mutant (Table 2). This effect indicates that C-H bond cleavage is also rate-limiting during flavin reduction in the mutant enzyme. The elevated substrate deuterium kinetic isotope effects in the mutein may be due to longer donor-acceptor distances in the mutein, allowing tunneling to occur only with the protiated substrate, compared to wild-type enzyme where tunneling may occur with both isotopes due to the short distances between the donor and acceptor, resulting in lower substrate deuterium kinetic isotope effects (25). The solvent kinetic isotope effect on k_{red} with the E417Q mutant with both the protiated and deuterated substrates is the same, with an average value of 0.94 ± 0.04 (Table 2), indicating that solvent-exchangeable protons are not in flight during flavin reduction even in this slow mutant enzyme.

Product analyses

The dramatic decrease in the flavin reduction rate with the E417Q mutant enzyme has been used as evidence supporting a concerted mechanism for BBE catalysis (9). The decrease in the rate of flavin reduction could, however, also result from improper positioning of the substrate in the active site of the mutant enzyme. Hydride transfer to the flavin cofactor requires precise positioning of the substrate by the flavin cofactor (26). Indeed, as demonstrated previously, the mutant enzyme yields two products, coreximine in addition to scoulerine (see Scheme 3 for structure of coreximine) (9). Coreximine could only be produced if the substituted benzene ring of the substrate were more flexible than in the wild-type enzyme; coreximine's production requires a 180° rotation of the aromatic ring in the active site (9), demonstrating that substrate binding in the mutant and wild-type enzyme are not the same.

A stepwise mechanism involves formation of a discrete iminium intermediate. Rapid-quench analyses of the reaction with the WT and E417Q enzymes (10 μM) were performed to obtain direct evidence for this intermediate. The imine would be expected to hydrolyze to its demethylated form, norreticuline, after the quench in 0.7 M NaOH. The lower limit for detection of norreticuline with HPLC analysis after rapid-quench was approximately 350 nM. We detected only the substrate and the product (*S*)-scoulerine with WT BBE and the products (*S*)-scoulerine and (*S*)-coreximine with the E417Q mutant.

Continuous-flow mass spectrometry (MS) experiments were also used in an effort to detect the intermediate directly; these also failed to identify any intermediate (Figure 1S). The only major peaks resulting from the reaction of wild-type or E417Q BBE with reticuline corresponded to the masses of the substrate reticuline and the products scoulerine and coreximine. The iminium intermediate would have the same m/z value as the product

scoulerine (m/z 328.1549), which complicated its detection by MS. However, MS/MS analyses of the m/z 328.1537 product peak from the WT and E417Q mutant reactions after various time periods yielded the same m/z product ions, indicating that the parent peak likely belonged to the products scoulerine and coreximine, and not the iminium intermediate. Norreticuline was not observed in MS spectra of the reactions above the levels observed in the MS spectra of the substrate reticuline. These results suggest that any iminium intermediate is short-lived and quickly cyclizes to the final product.

Conclusions

The combination of substrate and deuterium solvent isotope effects described here for wild-type and mutant BBE demonstrates that deprotonation is not occurring before or during C-H bond cleavage, suggesting that the concerted mechanism in Scheme 2 is unlikely. It is possible that deprotonation of the substrate phenol is unnecessary for catalysis. Even a protonated aromatic hydroxyl group functions as an electron-donating group, making the adjacent ortho carbon atoms more nucleophilic. A hydroxyl group must be present at C3' for cyclization of the substrate to occur by BBE in that its substitution with a methoxy group prevents cyclization (8). This could be due to the methoxy group being less electron-donating than a hydroxyl substituent or to disruption of the interaction between the glutamate and the substrate, causing altered binding. Still, a concerted attack of a methyl amine by the C2' atom and hydride transfer is less likely than attack on a methylene iminium ion intermediate by the C2' atom, indicating that a stepwise mechanism is the likely mechanism for catalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Prof. Peter Macheroux for the *Pichia pastoris* strains for expression of BBE and Sylvia Wallner for help with the fermentation protocols (University of Graz, Austria).

ABBREVIATIONS

BBE Berberine Bridge Enzyme

References

1. Ziegler J, Facchini PJ. Alkaloid Biosynthesis: Metabolism and Trafficking. *Annu Rev Plant Biol.* 2008; 59:735–769. [PubMed: 18251710]
2. Steffens P, Nagakura N, Zenk MH. Purification and characterization of the berberine bridge enzyme from berberis beaniana cell cultures. *Phytochemistry.* 1985; 24:2577–2583.
3. Taura F, Sirikantaramas S, Shoyama Y, Yoshikai K, Shoyama Y, Morimoto S. Cannabidiolic-acid synthase, the chemotype-determining enzyme in the fiber-type Cannabis sativa. *FEBS Lett.* 2007; 581:2929–2934. [PubMed: 17544411]
4. Sirikantaramas S, Morimoto S, Shoyama Y, Ishikawa Y, Wada Y, Shoyama Y, Taura F. The Gene Controlling Marijuana Psychoactivity. *J Biol Chem.* 2004; 279:39767–39774. [PubMed: 15190053]
5. Winkler A, Kutchan TM, Macheroux P. 6-S-Cysteinylation of Bi-covalently Attached FAD in Berberine Bridge Enzyme Tunes the Redox Potential for Optimal Activity. *J Biol Chem.* 2007; 282:24437–24443. [PubMed: 17573342]
6. Ralph EC, Hirschi JS, Anderson MA, Cleland WW, Singleton DA, Fitzpatrick PF. Insights into the mechanism of flavoprotein-catalyzed amine oxidation from nitrogen isotope effects on the reaction of N-methyltryptophan oxidase. *Biochemistry.* 2007; 46:7655–7664. [PubMed: 17542620]

7. Fitzpatrick PF. Oxidation of amines by flavoproteins. *Arch Biochem Biophys.* 2010; 493:13–25. [PubMed: 19651103]
8. Kutchan TM, Dittrich H. Characterization and Mechanism of the Berberine Bridge Enzyme, a Covalently Flavinylated Oxidase of Benzophenanthridine Alkaloid Biosynthesis in Plants. *J Biol Chem.* 1995; 270:24475–24481. [PubMed: 7592663]
9. Winkler A, Lyskowski A, Riedl S, Puhl M, Kutchan TM, Macheroux P, Gruber K. A concerted mechanism for berberine bridge enzyme. *Nat Chem Biol.* 2008; 4:739–741. [PubMed: 18953357]
10. Winkler A, Puhl M, Weber H, Kutchan TM, Gruber K, Macheroux P. Berberine bridge enzyme catalyzes the six electron oxidation of (S)-reticuline to dehydroscoulerine. *Phytochemistry.* 2009; 70:1092–1097. [PubMed: 19570558]
11. Rice K, Bossi A. Expedient synthesis of racemic and optically active N-norreticuline and N-substituted and 6'-bromo-N-norreticulines. *J Org Chem.* 1980; 45:592–601.
12. Meyers AI, Guiles J. The asymmetric total synthesis of (+)-reticuline. *Heterocycles.* 1989; 28:295–300.
13. Schrittwieser JH, Resch V, Sattler JH, Lienhart WD, Durchschein K, Winkler A, Gruber K, Macheroux P, Kroul W. Biocatalytic Enantioselective Oxidative C-C Coupling by Aerobic C-H Activation. *Angew Chem Int Ed.* 2011; 50:1068–1071.
14. Winkler A, Hartner F, Kutchan TM, Glieder A, Macheroux P. Biochemical Evidence That Berberine Bridge Enzyme Belongs to a Novel Family of Flavoproteins Containing a Bi-covalently Attached FAD Cofactor. *J Biol Chem.* 2006; 281:21276–21285. [PubMed: 16728404]
15. Dittrich H, Kutchan TM. Molecular Cloning, Expression, and Induction of Berberine Bridge Enzyme, an Enzyme Essential to the Formation of Benzophenanthridine Alkaloids in the Response of Plants to Pathogenic Attack. *Proc Natl Acad Sci U S A.* 1991; 88:9969–9973. [PubMed: 1946465]
16. Steffens P, Nagakura N, Zenk MH. The berberine bridge forming enzyme in tetrahydropyridoberberine biosynthesis. *Tetrahedron Lett.* 1984; 25:951–952.
17. Kurtz KA, Rishavy MA, Cleland WW, Fitzpatrick PF. Nitrogen isotope effects as probes of the mechanism of D-amino acid oxidase. *J Am Chem Soc.* 2000; 122:12896–12897.
18. Adachi MS, Juarez PR, Fitzpatrick PF. Mechanistic Studies of Human Spermine Oxidase: Kinetic Mechanism and pH Effects. *Biochemistry.* 2009; 49:386–392. [PubMed: 20000632]
19. Adachi MS, Torres JM, Fitzpatrick PF. Mechanistic Studies of the Yeast Polyamine Oxidase Fms1: Kinetic Mechanism, Substrate Specificity, and pH Dependence. *Biochemistry.* 2010; 49:10440–10448. [PubMed: 21067138]
20. Emanuele JJ, Fitzpatrick PF. Mechanistic studies of the flavoprotein tryptophan 2-monooxygenase. 2 pH and kinetic isotope effects. *Biochemistry.* 1995; 34:3716–3723. [PubMed: 7893668]
21. Dunn RV, Marshall KR, Munro AW, Scrutton NS. The pH dependence of kinetic isotope effects in monoamine oxidase A indicates stabilization of the neutral amine in the enzyme-substrate complex. *FEBS Journal.* 2008; 275:3850–3858. [PubMed: 18573102]
22. Basran J, Sutcliffe MJ, Scrutton NS. Optimizing the Michaelis Complex of Trimethylamine Dehydrogenase. *J Biol Chem.* 2001; 276:42887–42892. [PubMed: 11553643]
23. Cook PF, Cleland WW. pH variation of isotope effects in enzyme-catalyzed reactions. 1 Isotope- and pH-dependent steps the same. *Biochemistry.* 1981; 20:1797–1805. [PubMed: 7013800]
24. Sobrado P, Fitzpatrick PF. Solvent and Primary Deuterium Isotope Effects Show That Lactate CH and OH Bond Cleavages Are Concerted in Y254F Flavocytochrome b2, Consistent with a Hydride Transfer Mechanism†. *Biochemistry.* 2003; 42:15208–15214. [PubMed: 14690431]
25. Nagel ZD, Klinman JP. Update 1 of: Tunneling and Dynamics in Enzymatic Hydride Transfer. *Chem Rev.* 2010; 110:PR41–PR67. [PubMed: 21141912]
26. Fraaije MW, Mattevi A. Flavoenzymes: diverse catalysts with recurrent features. *Trends Biochem Sci.* 2000; 25:126–132. [PubMed: 10694883]

Figure 1.
The effects of pH on $k_{\text{cat(app)}}$ at 25 °C. The curve is a fit of the data to eq 1.

Figure 2. Reduction of wild-type BBE by protiated (circles) and methyl-deuterated (squares) reticuline at 25 °C. Curves are fits to eq 2. Only 10% of the data points are shown for clarity.

Figure 3. The effects of pH on k_{red} at 25 °C with protiated (circles) and methyl-deuterated (squares) reticuline in H_2O and with protiated reticuline in D_2O (open circles). The curves are fits to eq 1.

Figure 4.
The effects of pH on k_{red} at 25 °C with the BBE E417Q mutant. The curve is a fit to eq 1.

Scheme 1.
Reaction catalyzed by BBE.

Scheme 2.
Proposed mechanisms for the BBE-catalyzed reaction (8, 9)

Scheme 3.
BBE-catalyzed oxidation of various substrate analogs (8–10).

Table 1Kinetic Parameters of Wild-Type BBE^a

parameter	value
$k_{\text{cat(app)}}$	$5.4 \pm 0.3 \text{ s}^{-1}$
K_{m}	$0.27 \pm 0.03 \text{ }\mu\text{M}$
$k_{\text{cat}}/K_{\text{m(app)}}$	$20 \pm 2 \text{ }\mu\text{M}^{-1} \text{ s}^{-1}$
k_{cat}	$10.5 \pm 0.7 \text{ s}^{-1}$
$K_{\text{m(O}_2\text{)}}$	$280 \pm 70 \text{ }\mu\text{M}$
$k_{\text{cat}}/K_{\text{m(O}_2\text{)}}$	$37 \pm 9 \text{ mM}^{-1} \text{ s}^{-1}$
k_{red}	$98 \pm 4 \text{ s}^{-1}$

^a All kinetic constants were measured at 25 °C in 50 or 100 mM CHES, pH 9.0 with wild-type BBE. The $k_{\text{cat(app)}}$ and K_{m} values were determined in air-saturated buffer and k_{red} was determined in anaerobic solutions.

Table 2Kinetic Isotope Effects for Wild-Type and E417Q BBE^a

	Wild-Type	E417Q
$\text{D}_2\text{O}(k_{\text{cat}})_{\text{H}}$	1.4 ± 0.1	N.D.
$\text{D}_2\text{O}(k_{\text{red}})_{\text{H}}$	1.00 ± 0.02	0.96 ± 0.05
$\text{D}_2\text{O}(k_{\text{red}})_{\text{D}}$	0.88 ± 0.10	0.91 ± 0.03
$\text{D}(k_{\text{red}})_{\text{H}_2\text{O}}$	3.5 ± 0.3	6.7 ± 0.3
$\text{D}(k_{\text{red}})_{\text{D}_2\text{O}}$	3.2 ± 0.3	6.3 ± 0.2

^a Conditions: 25 °C in either 50 or 100 mM CHES at pH 9.0 or pD 9.5. N.D. - not determined. The $\text{D}_2\text{O}(k_{\text{red}})_{\text{H}}$ values were determined from the rate constant for flavin reduction in the pL-independent region measured in H_2O and D_2O .