Biochemical Evidence That Berberine Bridge Enzyme Belongs to a Novel Family of Flavoproteins Containing a Bi-covalently Attached FAD Cofactor*

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Berberine bridge enzyme (BBE) is involved in the transformation of (S)-reticuline to (S)-scoulerine in benzophenanthridine alkaloid biosynthesis of plants. In this report, we describe the high level expression of BBE encoded by the gene from Eschscholzia californica (California poppy) in the methylophytic yeast Pichia pastoris employing the secretory pathway of the host organism. Using a two-step chromatographic purification protocol, 120 mg of BBE could be obtained from 1 liter of fermentation culture. The purified protein exhibits a turnover number for substrate conversion of 8.2 s⁻¹. The recombinant enzyme is glycosylated and carries a covalently attached FAD cofactor. In addition to the previously known covalent attachment of the 8α-position of the flavin ring system to a histidine (His-104), we could also demonstrate that a covalent linkage between the 6-position and a thiol group of a cysteine residue (Cys-166) is present in BBE. The major evidence for the occurrence of a bi-covalently attached FAD cofactor is provided by N-terminal amino acid sequencing and mass spectrometric analysis of the isolated flavin-containing peptide. Furthermore, it could be shown that anaerobic photoradiation leads to cleavage of the linkage between the 6-cysteinyl group yielding 6-mercaptotetradoxatin and a peptide with the cysteine residue replaced by alanine due to breakage of the C–S bond. Overall, BBE is shown to exhibit typical flavoprotein oxidase properties as exemplified by the occurrence of an anionic flavin semiquinone species and formation of a flavin N(5)-sulfite adduct.

Many alkaloids possess potentially useful pharmaceutical properties, which have been exploited in traditional medicine for centuries. Among these, benzophenanthridines have an antimicrobial activity that prompted the elucidation of its biosynthesis in plants, most notably the California poppy (Eschscholzia californica), a plant used by American Indians as a traditional medicine (1). The biosynthesis of these alkaloids leads from the aromatic amino acid l-tyrosine to a central metabolite, (S)-reticuline, a compound that yields isoquinoline derivatives of diverse structure, such as protopine, sanguinarine, and berberine, in a series of enzyme-catalyzed transformations (2). Initially, (S)-reticuline is converted to (S)-scoulerine by berberine bridge enzyme (BBE). This FAD-dependent oxidase affords the transformation of the N-methyl group of (S)-reticuline into the berberine bridge carbon (C-8) of (S)-scoulerine, shown in Scheme 1.

The transformation involves the oxidation of the N-methyl group to the methylamine iminium ion with subsequent cyclization to the protoberberine carbon skeleton (3, 4). It has been suggested that the substrate-derived electrons are passed on to the covalently attached FAD cofactor either in two one-electron steps or in a single two-electron reduction step, e.g., via transfer of a hydride (5). The reduced cofactor is then regenerated by oxidation with dioxygen to yield oxidized FAD and hydrogen peroxide (see reaction scheme (Scheme 1)). The structural requirements for formation of the berberine bridge are (i) (S)-configuration at C-1 in the N-methyltetrahydrobenzylisoquinoline ring system and (ii) hydroxylation of the carbon ortho- to the 2′-carbon of the benzyl moiety (5). This latter requirement was interpreted as evidence for the involvement of an active site base, which deprotonates the hydroxy group to facilitate nucleophilic attack of the C-2′ on the methylene iminium ion to generate the carbon–carbon bond (5).

BBE was first isolated by Zenk and collaborators (6) from plant cell cultures producing only minute amounts of a homogenous protein with a molecular mass of 52 ± 4 kDa in a tedious eight-step purification procedure. The cloning of the gene encoding BBE from the California poppy (E. californica) paved the way for the heterologous expression of the enzyme in Saccharomyces cerevisiae and Spodoptera frugiperda (7, 8). The latter expression system produced ~4 mg of active enzyme per liter of insect cell culture, allowing some basic characterization of its substrate specificity and spectroscopic properties (5). However, the level of protein expression achieved in this system is clearly not sufficient to warrant a detailed investigation of the biochemical, kinetic, and structural properties of the enzyme. The difficulties of achieving high expression of BBE are high-

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² The abbreviations used are: BBE, berberine bridge enzyme; E.C., enzynatic classification; ESI-MS, electrospray ionization mass spectrometry; GOOX, glucooligosaccharide oxidase; PAS, periodic acid-Schiff; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Endo H, endoglycosidase H₁.
lighted by the fact that the gene possesses an N-terminal signal peptide as well as a vacuolar sorting determinant (7, 9). In addition, active enzyme requires covalently bound FAD and is N-glycosylated. Here, we report the construction of a new expression system for BBE in the methylotrophic yeast *Pichia pastoris* by using the secretory pathway of this organism. This approach yields large amounts of highly active BBE enabling us to study some basic properties of the enzyme. In the course of this characterization, we could demonstrate that the enzyme contains an FAD cofactor, which is covalently linked to a histidine (His-104) and a cysteine (Cys-166) residue, as recently reported for glucosoligosaccharide oxidase from *Acremonium strictum* (10, 11).

**EXPERIMENTAL PROCEDURES**

Reagents—All chemicals were of the highest grade commercially available and purchased from either Sigma-Al- drich, Fluka, or Merck. Restriction enzymes were obtained from Fermentas, Endo Hf from New England Biolabs, and phenyl-Sepharose 6 FF (high substitution) was purchased from Amersham Biosciences. All chemicals were from the natural product collection of the Department of Natural Product Biotechnology of the Leibniz Institute of Plant Biochemistry, Halle/Saale, Germany.

**Cloning of *E. californica* BBE**—The coding sequence of BBE from *E. californica* cloned into pUC18 was used for PCR reactions (7). Two different constructs were generated using PCR with especially designed forward primers in combination with a common reverse primer and pUC18 BBE as template. BBE (forward (5′), AAAATCGACAAAAATGAAAAACATTAAGATTGAGAAGAGGCTGAAGCTGGTAATGATCTCTCTTTTCTTTTGTGG; shared reverse (3′), CATGCGCCGCCCTATATTTAAACCTTCTCCACATC) represent a construct with the native BBE coding sequence and one where the native endoplasmic reticulum-targeting signal sequence was deleted. Both constructs were cloned into pCR® 4Blunt-TOPO® vectors (Invitrogen), and amplification of the correct variant was verified by sequence analysis. For expression analysis in *P. pastoris* the constructs were cloned into the expression vectors pPICZ B and pPICZA B using EcoRI (present on the vector)/NotI and XhoI/NotI for BBE and BBE-ER, respectively. Transformation into *P. pastoris* KM71H was carried out as outlined in the EasySelect™ Pichia Expression Kit (Invitrogen) following the protocol for lithium chloride transformation with 5 μg of Sacl-linearized DNA. The presence of the expression cassette in the genome of *P. pastoris* was verified by colony PCR as described in a previous study (12).

**Expression and Purification of BBE**—For comparison of expression by the two constructs, single transformants with an integrated BBE coding sequence were used to inoculate 25 ml of buffered minimal dextrose medium in 100-ml shake flasks. Generation of biomass and induction with methanol was carried out essentially as described previously (12). After a 100-h induction period, the fermentation supernatant was analyzed by SDS-PAGE, and activity assays for expression of BBE were carried out. Large scale expression of BBE for protein purification was carried out in a BBI CT5–2 fermenter (Sartorius) with a digital control unit using the MFCs win process control system. Cultivation was conducted with a glycerol batch phase followed by a glycerol-fed batch and after generation of enough biomass protein production was initiated by addition of methanol. The different stages followed the basic outline presented in *Pichia Fermentation Process Guidelines* (Invitrogen). After 90-h methanol induction, the fermentation was stopped and the cells were separated from the medium by centrifugation. For purification of BBE, ammonium sulfate was added to the supernatant to a final concentration of 0.5 M, and the pH was adjusted to 7.5. After an additional centrifugation step, the resulting solution was then loaded onto a XK50/20 phenyl-Sepharose 6 FF (high substitution) column with a 200-ml bed volume equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 0.5 M ammonium sulfate (buffer A). After complete loading and washing with buffer A, BBE was step-eluted by a 80%/20% mixture of 20% ethanol/buffer A. BBE-containing fractions (as determined by activity assays) were pooled and concentrated up to 30 mg/ml using the Centriprep system from Amicon (molecular mass cut-off, 10 kDa). Aliquots of 2 ml from the resulting deeply yellow solution were then loaded onto a HiLoad™ 16/60 Superdex™ 75 prep grade (Amersham Biosciences) gel-filtration column equilibrated with the storage buffer of BBE (150 mM NaCl, 50 mM Tris/HCl, pH 9.0) and eluted at a flow rate of 1 ml/min. Fractions of sufficient purity and BBE concentration (analyzed by SDS-PAGE) were pooled and again concentrated by ultrafiltration as described above. Aliquots of ~40 μl were stored at ~80 °C.

**Activity Assay**—Expression of BBE during fermentation and fractions containing the enzyme during purification were followed by conversion of (S)-reticuline to (S)-scoulerine. Reaction mixtures consisted of 1 μl of sample, 1 μl of 10 mM (S)-reticuline in methanol and 3 μl of 0.1 M Tris/HCl (pH 9.0) and were incubated at 37 °C for 10 min. For visualization of conversion the reaction mixtures were separated on tlc using CH2Cl2/MeOH/25% NH4OH (90/9/1) as the mobile phase and authentic standards for substrate and product as reference. BBE turnover rates were determined by following conversion of (S)-reticuline to (S)-scoulerine by HPLC analysis of the reaction mixture. A 100-μl assay consisted of 98.5 μl of 0.1 M Tris/HCl (pH 9.0), 1 μl of 10 μM (S)-reticuline in methanol, and 0.5 μl of BBE (200 nm) resulting in a final substrate concentration of 100 μM, which is ~30 times the reported *Km* value for (S)-reticuline (7). The reaction was incubated at 37 °C, and aliquots of 1 μl were removed at various time points and loaded onto an Atlantis® dC18 column (5 μm, 4.6 × 250 mm, Waters). Substrate and product were separated during an isocratic elution at 67%
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MeOH/33% 50 mM potassium phosphate buffer, pH 7.0, for 8 min.

SDS-PAGE—Protein samples were separated by SDS-PAGE (12.5%) under reducing conditions as described by Laemmli (13). Gels were stained with either Coomassie Brilliant Blue R or PAS stain to visualize N-glycosylation.

PAS Staining—After fixing the SDS gel in 50% MeOH for half an hour, it was washed with ddH2O twice. Oxidation was carried out by immersing the gel in 1% w/v periodic acid in 3% v/v acetic acid for 30 min. After two washing steps with ddH2O, it was stained using Schiff’s reagent (Sigma) for 1 h followed by reduction with 0.5% w/v sodium metabisulfite for 1 h. After additional washing steps with water the gel was stored in 5% acetic acid.

Deglycosylation—20 μl of BBE (1 mg/ml) was denatured by addition of 2.2 μl of 10× denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and heating to 95°C for 10 min. After cooling to 37°C 2.5 μl of 10× reaction buffer (500 mM sodium citrate, pH 5.5) was added, and deglycosylation was initiated by adding 1 μl of Endo Hf (1000 New England Biolabs units). After overnight incubation in the dark at 37°C the reaction mixture was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R or PAS stain. Coomassie-stained bands were excised and used for in-gel digestion and subsequent MALDI-TOF MS analysis to identify N-glycosylated residues of BBE.

Protease Digestion of BBE and Isolation of the Flavin-linked Peptide by HPLC—0.5 mg of purified BBE was denatured in the presence of 4 M urea, 50 mM Tris/HCl, 75 mM NaCl, pH 8.5, for 45 min at 65°C. The denatured sample was then diluted with 50 mM Tris/HCl, 1 mM CaCl2, pH 8.0, to 2 M urea and cooled to 37°C prior to addition of 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at a protease:protein ratio of 1:50 (w/w). The digest was then performed at 37°C prior to addition of L-1-tosylamido-2-phenylethyl chloride, pH 5.5) was added, and deglycosylation was initiated by addition of 2.2 μl of 10× denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) and heating to 95°C for 10 min. After cooling to 37°C 2.5 μl of 10× reaction buffer (500 mM sodium citrate, pH 5.5) was added, and deglycosylation was initiated by adding 1 μl of Endo Hf (1000 New England Biolabs units). After overnight incubation in the dark at 37°C the reaction mixture was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R or PAS stain. Coomassie-stained bands were excised and used for in-gel digestion and subsequent MALDI-TOF MS analysis to identify N-glycosylated residues of BBE.

UV-visible Spectroscopy and Fluorescence—Absorption spectra were recorded with a Specord 205 spectrophotometer (Analytik Jena) at 25°C using 1-cm quartz cuvettes. Fluorescence measurements with suitable quartz cuvettes were carried out with a Shimadzu RF-5301 PC using excitation and emission slits of 20 nm and an excitation wavelength of 450 nm.

MALDI-TOF MS—A MALDI Micro MX (Waters) time-of-flight instrument was used in reflectron mode with 2.3 mM m/z of the corresponding peak ratio (1.5:1) was measured and determined by the characteristic absorption at 440 nm, and the corresponding peak was collected, vacuum-dried, and redissolved in 50 μl of 50% acetic acid for subsequent N-terminal sequencing and MS analysis.

Protein Quantification and Calculation of the Extinction Coefficient—Protein concentration of purified BBE was determined by the characteristic absorption of bound FAD. The extinction coefficient was calculated using the value of 6-S-cysteinyl FMN for denatured BBE (16). This leads to an ε445 of 11,600 M⁻¹ cm⁻¹ for native heterologously expressed BBE.

Comparative Modeling—The amino acid sequence of BBE was submitted to the Robetta server (17), and a model was generated using glucoamylase oxidase from A. strictum as a template (pdb code: 2AXR). The two proteins share a sequence.
identity of 21%. The FAD cofactor of the oxidase was combined with the newly generated model of BBE, and the supposed covalent linkages with the protein backbone were introduced using the program SYBYL v7.1 (Tripos Inc., St. Louis, MO). A rough geometry optimization to remove unfavorable contacts between the cofactor and the surrounding amino acids was performed with the same program.

**N-terminal Sequencing Analysis**—Purified BBE was electrophoresed using SDS-PAGE, and the separated proteins were transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore) using 10 mM CAPS buffer (pH 11, 10% methanol) in a tank transfer system. The transfer was performed at a constant current of 150 mA for 1 h with ice cooling. After transfer, protein was stained with Ponceau-S (0.5% Ponceau-S red, 1% acetic acid) for 1 min, and the band corresponding to BBE was excised. After destaining with ddH2O, the membrane was air-dried and subsequently analyzed by N-terminal sequencing on a 494-HT Procise Edman sequencing system (Applied Biosystems). The same facility was used to sequence the flavin-containing peptide by direct injection of the solution obtained after HPLC purification.

**ESI Mass Spectrometry**—High resolution MS spectra of the HPLC-purified peptide were recorded on a Finnigan LTQ FT instrument (Thermo Electron) in positive-ion ESI mode.

**RESULTS**

Cultures of *P. pastoris* cells carrying the BBE gene from the California poppy *E. californica* express and secrete large quantities of the protein upon induction with methanol (Fig. 1A). BBE is the dominant protein in the growth medium with expression reaching a maximum after ~85 h (Fig. 1A, lane 8). Activity of the expressed protein could be demonstrated by incubation of the substrate (S)-reticuline with growth medium and detection of product (S)-scoulerine by tlc as described under “Experimental Procedures.” Semi-quantitative analysis of activity indicated a higher expression level of BBE from the construct with the α-factor replacing the native endoplasmic reticulum secretion signal sequence. Therefore, this system was used for the large scale expression of BBE, and all further experiments were carried out with this enzyme. Nevertheless, it should be pointed out that *P. pastoris* also recognizes the native secretion signal of BBE from *E. californica* and secretes active enzyme into the fermentation broth (results not shown). BBE can be purified in a convenient and rapid two-step purification procedure leading to a homogenous, and intensely yellow, protein preparation with an estimated molecular mass of ~62 kDa (Fig. 1B). Without any optimization, the *P. pastoris* expression system produces ~120 mg of purified BBE per liter of culture medium and hence ~30-fold as much as previously achieved in insect cell culture (8).

Activity of BBE was monitored by incubation of the enzyme with (S)-reticuline and time-dependent HPLC analysis of the conversion of the substrate to (S)-scoulerine, as shown in Fig. 2. The initial velocity of the reaction allowed estimating a turnover rate of 8.2 s⁻¹, which is in the same range as determined for the enzyme expressed in insect cell culture (~6.8 s⁻¹ (8)).

To ensure secretion of the protein, BBE is initially expressed as an N-terminal fusion protein with the *Saccharomyces cerevisiae* α-factor prepro signal sequence, including an EAAE spacer (Ste13 cleavage site), which can help to recognize the Kex2 site at the end of the signal sequence efficiently during processing in the secretory pathway of the host cells (18). N-terminal sequencing of purified protein showed that the Kex2 cleavage site, which can help to recognize the Kex2 cleavage site, was recognized and cleaved efficiently but not the Ste13 site, resulting in isolated BBE having four additional amino acids at the N-terminal end and starting with the sequence EAEAGNDLL. Based on the integrated full-length cDNA of BBE, the total length of the protein comprises 519 (including the 4 extra amino acids EAEA) amino acids with a theoretical
molecular mass of 58,599 Da (including the covalently attached FAD cofactor). This expected mass is clearly lower than the estimated mass suggested by SDS-PAGE (Fig. 1, A and B). The higher than expected molecular mass of expressed BBE can be explained by assuming a post-translational modification of the protein such as glycosylation. The amino acid sequence of BBE possesses three potential N-glycosylation sites, one at the N terminus (N38) and two at the C terminus (N423 and N471) (7). As shown in Fig. 3, treatment of BBE with Endo Hf leads to cleavage of glycosylation leaving only an N-acetylglucosamine attached to the asparagine residues and reducing the molecular mass of the protein by ~4 kDa (as visualized by SDS-PAGE). To identify the sites of glycosylation, both recombinantly expressed and Endo Hf-treated BBE were subjected to trypsinolysis and subsequent peptide mapping by MALDI-TOF mass spectrometry. A compilation of resulting peptide masses is shown in Table 1. The peptides identified by their expected masses gave rise to a sequence coverage of 78.4%, clearly identifying the expressed and purified protein as BBE.

Therefore, it can be concluded that N-glycosylation occurs at Asn-38, partially at Asn-471 but not at Asn-423. BBE was shown to carry a covalently attached FAD moiety, which is bound via its 8-methyl group to a histidine residue contained in a consensus sequence comprising amino acids 100–110 of the protein (5, 19). The recombinant BBE exhibits absorbance maxima at 380 and 445 nm, typical for a flavin-containing protein and very similar to the absorbance spectrum previously reported for recombinant BBE obtained from insect cell culture (5). HPLC analysis of denatured recombinant protein confirmed the presence of covalently bound FAD (concentration of non-covalently bound FMN/FAD is <1% of the protein concentration). However, when BBE was denatured by treatment with SDS, the absorbance spectrum had only a single absorbance maximum at 440 nm (Fig. 4, dashed line) with the short wavelength absorbance peak disappearing upon denaturation. Because covalent attachment of the FAD cofactor to a histidine via its 8-methyl group yields an absorbance spectrum that features two peaks at ~350 and ~450 nm (20), similar to the unsubstituted 7,8-dimethylisoalloxazine ring system, these observed changes were unexpected and indicate that the isoalloxazine ring system is subject to further chemical modifications (see below). Moreover, an expected tryptic fragment carrying the flavinylated histidine residue (SGGHSEYGLSYTS-DTPFILDLMLNR) with a theoretical mass of 3781.57 Da could not be identified in the peptide mapping experiment described above (see also Table 1). Therefore, tryptic peptide fragments obtained from BBE digestion were subjected to

### Table 1

| Experimental mi mass | Calculated mi mass | Start | End | Sequence | % of total sequence |
|----------------------|--------------------|-------|-----|----------|---------------------|
| 1965.90              | 1965.93            | (−4)  | 37  | EAEA*GNDLLSC*LTFGVVR | 3.3                |
| 1914.99              | 1914.83            | 38    | 52  | N*HTVFSA5DSDNSFR    | 3.0                |
| 3062.80              | 3062.73            | 53    | 80  | FLHLISQPLNFSLKPSAILPGSK | 5.3               |
| 961.50               | 961.49             | 81    | 88  | EELSTIR             | 1.7                |
| 719.10               | 719.93             | 98    | 98  | GSWTIR             | 1.2                |
| 3000.32              | 3000.44            | 101   | 127 | SGGHSEYGLSYTS-DTPFILDLMLNR | 5.2               |
| 3352.39              | 3351.60            | 128   | 158 | VSIDTESETAWVESGTGLGELYAITESK | 5.8               |
| 2698.21              | 2698.23            | 159   | 185 | LGFTAGWC*PTVGTHGSHGGGGFGMMSR | 4.7               |
| 2372.19              | 2372.21            | 187   | 209 | YGLADN6VYDAILDANGAILD | 4.1                |
| 1422.60              | 1422.68            | 210   | 221 | QAMGEDFWAIR         | 2.5                |
| 6378.68              | 1378.69            | 222   | 235 | GGGGGWGAIAYWK       | 2.4                |
| 621.00               | 621.37             | 245   | 249 | VTVFR               | 1.1                |
| 1410.72              | 1410.76            | 253   | 265 | NVAIDEATSLH            | 2.4                |
| 2621.25              | 2621.24            | 266   | 288 | WQVFVEELEDTSVLGGADEK | 4.5                |
| 1676.90              | 1676.90            | 289   | 302 | QVWLMGLFHFGLK       | 2.9                |
| 968.50               | 968.52             | 371   | 378 | AFYGILLER           | 1.7                |
| 1766.77              | 1766.85            | 382   | 398 | EPNGFIALNGGGGQMSK   | 3.1                |
| 1303.60              | 1303.64            | 399   | 409 | ISSDFTPFPRH         | 2.3                |
| 1837.90              | 1837.92            | 414   | 428 | LMVEYIWAVN*Q5EQK    | 3.2                |
| 1180.60              | 1180.59            | 431   | 439 | TEFDDLWLEK         | 2.0                |
| 1354.67              | 1354.71            | 440   | 450 | VYEIFKPVYSK         | 2.4                |
| 2099.11              | 2099.06            | 453   | 472 | LGYWNNHIDLLDGGFDNSK | 3.6                |
| 1215.60              | 1215.67            | 473   | 483 | TVVNASIEIR         | 2.1                |
| 1637.70              | 1637.72            | 484   | 496 | SWGESYFLSYER        | 2.8                |
| 3014.26              | 3014.47            | 502   | 527 | TLDIPVINQHFSIPMPANFDYLEK | 5.2        |

### Notes:
- **a** New N terminus due to incomplete processing of the α-factor signal peptide (EAEA) followed by the native sequence of the processed protein (GNDLL...). The numbering of BBE residues equals those published before (7).
- **b** Cysteines are carbamidomethylated.
- **c** Asparagine residue is glycosylated. Observed mass equals the expected peptide mass + 203.1 Da (N-acetylglucosamine) after Endo Hf treatment.
- **d** Both peptides involved in covalent attachment of the flavin were detected with signal intensities lower than 5% compared to the strongest signal. Their presence can be attributed to a part of the protein preparation with non-covalently attached FAD (see "Results" and "Discussion").
- **e** Peptide can be identified without a post-translational modification at the N-glycosylation consensus.
- **f** Ambiguous results concerning glycosylation of this asparagine residue did not allow a definite statement of glycosylation status.
HPLC, and a single fragment with an absorbance spectrum similar to that of denatured BBE was isolated (Fig. 5). The isolated tryptic peptide was analyzed by N-terminal amino acid sequencing, MALDI, and ESI-MS. The amino acid sequence determined for the peptide did not correspond to a single tryptic fragment, however, closer inspection revealed that the obtained sequence was composed of two tryptic fragments comprising amino acids 101–127 and 159–185. The amino acids that could be identified by N-terminal sequencing are highlighted in the sequences shown in Fig. 5. This finding indicates that the flavin is attached covalently to both peptides, as shown here to His-104 and to Cys-166 via the 8α- and 6-position of the isalloxazine ring system, respectively. Attempts to determine a molecular mass of the peptide by MALDI-MS produced a series of non-interpretable signals, probably due to photo-induced fragmentation of the peptide. On the other hand, ESI-MS generated two signals at 6420.8 and 6073.8 m/z, which correspond to the flavin-dipeptide shown in Fig. 5 where R is either the complete ribityl phosphate-AMP side chain (calculated mass of 6420.8 Da) or the cyclized ribityl phosphate side chain (calculated mass of 6073.7 Da). Hence, ESI-MS is in full agreement with the postulated bi-covalent attachment via the 8α- and 6-position of the FAD moiety. The UV-visible absorbance spectrum of denatured BBE is also compatible with an additional covalent linkage to the 6-position, because 6-cysteinylflavin possesses a dominant peak at 440 nm with a second, less pronounced peak at 340 nm (21). It should be noted, however, that 8-histidyl-6-cysteinyllflavin model compounds have not been synthesized so far, and therefore their spectral properties are not available for comparison.

Furthermore, this compound is also known to undergo photo-degradation to the 6-thio-flavin and alanine (21). Irradiation of BBE leads to complete bleaching of the sample with no residual UV-visible absorbance (data not shown). However, when dissolved oxygen is removed from the sample prior to photoirradiation, we could observe reduction of the flavin compatible with the generation of an anionic flavin semiquinone, typically observed for flavin-containing oxidases (22). As shown in Fig. 6A, the flavin was successively reduced to a species with an absorbance maximum at 372 nm, whereas the absorbance at longer wavelength decreases. These spectral changes are clearly indicative for the generation of the anionic flavin semiquinone as reported for several other oxidases (23, 24). The flavin semiquinone was then apparently further reduced to the hydroquinone form as shown in Fig. 6B. However, it should be pointed out that the observed spectral changes proceed non-isosbestically, and therefore it can be assumed that reduction is not the only process occurring. This interpretation is also supported by the spectrum obtained after re-oxidation, as shown in Fig. 6C, because the original oxidized BBE spectrum was not regenerated. Instead, the spectrum features a sharp absorbance maximum at 443 nm and a long wavelength absorbance from 550 to 880 nm peaking at 720 nm. These spectral properties are in accordance with the generation of a 6-thio-FAD from the flavin cofactor bound to BBE (21). The conversion of 6-cysteinyllflavin to the 6-thio-species is also accompanied by the generation of an anionic flavin semiquinone, typically observed for flavin-containing oxidases (22, 23). Hence, some of the BBE-bound cofactor was apparently destroyed during irradiation, but not completely as observed before under aerobic conditions.

The sample from the anaerobic photoreduction was digested with trypsin, and the peptide fragments were analyzed by MALDI MS. A new tryptic peptide with a monoisotopic mass of 2609.1 Da was found, which corresponds to a peptide fragment comprising amino acids 159–185 with cysteine (Cys-166) converted to alanine (data not shown). This result provides further support for photocleavage of the 6-cysteinyllflavin linkage between Cys-166 and the flavin.

Flavin-dependent oxidases are known to share several salient characteristics such as the stabilization of the anionic (red) flavosemiquinone, formation of an N(5)-sulfite adduct, as well as preferential binding to the anionic forms of modified flavins bearing an ionizable group at the 8- or 6-position of the flavin.
isoalloxazine ring system, e.g. mercapto- and hydroxyflavins (22). These properties are connected to the ability of the protein to stabilize a negative charge at the N(1)–C(2) = O locus of the flavin ring system, which is also relevant for catalysis, because the flavin cofactor serves as an electron sink during substrate oxidation. Structurally, the stabilizing effect is exerted by a positively charged amino acid residue, arginine or lysine, or an α-helix placed in the direct vicinity of the N(1)–C(2) = O locus of the flavin cofactor (25). The photoreduction experiment described above already indicated that BBE behaves like a typical oxidase, because the flavin semiquinone species was observed during anaerobic reduction. Furthermore, the resulting 6-thio-species obtained after re-oxidation is clearly bound in its deprotonated form to BBE, hence the enzyme is capable of stabilizing a negatively charged species through delocalization of the charge to the N(1)–C(2) = O locus. In an additional experiment, we have examined the ability of BBE to form the N(5)-sulfite adduct by measuring the rate of reaction of BBE and sulfite as a function of the sodium sulfite concentration. As shown in Fig. 7 (main panel), sulfite binds to BBE generating the N(5)-sulfite adduct as reported earlier for several other flavoprotein oxidases (24, 26). The secondary rate constant for the reaction of the flavin cofactor with sulfite was determined to $k_{on} = 8.1 \times 10^{-5} \text{ mm}^{-1} \text{s}^{-1}$. The plot of the observed rate of reaction versus sulfite concentration produces a linear dependence with a $y$-axis intercept reflecting $k_{off} (= 4.3 \times 10^{-4} \text{s}^{-1})$. This allows the calculation of the dissociation constant $K_d = 5.3 \text{ mm}$ for the N(5)-sulfite adduct.

**DISCUSSION**

Mechanistic and structural studies of low abundance proteins depend on the ability to express large amounts of soluble and active enzyme in a suitable host organism. This task is often confounded by the presence of targeting presequences, the necessity for post-translational modifications (e.g. glycosylation), or the requirement for the provision of catalytically essential cofactors. In the case of berberine bridge enzyme
(BBE), heterologous expression in a bacterial host\(^3\) as well as in \(S.\) \textit{cerevisiae} were not met with much success leading either to insoluble protein or to very small amounts of soluble and active enzyme \((7)\). Higher amounts of active BBE could be generated in insect cell culture using the baculovirus expression system \((8)\), however, the amounts obtained were still not sufficient for a detailed biochemical, mechanistic, and structural investigation of the enzyme. Therefore, we have attempted expression of the protein in \(P.\) \textit{pastoris}, a fungal host that has been successfully used for numerous proteins requiring correct processing of targeting sequences as well as typical eukaryotic post-translational modifications. The expression described here yields \(\sim\)30-times more protein with a specific activity similar to that reported for the enzyme isolated from insect cell culture \((8)\). Moreover, the UV-visible absorbance spectrum of the enzyme derived from \(P.\) \textit{pastoris} KM71H is identical to the one reported for BBE expressed in insect cell culture \((5)\). Another characteristic of BBE is a covalently linked FAD cofactor, which was found to be bound via the 8-methyl group of the isoalloxazine ring system to a histidine identified as His-104. To verify a covalent linkage between BBE and the flavin, purified BBE was subjected to tryptic digestion followed by separation of the obtained peptide fragments by HPLC. A single peptide was isolated having the same UV-visible absorbance properties as the SDS-denatured BBE \((\text{see Figs. 4 and 5})\) indicating that this peptide carries the flavin cofactor. The peptide could be successfully analyzed by Edman sequencing, and its mass was determined by ESI-MS. This analysis revealed that the HPLC-purified peptide is not simply the His-104-containing tryptic fragment comprising amino acids 101–127 \((\text{see Table 1})\). Instead, the obtained amino acid sequence and its mass indicate that a second tryptic peptide is covalently linked to the flavin and is identified as a fragment containing amino acid residues 159–185 \((\text{Table 1})\). Most strikingly, this tryptic fragment carries a cysteine residue \((\text{Cys-166})\), which we propose is engaged in a second covalent linkage to the flavin. This conclusion is supported by the finding that the flavin-containing peptide is non-fluorescent as was also reported for the first 6-cysteinyl-linked flavin in trimethylamine dehydrogenase \((27)\). It should be noted here that isolated BBE shows a very weak fluorescence emission peaking at around 520 nm \((\text{data not shown})\), which is probably due to traces of monosubstituted 8a- and non-covalently bound FAD. This interpretation is supported by the results from the peptide mapping where traces of the tryptic peptides involved in the covalent linkage \((\text{amino acids 101–127 and 159–185})\) to the flavin moiety could be detected \((\text{see Table 1})\). Furthermore, HPLC analysis of our tryptic digests have demonstrated the presence of non-covalently bound FMN and FAD. Both results suggest that the isolated BBE also contains non-covalently or monosubstituted FAD, which gives rise to the observed fluorescence emission.

To obtain further support for the existence of a covalent linkage between Cys-166 and the flavin, we have studied the UV-visible absorbance changes upon photoreduction of BBE. It is known for both free 6-cysteinyl flavins and the 6-cysteinyl-linked FAD cofactor in trimethylamine dehydrogenase that photoreduction results in cleavage of the C–S bond giving rise to characteristic spectral changes \((21, 27)\), which are indicative of the generation of 6-thioflavin. Anaerobic photoreduction of BBE precipitates identical spectral changes as those reported earlier for the generation of a 6-thioflavin species from either 6-cysteinylflavins or the 6-S-linked flavin in trimethylamine dehydrogenase \((\text{see Fig. 6C})\) strongly supporting the presence of a 6-S-linked flavin in BBE. To substantiate this claim, we have subjected photoirradiated BBE to a tryptic digest followed by MALDI-MS analysis. The peptide that results from the photocleavage process, \textit{i.e.} the fragment comprising amino acids 159–185, should now have a reduced mass due to the conversion of Cys-166 to an alanine. In fact, this altered peptide could only be observed when BBE was photoirradiated prior to analysis demonstrating that generation of the 6-thio flavin species is accompanied by the occurrence of alanine instead of cysteine in the peptide fragment. In this context, it is interesting to note that BBE is expressed in the aerial, \textit{i.e.} light-exposed parts of \(E.\) \textit{californica} and \(Papaver somniferum\), and hence it can be concluded that these plants possess efficient protection mechanisms to prevent photodegradation of the covalently bound BBE cofactor.

Recently two homologous proteins, glucooligosaccharide oxidase \((\text{GOOX})\) and \(\Delta^1\)-tetrahydrocannabinolic acid synthase, have been described in the literature, both of them having a similar UV-visible absorbance spectrum as BBE \((10, 28)\). These proteins show 20.7 and 37.8% sequence identity, respectively, to BBE and also share a conserved cysteine residue in the corresponding position of Cys-166 \((10)\). In the case of GOOX it was also noted that mutation of the histidine, to which the flavin is covalently attached via the 8a-methylene group, does not abolish covalent attachment of the flavin indicating that another
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covalent linkage exists (10). This could be confirmed by the elucidation of the three-dimensional structure of GOOX by x-ray crystallographic analysis (11) (pdb code: 2AXR). This structure reveals the existence of a cysteine-linkage between the protein and the 6-position of the flavin, supporting our experimental findings with BBE. Based on these results, we propose that the FAD cofactor in BBE is linked to both His-104 and Cys-166 via positions 8α and 6 of the flavin isoalloxazine ring system, respectively, as shown in Fig. 8.

Based on sequence alignments, several other proteins possess a conserved histidine and cysteine residue and may therefore also feature a bi-covalently attached FAD cofactor (10). This group of BBE-like proteins comprises so far several oxidases of bacterial and plant origin, such as GOOX from A. strictum and hexose oxidase from the seaweed Chondrus crispus (10, 29). In addition, another group of plant proteins known to act as pollen allergens also shares the histidine and cysteine motifs and, hence, the bi-covalent attachment of FAD of BBE-like proteins (30, 31). Except for the latter group, where a catalytic role of the protein could not be identified so far, the bi-covalently attached FAD cofactor is required for the oxidation of the substrate of the enzyme. Therefore, the redox potential of the flavin cofactor is modulated by the protein, such that it is more positive than the corresponding redox couple of (reduced) substrate/oxidized) substrate. For example, in the case of lactate oxidase, a paradigm for this family of flavin-dependent oxidases, the redox potential for the FMN/FMNH−couple is −149 mV, whereas the redox potential for lactate/pyruvate is −189 mV (32), and hence the flavin cofactor can serve as the electron acceptor in the substrate oxidation reaction. Because the redox potential for the free FMN/FMNH−couple is −208 mV, the cofactor binding pocket in the active site of an enzyme must provide an environment that leads to the required alteration of the redox potential necessary for catalysis. On the other hand, the redox potential can also be modified by derivatization of the isoalloxazine ring system, for example introduction of a 6-S-cysteiny1 residue increases the redox potential by 54 mV to −154 mV (21). Hence, it is plausible that formation of a 6-s-cysteine-linkage in BBE and BBE-like proteins has the purpose of increasing the redox potential of the cofactor by chemical modification and is potentially further tuned by environmental changes in the FAD binding pocket. Therefore, it will be very interesting to characterize the functional role of FAD attachment via the 8α- and 6-position to BBE by investigating His-104 and Cys-166 single and double mutant proteins in conjunction with the determination of their catalytic and redox properties.

Flavoprotein oxidases are known to share several properties reflecting the ability of the redox cofactor to act as an acceptor of substrate-derived electrons (22). These properties encompass the ability to stabilize the (red) anionic flavin semiquinone, an N(5)-sulfite adduct as well as the deprotonated form of an artificial flavin bearing an ionizable group at positions 6 and 8 (e.g. 8- or 6-mercaptopflavin). As far as the stabilization of the anionic flavin semiquinone is concerned, anaerobic photoreduction of BBE results in the generation of a species that bears characteristics of such a species, reflected by the occurrence of a short wavelength peak at 370 nm accompanied by the bleaching of the absorbance at around 450 nm (see Fig. 6A). Furthermore, photoreduction of the BBE cofactor results in cleavage of the 6-cysteinyl linkage leading to the 6-mercaptopflavin species, as becomes evident after re-oxidation (see Fig. 6C). As is obvious from the presence of the long wavelength absorbance at 720 nm, the 6-mercaptop-FAD is stabilized in its deprotonated form by BBE. Additional support for the stabilization of a negative charge on the isoalloxazine ring system is provided by its ability to form N(5)-sulfite adducts. As shown in Fig. 7, BBE is able to form such an adduct, albeit with a dissociation constant that is 4–5 orders of magnitude higher than for glycolate and lactate oxidase (24, 32). However, it is important to bear in mind that BBE possesses a novel bi-covalently linked FAD and not a dissociable cofactor, as the before mentioned model flavoprotein oxidases, and that these comparisons should therefore be regarded with great caution.

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