6-S-Cysteinylation of Bi-covalently Attached FAD in Berberine Bridge Enzyme Tunes the Redox Potential for Optimal Activity*

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A mutagenic analysis of the amino acid residues His-104 and Cys-166, which are involved in the bi-covalent attachment of FAD to berberine bridge enzyme, was performed. Here we present a detailed biochemical characterization of the cysteine link to FAD observed in this recently discovered group of flavoproteins. The C166A mutant protein still has residual activity, but reduced to ~6% of the turnover rate observed for wild-type berberine bridge enzyme. A more detailed analysis of single reaction steps by stopped-flow spectrophotometry showed that the reductive half-reaction is greatly influenced by the lack of the 6-S-cysteinyl linkage, resulting in a 370-fold decrease in the rate of flavin reduction. Determination of the redox potentials for both wild type and the C166A mutein revealed that the difference in the redox potential observed can fully account for the change in the kinetic properties. The wild-type protein exhibits a midpoint potential of +132 mV, which is the highest redox potential determined for any flavoenzyme so far. Removal of the cysteine linkage to FAD in the C166A mutein leads to a redox potential of +53 mV, which is in the expected range for flavoproteins with a single covalent attachment of FAD to a His residue via its 8-α position. We also show that the biochemical properties of the mutein resemble that of typical flavoprotein oxidases and that deviations from this behavior observed for the wild type are due to the FAD-6-S-cysteinyl bond. In addition, rapid reaction stopped-flow experiments give no indication for a radical mechanism supporting the direct transfer of a hydride from the substrate to the cofactor.

Berberine bridge enzyme (BBE)² (EC 1.21.3.3) is a central enzyme of alkaloid biosynthesis in plant species capable of producing protopine, protoberberine, and benzophenanthridine alkaloids. Because of the challenging chemical reaction it catalyzes and its involvement in the production of pharmaceutically important chemicals, it has long been of interest to get a detailed understanding of the processes occurring at the molecular level (1, 2). However, an in-depth biochemical characterization was limited for a long time by the rather low amount of protein available from expression in plant and insect cell cultures. Recently, we have developed an expression system in Pichia pastoris that enabled us to obtain sufficient quantities of purified BBE from Eschscholtzia californica, allowing a more detailed characterization. In the course of these studies it was discovered that BBE belongs to a novel group of flavoproteins containing a bi-covalently attached flavin cofactor (3–7).

In addition, sequence alignments reveal that several other proteins reported to possess an 8-α-histidyl bond have the Cys residue involved in binding to position 6 of the cofactor conserved, e.g. Δ¹-tetrahydrocannabinolic acid synthase from Cannabis sativa (8). Most of these enzymes with a confirmed or proposed bi-covalently attached flavin have a function in specialized metabolic pathways and catalyze reactions with rather challenging chemistry or substrates.

The occurrence of this bi-covalent FAD attachment raises the question of its role in enzyme catalysis. Several explanations for the occurrence of covalent flavinylation have been given for different enzymes, ranging from prevention of cofactor modification in the case of trimethylamine dehydrogenase (4, 9) or increasing the redox potential of flavins modified in position 8α (10, 11) to facilitating electron transfer from the flavin to other redox centers present in p-cresol methylhydroxylase (12). In addition to this variety of explanations for the existence of a covalent linkage, the situation is further complicated by the occurrence of covalent flavoenzymes having isoenzymes bearing a dissociable cofactor but still exhibiting a similar kinetic behavior (13, 14). This clearly shows that the explanations for the covalent attachment of flavins to proteins might be manifold and very much depend on the enzyme and the environment where it occurs in nature. As far as bi-covalently flavinylated enzymes are concerned, so far no studies addressing the importance of this type of linkage have been performed.

To provide answers to these issues, we have mutated the residues involved in generation of the bi-covalent linkage of FAD to BBE and have studied the influence on the spectral and kinetic parameters as well as the redox potential. Here, we demonstrate that the 6-S-cysteinyl bond is responsible for the atypical behavior of wild-type BBE compared with other related flavoprotein oxidases (7). In addition, we show that this linkage modulates the flavin redox potential, strongly...
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affecting the rate of flavin reduction in the oxidation of the substrate (S)-reticuline.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes—Polyacrylamide gel electrophoresis-purified oligos were obtained from VBC-Biotech, and the QuikChange™ XL kit for site-directed mutagenesis was from Stratagene. Milk xanthine oxidase was of Grade I purity and purchased from Sigma. Thymolindophenol was from TCI Europe. (S)-Reticuline was from the natural product collection of the Department of Natural Product Biotechnology of the Leibniz Institute of Plant Biochemistry, Halle/Saale, Germany. Sequencing grade modified trypsin for MALDI mass spectrometry sample preparation was from Promega. All other chemicals were purchased from Sigma-Aldrich and of the highest quality available.

Site-directed Mutagenesis—Mutagenesis was carried out directly with the expression vector pPICZα BBE-ER described in Ref. 7 in order to remove the sites of covalent linkage for FAD (His-104 and Cys-166). Replacement of His-104 was accomplished using polymerase chain reaction-based mutagenesis using the oligonucleotides 5’-GATTAAGAGTGGTG-GTXXXXAGTTATGAGGTATCTTAC-3’ (sense) and the complementary antisense primer where XXX substitutes the replaced codon CAT with either GCT (H104A) or ACT (H104T). The second site of covalent attachment was replaced with an alanine using the primer pair 5’-TTACGGCTGGTG-GGCGTCCAACCGTGGTACTGG-3’ and its complementary oligonucleotide. The underlined nucleotides represent the mutated codon replacing TGT (C166A). Mutagenesis was carried out as described in the QuikChange™ XL Site-directed Mutagenesis kit (Stratagene) and the introduction of the correct mutation verified by plasmid sequencing. Generation of the double mutant (H104T,C166A) was accomplished using the same procedure described above with pPICZα BBE-ER C166A as the template for polymerase chain reaction and the primer pair described for the H104T mutant.

Transformation, Expression, and Purification—All four mutated vectors were transformed into the P. pastoris strain KM71H, generating transformants that, after verification of vector integration into the Pichia genome by colony polymerase chain reaction, were then used for expression of the muteins. All experiments, including scale up from small scale expression trials to the final protein production in a BBI-CT5-2 fermenter and the two-step purification procedure using a hydrophobic interaction chromatography step followed by gel filtration, were performed as described for the wild-type enzyme (7).

Analytical Experiments—SDS-polyacrylamide gel electrophoresis was carried out with 12.5% separating gels and 5% stacking gels as described by Laemmli (15). In-gel fluorescence for detection of covalently bound flavin was performed after fixing the gel in 5% (v/v) acetic acid for 5 min (16) followed by visualization in a standard UV-transillumination chamber at 302 nm. Staining of gels was conducted with Coomasie Brilliant Blue R. MALDI time-of-flight mass spectrometry was used to verify expression of the correct muteins essentially as described previously (7). Absorption spectra were recorded with a Specord 205 spectrophotometer (Analytik Jena) using 1-cm quartz cuvettes. All measurements were performed in 150 mM NaCl, 50 mM Tris/HCl, pH 9.0, at 25 °C unless stated otherwise. The extinction coefficient of covalently bound FAD was estimated by unfolding the enzyme with 0.5% (w/v) SDS and comparing the obtained flavin spectrum with that of free FAD in the case of the C166A mutein.

Anaerobic Photoreduction—Photoreduction was carried out as outlined in a previous study (17). Briefly, special quartz cuvettes were rendered anaerobic by repeated cycles of evacuation and flushing with oxygen-free nitrogen. EDTA and 5-deazariboflavin were stored in the side arm of the cuvette during this process and illuminated for 5 min prior to mixing with the enzyme solution (final concentrations 1 mM and 1 μM, respectively). A conventional slide projector was used for photoirradiation of the sample, which was cooled to 15 °C during this process. All spectra monitoring the progress of reduction were measured at the same temperature.

Steady-state Kinetic Analysis—To compare steady-state turnover rates of the mutein with wild-type BBE, the conversion of (S)-reticuline to (S)-scoulerine was followed by high performance liquid chromatography analysis of the reaction mixture as described previously (7). In the case of BBE-ER C166A, the assay mixture consisted of 98 μl of 0.1 mM Tris/HCl, pH 9.0, 1 μl of 10 mM (S)-reticuline in methanol, and 1 μl of C166A mutein (1.95 μM), resulting in a final substrate concentration that is ~30 times the reported K_m for (S)-reticuline in the case of wild-type BBE.

Transient Kinetics—Reductive half-reactions were analyzed with a stopped-flow device from Hi-Tech (SF-61DX2) positioned in a glove box from Belle Technology. Changes in flavin absorbance were either followed with a KinetaScan T diode array detector (MG-6560) or a PM-61s photomultiplier in the case of single wavelength kinetic traces. All concentrations stated in this context are final values after mixing in the flow cell. Samples were rendered anaerobic by flushing with oxygen-free nitrogen followed by incubation in the glove box environment of ~0.8 ppm oxygen. Apparent rate constants for the reductive half-reaction were measured at five different concentrations of (S)-reticuline for an accurate estimation of the maximal rate of flavin reduction. Fitting of the obtained transients at 445 nm was performed with SpecFit 32 (Spectrum Software Associates) using a function of two exponentials. Rates for the oxidative half-reaction were measured by mixing substrate-reduced enzyme solution with air-saturated buffer (21% oxygen). Lag phases in the reoxidation process were prevented by using substoichiometric amounts of (S)-reticuline for the reduction of enzyme-bound flavin.

Redox Potentials—Determination of the redox potential was carried out by the dye-equilibration method using the xanthine/xanthine oxidase electron delivering system as described by Massey (18). Concentrations of protein and redox indicator were chosen so that the absorbance changes at the wavelength employed for interpretation of the data were roughly in the same absorbance range. Reactions were carried out in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C containing benzyl viologen (5 μM) as a mediator, 250 μM xanthine, and xanthine oxidase in catalytic amounts (1–8 nM). A typical reduction
lasted for ~60 min in order to ensure equilibration between dye and protein. For maintaining anaerobic conditions throughout the experiment, the reduction was carried out in a stopped-flow device (SF-61D2X2, Hi-Tech) positioned in an anaerobic glove box (~0.8 ppm oxygen; Belle Technology) after rendering all solutions anaerobic by repeated evacuation and flushing with nitrogen. Spectra during the course of reduction were recorded with a KineteScan™ diode array detector (MG-6560) from Hi-Tech. Dyes used for analysis and their corresponding redox potentials were 2,6-dichloro indophenol (Em = +217 mV), thymolindophenol (Em = +174 mV), methemoglobin (from beef, Em = +144 mV at I = 0.2 m), toluylene blue (Em = +115 mV), thionin acetate (Em = +64 mV), and toluidine blue (Em = +34 mV). The reduction oxidation potentials were calculated from plots of log([ox]/[red]) of BBE or the C166A mutant protein versus log([ox]/[red]) of the dye according to Minnaert (19).

RESULTS

Expression of BBE Muteins—All variants of BBE generated by site-directed mutagenesis were expressed exactly in the same way as wild-type BBE. However, only the C166A mutein could be detected in the fermentation supernatant in similar amounts as reported for the wild-type protein (7). All muteins in which His-104, which is involved in covalently linking FAD via its 8-α-methyl group, is exchanged are expressed in very low levels. Therefore, the H104A and H104T single and H104T,C166A double mutants could not be isolated in amounts necessary for a basic biochemical characterization. Concentrated fermentation supernatants did not show any significant difference with respect to the negative control on SDS-PAGE and Western blotting. It was only after tryptic in-gel digestion of gel pieces from the expected region of BBE muteins on SDS gels that the strongest peptide signals from a typical BBE digest could be observed (results not shown). The very low expression level already detected in small scale expression trials could not be increased by scaling up to a fermenter.

General Properties—The C166A mutein could be purified according to the protocol established for wild-type BBE (7). After applying the two-step purification procedure a bright yellow protein sample was obtained. Because wild-type BBE binds FAD in a bi-covalent manner via its His-104 and Cys-166 residues, it was expected that the C166A mutant protein still binds FAD covalently. To test this assumption, the protein sample was precipitated with 13% (w/v) ice-cold trichloroacetic acid, and after centrifugation no free flavin was detected in the supernatant, indicating a covalent linkage between cofactor and protein. In addition, in-gel fluorescence of unstained SDS gels showed that the flavin emission co-migrates with the protein band of C166A, verifying the covalent linkage. Loading the same amount of wild type and C166A mutant protein indicates that the flavin cofactor of the mutein exhibits stronger fluorescence intensity, which would be expected for the removal of the FAD 6-S-cysteinyl bond (20).

Spectral Data—A comparison of the spectral properties of the covalently attached flavin cofactors is shown in Fig. 1. In its native form the mutein already shows a significant difference compared with the wild type. Whereas the changes around the 450 nm absorption maximum only involve a more detailed fine-structurating of the peak, the absorption at 370 nm changes substantially to a broad maximum ranging from 350–370 nm. Comparison of the denatured spectra reveals a remarkable difference in both absorption maxima. Whereas the spectral characteristics of denatured wild-type BBE already indicate the modification of the flavin in its C6 position, these influences are completely absent in the C166A mutant. For the mutein, the spectral properties after denaturation resemble more that of free FAD (~450 nm absorption) and the strong hypsochromic shift in the second absorption band (~350 nm) is indicative for an 8-α-modification of the flavin cofactor (21).

In combination with the verification that C166A contains a covalently attached FAD cofactor, this clearly demonstrates that the bi-covalent linkage via His-104 and Cys-166 observed in wild-type BBE has been replaced by a single amino acid linkage involving His-104 and the 8-α-methyl group of FAD. In addition, MALDI mass spectrometry analysis of tryptic fragments obtained from C166A BBE digestion shows that the peptide harboring Ala-166 can be identified without any modification. Assuming that the absorption coefficient of the unfolded enzyme is comparable with that of free FAD, the extinction coefficient for native C166A mutein can be estimated to 11,700 M⁻¹ cm⁻¹ at 444 nm.

Kinetic Characterization—The steady-state turnover rate for C166A BBE was determined by high performance liquid chromatography analysis of substrate conversion as reported for wild-type enzyme (7). A kcat of 0.48 ± 0.05 s⁻¹ was determined for the mutein under identical reaction conditions as used for determination of the wild-type turnover rate. Comparison of the turnover rates shows that the mutein retains ~6% of the wild-type activity. In the following experiments the catalytic properties were addressed in more detail to allow determination of the reaction step responsible for the deterioration in enzymatic turnover.

For the majority of flavoprotein oxidases the rate-limiting step is the reductive half-reaction. To determine whether this
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step is affected by the introduced mutation, the reductive rates for both wild-type and C166A BBE were measured. Both enzymes were mixed with various concentrations of substrate (S)-reticuline under anaerobic conditions, and the reduction of the cofactor was followed at 445 nm. The obtained transients had a biphasic characteristic consisting of a substrate-dependent fast phase and a second phase that did not correlate with the substrate concentration and could not be assigned to a specific process. A plot of the concentration-dependent phase as a function of (S)-reticuline concentration yields a hyperbolic dependence for both wild-type and mutant protein (Fig. 2).

At substrate saturating conditions a limiting reductive rate for wild-type BBE of 103 ± 4 s⁻¹ is observed. This is >360-fold faster than for the C166A mutein with 0.28 ± 0.02 s⁻¹. The $K_d$ value is only slightly affected and changes from 8.7 ± 0.8 μM for the wild type to 17 ± 3 μM for the mutein. In comparison with data obtained from steady-state experiments this shows that for the wild-type enzyme another step in catalysis must be responsible for slowing down the overall turnover rate. However, for the C166A mutein the rate of the reductive half-reaction is in the range of the rate-limiting step in enzyme turnover.

Characterization of the spectral changes accompanying the reduction of the cofactor by the substrate revealed no indication of a radical species. As depicted in Fig. 3A, the flavin of wild-type BBE is directly converted to its dihydro-reduced form, exhibiting a broad absorption maximum ~400 nm. Also, the C166A mutein is reduced without detection of radical intermediates to a species with the absorption maximum shifted to ~365 nm (Fig. 3B).

After reduction of the cofactor by the substrate, the flavin is regenerated by reaction with molecular oxygen, resulting in the formation of hydrogen peroxide and the reoxidation of FAD. The oxidative half-reaction was studied at a single oxygen concentration (10.5% O₂, 122 μM O₂) for both wild-type and mutant protein. Regeneration of oxidized FAD was monophasic with observed rates for wild-type and C166A BBE of (0.5 ± 0.1) × 10⁻³ and (1.0 ± 0.1) × 10⁻⁵ M⁻¹ s⁻¹, respectively. These values are in the range of values obtained for other flavoprotein oxidases (22). Using an oxygen concentration of ~200 μM (the approximate concentration of oxygen at 37 °C), the observed rate for reoxidation of reduced FAD amounts to 10 ± 2 s⁻¹, and therefore it can be concluded that in the case of wild-type BBE the oxidative half-reaction is the rate-limiting step in catalysis ($k_{cat} = 8.2$ s⁻¹) (7).

**Photoreduction**—Reduction of wild-type BBE by light in the presence of EDTA and 5-deazariboflavin followed by reoxidation resulted in a rather atypical reoxidized flavin spectrum that was attributed to the 6-S-cysteinyl link between FAD and Cys-166 (7). To confirm this interpretation, the C166A mutant was treated in exactly the same way that resulted in spectral changes depicted in Fig. 4. Light illumination leads to the formation of the red anionic semiquinone followed by full reduction, as observed for the wild-type protein. However, upon admission of oxygen virtually the same spectrum as at the beginning of.

**FIGURE 2. Observed rates obtained from fitting the transients of the reductive half-reaction experiments.** A hyperbolic dependence upon substrate concentration was observed in both cases. Extrapolation to saturating substrate concentrations yields a $k_{cat}$ of 103 ± 4 s⁻¹ for wild-type BBE with a $K_d$ of 8.7 ± 0.8 μM. In the case of the C166A mutant, $k_{cat}$ is 0.28 ± 0.02 s⁻¹ and the $K_d$ is 17 ± 3 μM. Error bars express the standard deviation of five individual measurements.

**FIGURE 3. Spectral changes observed during the anaerobic reduction of 11 μM wild-type BBE (A) and 7 μM C166A BBE (B) with excess (S)-reticuline (~20 μM). Solid lines represent the first spectrum after mixing with substrate.** For the time course of reduction, only selected spectra with decreasing absorbance at 450 nm are shown.
the experiment was regenerated, further substantiating that the unexpected changes for the wild type are indeed based on the cleavage of the 6- S-cysteinyl bond.

**Redox Potential Determination**—To assess the effect of the covalent modification by Cys-166, the redox potential of the FAD was determined for both wild-type and the C166A mutant protein. Reduction was achieved by the xanthine/xanthine oxidase system in the presence of suitable redox indicators. For the wild-type enzyme a direct two-electron reduction process could be observed in the presence of tolulene blue (\( E_{m} = +115 \) mV). Plotting \( \log(\text{BBEox/BBEred}) \) versus \( \log(\text{dyeox/dyered}) \) as described by Minnaert (19) allowed the estimation of the redox potential to 132 ± 4 mV (Fig. 5A). The obtained value was verified using beef methemoglobin as indicator (at ionic strength 0.2 M, \( E_{m} = -144 \) mV for the homologue from horse) (23), which also resulted in parallel reduction of both proteins and yielded a similar potential (–135 mV). This unusually high redox potential was further verified by obtaining the higher and lower limits for the potential of wild-type BBE using the dyes 2,6-dichloro indophenol and thionin acetate (\( E_{m} = +217 \) mV and \( E_{m} = +64 \) mV), which were reduced before and after the enzyme cofactor, respectively. For the C166A mutant protein the potential was determined to 53 ± 2 mV after reduction in the presence of thionine acetate. As depicted in Fig. 5B, the spectral changes observed again represent a simultaneous two-electron transfer to the flavin. The same redox potential was obtained after performing the experiment in the presence of tolulene blue (\( E_{m} = +34 \) mV). The slopes for the two linear fits in the insets of Fig. 5 are 0.92 and 0.99 for wild-type BBE and mutein, respectively, which is close to the theoretical value of 1 expected for a simultaneous two-electron transfer and indicates that the reaction was in equilibrium.

**DISCUSSION**

BBE is a member of the recently discovered group of bi-covalently flavinylated enzymes, and so far it is the only protein characterized in detail with respect to its biochemical properties in relation to other flavoprotein oxidases. To gain further insight into the role of bi-covalent linkage of the cofactor, via a 6-S-cysteinyl and an 8-α-histidyl bond, we have addressed the issue by site-directed mutagenesis of the amino acid residues involved in the covalent attachment.

Replacement of His-104, which is the residue forming a covalent bond with the 8-α-methyl group of FAD, by either Ala or Thr did not allow the isolation of mutein forms of BBE. In both cases only traces of the protein could be detected in the fermentation supernatant, preventing even a basic characterization with respect to the spectral and kinetic properties. This observation was rather unexpected because for related flavoproteins like vanillyl alcohol oxidase, where FAD is also linked via its 8-α-methyl group to a His of the protein, the removal of the covalent bond resulted in the expression of protein with a dis-
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Scheme 1. Reaction mechanism of the BBE-catalyzed reaction based on the proposal of Ref. 2. The possibility of sequential transfer of electrons to the flavin cofactor could be ruled out by spectral characterization of FAD reduction upon incubation with substrate (compare Fig. 3). No transient formation of any radical flavin intermediate was observed for either wild-type BBE or the C166A mutant. The direct conversion to the fully reduced cofactor is indicative for a hydride transfer between substrate and flavin.

For enzymes belonging to the group with a bi-covalent flavin attachment similar results have been published (4–6). In the case of glucooligosaccharide oxidase it is reported that replacement of His by various amino acids resulted in the isolation of proteins still bearing a covalently linked cofactor via the second site of attachment. However, not much information regarding the expression level and the spectral properties of these muteins was presented in these reports (5, 6). For hexose oxidase from Chondrus crispus, which has been shown to be a bi-covalently flavinylated enzyme, spectral data indicative for the presence of a retained 6-S-cysteinyl linkage when replacing His with another amino acid are reported; however, the expression level is reduced from 3 g to ∼50 mg for this mutein (6). Considering that wild-type BBE can only be expressed in comparatively low levels of ∼120 mg/liters of fermentation medium, this could explain the failure with the isolation of His-104 muteins. However, this amino acid replacement will be further investigated for BBE, especially because there are also reports in literature of proteins, putatively also belonging to the same group of enzymes, in which the replacement of His results in protein preparations without covalently attached flavin (8). A better understanding of the importance of the 8-α-histidyl linkage for bi-covalent flavinylation is required before assumptions regarding its functional role can be made.

The second amino acid involved in forming a link between protein and cofactor (Cys-166 for BBE) was substituted with an Ala residue. This replacement did not cause any problems for protein expression. The mutant protein was isolated in similar amounts as the wild type and could be purified using the same procedures as for wild-type BBE (7). However, the spectral properties of this BBE mutein were quite different from the wild type. In fact, all the properties of BBE that we attributed to the presence of a linkage of the cofactor to a Cys residue via position 6 of the isoalloxazine ring were absent in the mutein. Denaturation of the C166A mutant resulted in changes expected for a single covalent link via a His residue to position 8-α, as can be seen in the hypochromic shift of the near UV band observed for all proteins bearing this type of covalent attachment (11, 24). In addition, the result of the photoreduction experiment is quite similar to that of vannilyl alcohol oxidase, which is one of the best characterized enzymes of the p-cresol methylhydroxylase superfamily. Interestingly, many of the spectral properties of the C166A mutant protein (see Figs. 1 and 4) are almost identical to those observed for vannilyl alcohol oxidase (11, 25).

Prominent differences between wild-type and C166A mutein were also observed when the kinetic properties of both enzymes were compared. Steady-state turnover was already reduced by a factor of 17 for the mutant protein; however, the influence on the reductive half-reaction was even more pronounced. A 370-fold decrease in the rate of reduction under substrate-saturating conditions shows that the 6-S-cysteinyl bond has an important influence on the flavin cofactor and its involvement in catalysis (see Fig. 2). This is further supported by the fact that a switch in the rate-limiting step occurs upon removal of this covalent linkage. For the wild type, the reactivity of the flavin is enhanced to a point where reoxidation becomes the rate-limiting step in turnover. This is a rather unusual finding, but it should be pointed out that this is an observation based on a calculation taking into account the solubility of oxygen in aqueous buffer systems. How far this reflects the availability of oxygen in the plant organelles where BBE usually catalyzes its reaction (26, 27) is difficult to assess. In the case of the mutant protein it is the reductive half-reaction that determines the catalytic efficiency, a fact that is observed for most flavoprotein oxidases (28–30). Regarding the proposed reaction mechanism (2), the observed spectral characteristics of cofactor reduction depicted in Fig. 3 clearly favor the direct transfer of a hydride from (S)-reticuline to the cofactor. The direct two-electron reduction obtained cannot strictly rule out the alternative radical mechanism that was also proposed for other amine oxidases; however, the consecutive transfer of single electrons for this class of enzymes was recently criticized (31). Therefore our results support a hydride transfer mechanism from the N-methyl group of the substrate to the flavin cofactor (Scheme 1) (2). Presently, a more detailed understanding of the factors that promote the proposed hydride transfer mechanism is not possible because of the lack of a three-dimensional structure of BBE. Moreover, the role of amino acid residues in controlling the reactivity of the nitrogen lone pair as well as their participation in general acid catalysis, as shown in Scheme 1, depends on the availability of a high resolution structure of BBE in complex with an appropriate substrate mimic. These studies are currently in progress and are expected to provide further insights into the molecular mechanism of the enzyme.

In an attempt to better understand the decreased rate of flavin reduction for the mutein, we determined the redox potentials of wild-type BBE and the C166A mutein. It has been reported for several flavoenzymes that covalent modification is employed to increase the redox potential in the case of 8-α-histidyl flavinylation (10, 11) and also that enzymes bearing a 6-5-cysteinyl link have a relatively high
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midpoint potential (32). For wild-type BBE the midpoint potential reaches a value of +132 mV, which is higher than that observed for any other flavoenzyme without any additional cofactors so far. The redox potential of the C166A mutant ($E_m = +53$ mV) is in the range of that observed for other flavoprotein oxidases with a covalent link in position 8–$\alpha$, again demonstrating the similarities to vannilyl alcohol oxidase (11, 33). Therefore, our data clearly show that also for bi-covalently flavinylated proteins the modification in position 6 of the isoalloxazine ring system significantly increases the midpoint potential by a value of +79 mV. The effect is higher than expected from studies with free FMN and its 6-cysteinylated analogue (34). To the best of our knowledge, the flavoproteins with the highest potentials reported so far all belong to the $p$- cresol methylhydroxylase superfamily and have midpoint potentials up to 55 mV for vannilyl alcohol oxidase (11) and 84 mV in the case of $p$-cresol methylhydroxylase (35).

A closer comparison of the differences in the reductive rates with the changes in redox potential according to Equation 1 revealed that the decrease in the midpoint potential for the mutant can be fully correlated with the reduced rate of reduction.

$$\Delta(\Delta G) = -R \cdot T \cdot \ln \left( \frac{K_{\text{red}}\text{-wt}}{K_{\text{red}}\text{-C166A}} \right) \frac{K_{\text{red}}\text{-C166A}}{K_{\text{red}}\text{-C166A}}$$

$$= -2 \cdot F \cdot \Delta E_m(\text{wt} - \text{C166A}) \quad (\text{Eq. 1})$$

The correlation between thermodynamics and kinetics in Equation 1 ($R$, gas constant (J mol$^{-1}$ K$^{-1}$); $T$, absolute temperature (K); $F$, faraday constant (As mol$^{-1}$); $\Delta E_m$, midpoint potential (V); wt, wild type) has been adapted to describe only the reductive half-reaction of enzymatic catalysis, which is the major step influenced by the redox potential of the cofactor. Calculation of the expected change in redox potential using the experimentally determined rates of reduction and the corresponding $K_r$ values results in a potential difference of 84 mV, which is in very good agreement with our experimental data (79 ± 6 mV). Therefore, it can be concluded that the 6-$\gamma$-cysteinyl linkage in BBE increases the redox potential of the cofactor, thereby leading to improved catalytic efficiency of the enzyme. Based on this effect we propose that the family of bi-covalently flavinylated proteins has evolved in order to fine-tune the catalytic properties for optimized functioning.

In summary, our results clearly show that the bi-covalent attachment of FAD enhances the oxidative power of the cofactor and shifts the redox potentials to unprecedented high values. This increase in midpoint potential can be directly correlated to the acceleration of flavin reduction during turnover, and it is therefore tempting to assume that it might have evolved in order to facilitate conversion of the substrates accepted. Especially because a similar conclusion can be made for the 8-$\alpha$-histidyl linkage (11), it is tantalizing to think of the topic of covalent modification as sort of a modular system in which different modes of covalent linkage can be employed to achieve the optimal system for the reaction.

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