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# Isotope Effects Suggest a Stepwise Mechanism for Berberine Bridge Enzyme

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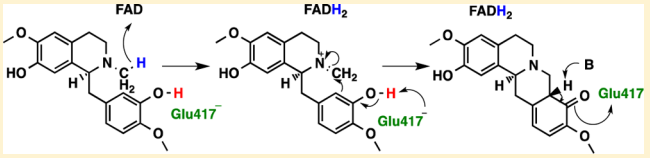
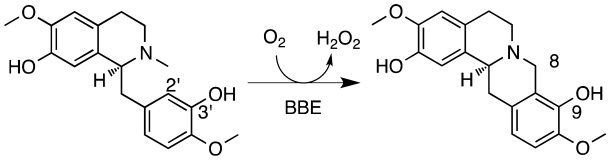
Supporting Information

**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 to discriminate between a concerted mechanism, in which deprotonation of the substrate phenol occurs before or during the transfer of a hydride 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 equal to 1 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 that catalyze similar reactions have been identified: cannabidiolic acid synthase and $\Delta^1$-tetrahydrocannabinolic acid synthase from *Cannabis sativa*.[3,4] Neither of these enzymes has been characterized enzymatically.

**Scheme 1. Reaction Catalyzed by BBE**




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 the transfer of a single electron 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 allowing its attack on the *N*-methylene group, yielding the cyclized product. In the concerted mechanism, deprotonation of the phenol occurs during the concomitant attack on the *N*-methyl group by C2′ and the transfer of a hydride to the flavin.

Several studies support each mechanistic proposal. Oxidation by BBE of (*S*)-*N*-methylcoclaurine yields a demethylated product, suggesting that the reaction proceeds via formation of a methylene iminium ion intermediate (Scheme 3).[8] BBE oxidizes the tetrahydroprotoberberine alkaloids (*S*)-coreximine, (*S*)-scoulerine, and (*S*)-norsteponine, forming a double bond between the nitrogen and C8 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 analogues have supported 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−$CH_3$ 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]

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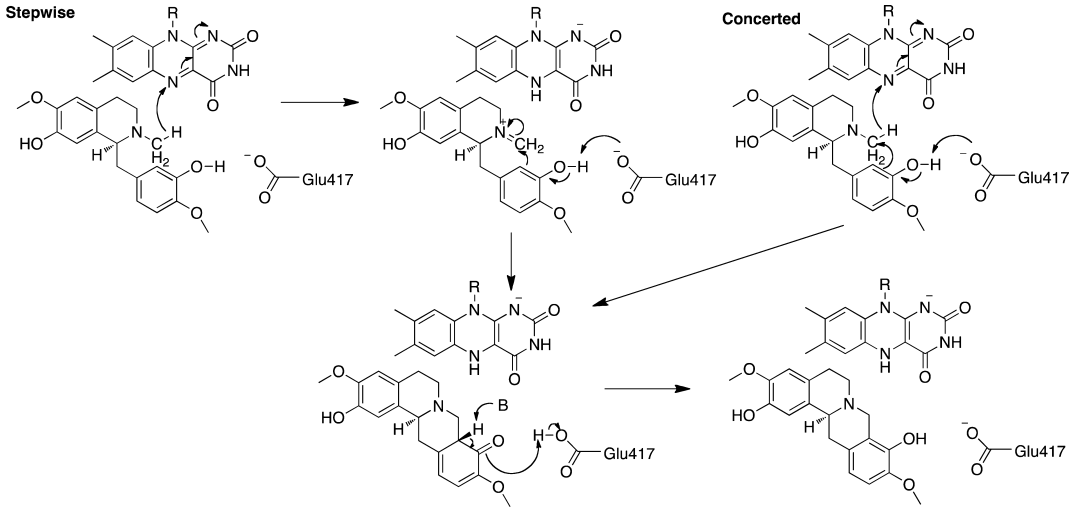
**Scheme 2. Proposed Mechanisms for the BBE-Catalyzed Reaction**[8,9]




This has been interpreted as additional evidence of 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 this work, 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-$CD_3$) was synthesized with the same protocol using ethyl formate-$d_1$ (CDN Isotopes, Quebec City, QC) and $BD_3$ in THF. $^1$H nuclear magnetic resonance (NMR) (Bruker Avance 500 MHz) analysis of the deuterated substrate yielded essentially the same NMR spectrum that was reported for protiated (*R*,*S*)-reticuline,[12] but with the singlet of the N-$CH_3$ 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 P. Macheroux (Graz University of Technology, Graz, Austria). Volumes were adjusted for a 2.5 L fermentation in a BioFlo/Celligen (Edison, NJ) model 115 fermentor. 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 that 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 with an increasing salt concentration [from 0 to 250 mM NaCl in 100 mM Tris buffer (pH 8.0)].

**Enzyme Assays.** High-performance liquid chromatography-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 $\mu$M) in the appropriate buffer (50 mM) at 25 °C. The assays were initiated by the addition of enzyme. The following buffers were used: MOPS at pH 6.75−7.75, Bicine at pH 7.75−8.75, and CHES at 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 (from 40 s to 20 min). Subsequently, the pH was neutralized with the same volume of either 1 N HCl or 1 N acetic acid before product formation was quantified by high-performance liquid chromatography (HPLC) analysis. HPLC analysis was performed as described previously,[14] but using a Phenomenex (Torrance, CA) C18 column (250 mm × 4.6 mm) and a Waters (Milford, MA) HPLC system. The substrate and product were separated using an isocratic elution of 65% methanol and 35% potassium phosphate (50 mM, pH 7.0).

Wild-type enzyme $k_{cat}$ values were determined in 50 mM CHES (pD 9.5) in 98−99% $D_2O$, and wild-type and mutant flavin reduction rate constants were measured in the appropriate buffer listed above (100 mM, pD 7−10) in 94−95% $D_2O$. All experiments were performed at saturating substrate concentrations [100−400 $\mu$M (*R*,*S*)-reticuline or 100−400 $\mu$M deuterated (*R*,*S*)-reticuline]. Solvent isotope effects were calculated by directly comparing the kinetic parameters obtained in $H_2O$ and $D_2O$. 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 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 mixture contained 50 mM CHES (pH 9) and 200 $\mu$M (*R*,*S*)-reticuline, and the reaction was initiated by the addition of BBE (final concentration of 0.25 or 0.125 $\mu$M). The oxygen concentration was varied from 20 to 1250 $\mu$M by bubbling the reaction solution with the appropriate concentration of oxygen for 15 min before the addition of enzyme.

Flavin reduction during substrate oxidation was monitored at 445 nm using an Applied Photophysics SX-20 MV stopped-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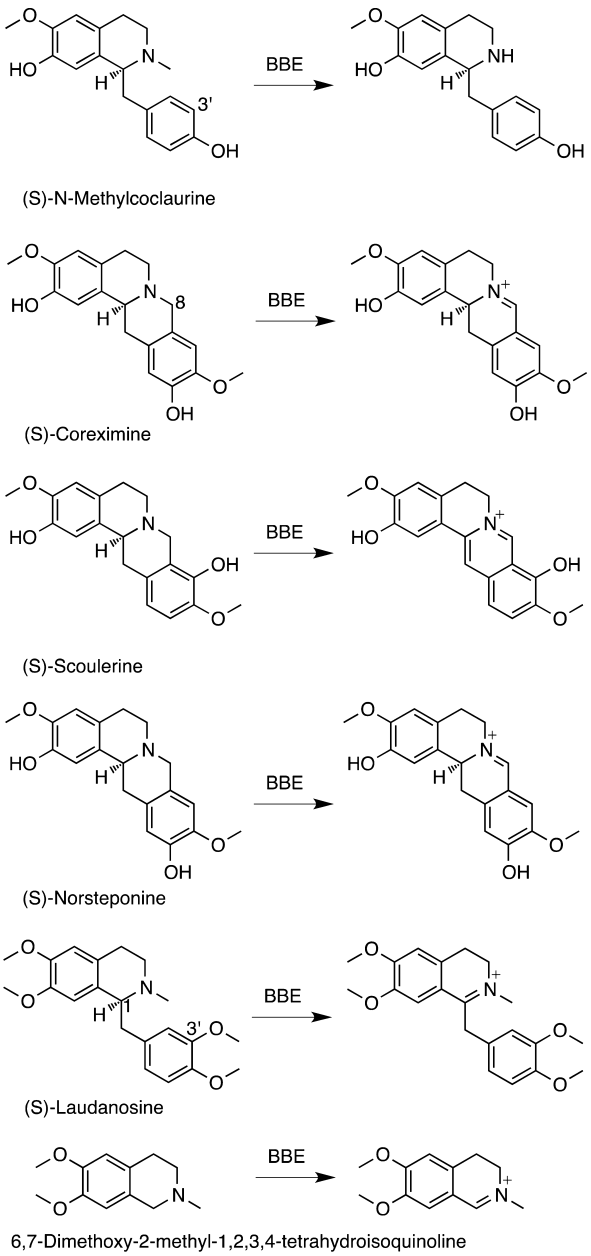
**Scheme 3. BBE-Catalyzed Oxidation of Various Substrate Analogues**[8−10]




flow spectrophotometer under anaerobic conditions. The system was made anaerobic by overnight incubation of the flow cell and syringes with the appropriate buffer (100 mM) containing 5 mM glucose. The buffer was deoxygenated in a tonometer by being exposed to 16 cycles of vacuum and oxygen-scrubbed argon; subsequently, glucose oxidase (final concentration of 30 nM) was added to maintain anaerobic conditions and remove any remaining oxygen. The following buffers were used: MES at pH 6.0, MOPS at pH 6.75−7.75, Bicine at pH 8.0−8.55, and CHES at pH 9−9.75. Substrate solutions were made anaerobic by being bubbled with oxygen-scrubbed argon for 5 min. Oxygen was removed from enzyme solutions when they were exposed 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 $\mu$M BBE, and 75−400 $\mu$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 $\mu$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 instrument. Reaction mixtures contained final concentrations of 10 $\mu$M BBE, 200 $\mu$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 nanoelectrospray 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 $\mu$L/min at 10 and 25 °C; the delay volume was approximately 0.04 $\mu$L, yielding reactions times of 0.25−2.5 s. The reaction mixtures consisted of final concentrations of 10 $\mu$M wild-type or E417Q BBE and 40 or 400 $\mu$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 KaleidaGraph (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 eq 1, which accounts for a single p$K_a$ value with rates increasing to a constant value at high pH:

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

where $Y$ 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 eq 2:

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

where $A$ is the absorbance at time $t$, $A_\infty$ 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 eq 3, which describes a single-exponential decay.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$$A = A_{\infty} + Ae^{-kt} \quad (3)$$

## RESULTS AND DISCUSSION

**Steady-State Kinetics.** BBE activity under steady-state 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 $k_{cat(app)}$ value consistent with the value previously reported at 37 °C.[14] The $K_{m(app)}$ value of (*S*)-reticuline for BBE has been most recently reported to be 3 $\mu$M[15] but has been previously reported to be as low as 0.14 $\mu$M,[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 ± 70 $\mu$M (Table 1) indicates that assays performed in air-saturated buffers ($[O_2] \approx 250$ $\mu$M) yield only apparent $k_{cat}$ values. The $k_{cat}/K_{O_2}$ value is consistent with the $k_{ox}$ rate constant for flavin reoxidation that has been reported previously.[5]

**Table 1. Kinetic Parameters of Wild-Type BBE**[a]

| | |
|---|---|
| $k_{cat(app)}$ | 5.4 ± 0.3 $s^{-1}$ |
| $K_m$ | 0.27 ± 0.03 $\mu$M |
| $k_{cat}/K_{m(app)}$ | 20 ± 2 $\mu M^{-1}$ $s^{-1}$ |
| $k_{cat}$ | 10.5 ± 0.7 $s^{-1}$ |
| $K_{m(O_2)}$ | 280 ± 70 $\mu$M |
| $k_{cat}/K_{m(O_2)}$ | 37 ± 9 $mM^{-1}$ $s^{-1}$ |
| $k_{red}$ | 98 ± 4 $s^{-1}$ |

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

Measurement of substrate deuterium and solvent kinetic isotope effects necessitates identification of a pH-independent region of activity for accurate analyses of results. The $k_{cat(app)}$–pH profile with wild-type BBE at 25 °C fit to a curve with a p$K_a$ of pH 7.8 ± 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 $k_{cat}/K_m$ could not be determined accurately because of the low (*S*)-reticuline $K_m$ value and the sensitivity limits of the assay.

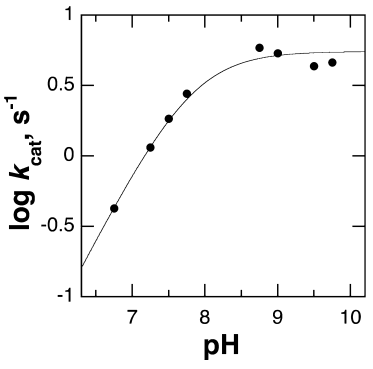


**Figure 1.** Effects of pH on $k_{cat(app)}$ at 25 °C. The curve is a fit of the data to eq 1.

**Table 2. Kinetic Isotope Effects for Wild-Type and E417Q BBE**[a]

| | wild type | E417Q |
|---|---|---|
| $^{D_2O}(k_{cat})_H$ | 1.4 ± 0.1 | not determined |
| $^{D_2O}(k_{red})_H$ | 1.00 ± 0.02 | 0.96 ± 0.05 |
| $^{D_2O}(k_{red})_D$ | 0.88 ± 0.10 | 0.91 ± 0.03 |
| $^{D}(k_{red})_{H_2O}$ | 3.5 ± 0.3 | 6.7 ± 0.3 |
| $^{D}(k_{red})_{D_2O}$ | 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. The $^{D_2O}(k_{red})_H$ values were determined from the rate constant for flavin reduction in the pL-independent region measured in $H_2O$ and $D_2O$.

**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 p$K_a$ value of 7.3 ± 0.1 (Figure 3). This p$K_a$ 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 p$K_a$ 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 p$K_a$ 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.

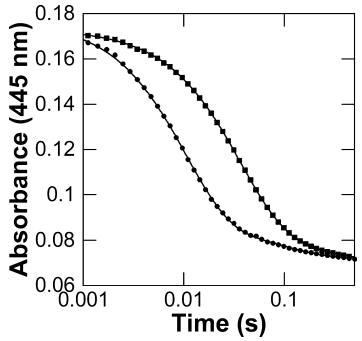


**Figure 2.** Reduction of wild-type BBE by protiated (●) and methyl-deuterated (■) reticuline at 25 °C. Curves are fits to eq 2. Only 10% of the data points are shown for the sake of clarity.

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 $^{D}k_{red}$ values are pH-independent with the wild-type enzyme (Figure 3 and Table 2), demonstrating that reduction is not limited by


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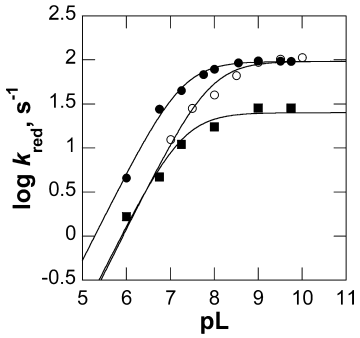


**Figure 3.** Effects of pH on $k_{red}$ at 25 °C with protiated (●) and methyl-deuterated (■) reticuline in $H_2O$ and with protiated reticuline in $D_2O$ (○). The curves are fits to eq 1.

substrate binding.[23] The $k_{red}$–pH profile with the deuterated substrate yielded a similar p$K_a$ value of 7.4 ± 0.1 (Figure 3).

The $k_{red}$ was measured at varying pD values; the p$K_a$ 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. The value is similar with the deuterated substrate (Table 2). Solvent isotope effects of ~1 on the rate of flavin reduction indicate that the substrate phenolic proton is not in flight during C–H bond cleavage. The $^{D}k_{red}$ did not change significantly in $D_2O$, consistent with C–H 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 p$K_a$ 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

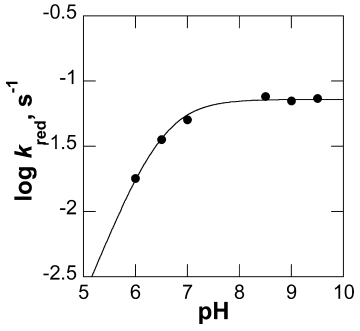


**Figure 4.** Effects of pH on $k_{red}$ at 25 °C with the BBE E417Q mutant. The curve is a fit to eq 1.

to the wild-type enzyme with which tunneling may occur with both isotopes because of 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. The transfer of a hydride 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 the structure of coreximine).[9] Coreximine could be produced only 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 that in the 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 of the Supporting Information). 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.

## ■ ASSOCIATED CONTENT

### Supporting Information

Figure 1S. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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