Phyceroerythrobilin is a linear tetrapyrrole molecule found in cyanobacteria, red algae, and cryptomonads. Together with other bilins such as phycocyanobilin it serves as a light-harvesting pigment in the photosynthetic light-harvesting structures of cyanobacteria called phycobilisomes. The biosynthesis of both pigments starts with the cleavage of heme by heme oxygenases to yield biliverdin IXα, which is further reduced at specific positions by ferredoxin-dependent bilin reductases (FDBRs), a new family of radical enzymes. The biosynthesis of phyceroerythrobilin requires two subsequent two-electron reductions, each step being catalyzed by one FDBR. This is in contrast to the biosynthesis of phycocyanobilin, where the FDBR phycocyanobilin:ferredoxin oxidoreductase (PcyA) catalyzes a four-electron reduction. The first reaction in phyceroerythrobilin biosynthesis is the reduction of the 15,16-double bond of biliverdin IXα by 15,16-dihydrobiliverdin:ferredoxin oxidoreductase (PebA). This reaction reduces the conjugated π-electron system thereby blue-shifting the absorbance properties of the linear tetrapyrrole. The second FDBR, phyceroerythrobilin:ferredoxin oxidoreductase (PebB), then reduces the A-ring 2,3,3',3''-diene structure of 15,16-dihydrobiliverdin to yield phyceroerythrobilin. Both FDBRs from the limnic filamentous cyanobacterium *Fremyella diplosiphon* and the marine cyanobacterium *Synechococcus* sp. WH8020 were recombinantly produced in *Escherichia coli* and purified, and their enzymatic activities were determined. By using various natural bilins, the substrate specificity of each FDBR was established, revealing conformational preconditions for their unique specificity. Preparation of the semi-reduced intermediate, 15,16-dihydrobiliverdin, enabled us to perform steady state binding experiments indicating distinct spectroscopic and fluorescent properties of enzyme-bilin complexes. A combination of substrate/product binding analyses and gel permeation chromatography revealed evidence for metabolic channeling.

Phycobilins are linear tetrapyrrole molecules that are important cofactors of the photoreceptor phytochrome and the cyanobacterial light-harvesting phycobiliproteins. One of the major pigments found in the phycobilisomes of certain cyanobacteria, red algae, and cryptomonads is phyceroerythrobilin (PEB). In these organisms PEB is covalently linked to the phycobiliprotein phycoerythrin (PE), a major constituent of the light-harvesting structures called phycobilisomes. These structures allow the organisms to efficiently absorb light in regions of the visible spectrum that are poorly covered by chlorophylls. Through resonance energy transfer the absorbed light energy is transferred to the photosynthetic reaction centers in the membrane. Freshwater cyanobacteria of the genus *Calothrix* (*Freymella*) are able to adapt their phycobiliprotein composition within the phycobilisome in response to different light conditions. In a process called complementary chromatic adaptation the organisms are able to adjust the quantities of phycocyanin and PE for maximal light harvesting efficiency. Not only is the synthesis of apophycobiliproteins and linker proteins regulated by light, but also the biosynthesis of the enzymes required for PEB synthesis. It has been demonstrated that the expression of the genes *pebA* and *pebB* encoding ferredoxin-dependent bilin reductases (FDBRs) in *Fremyella diplosiphon* (*Calothrix* or *Tolyphothrix* sp. PCC 7601) is up-regulated by green light, as is the expression of the *cpeA* genes encoding the α- and β-subunits of PE (1).

In a similar manner, marine cyanobacteria of the *Synechococcus* group are able to regulate phycourobilin to PEB ratios by adjusting the expression of phycoerythrins with different phycoerythrin content, PE(I) and PE(II) (2), or, as recently suggested, by lyses that mediate PEB isomerization on the phycobiliproteins (3). Because these organisms retain a fixed phycocyanin:PE ratio (4), they are not considered as classical chromatic adapters.

The biosynthesis of phycobilins proceeds via the heme biosynthetic pathway. The final product, heme, is cleaved by heme oxygenases to yield biliverdin IXα (BV), which is subsequently further reduced by a family of FDBRs (Fig. 1). These enzymes are characterized by a distinct double bond regiospecificity resulting in bilins with a wide variety of spectroscopic properties. Synthesis of phytocouromobilin (PΦB), the chromophore of plant phytochromes, is catalyzed by phytocouromobilin syn-

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FIGURE 1. Biosynthetic pathway of bilin pigment synthesis. In plants, red algae, cyanobacteria, and cryptomonads BV is reduced by FDBRs with high double bond regiospecificity to produce phytobilins. PEB synthesis is catalyzed by PebA and PebB and proceeds via the semi-reduced intermediate DHBV.

Phycoerythrobilin biosynthesis involves the two-electron reduction of biliverdin IXα (BV) by Phycocyanobilin:ferredoxin oxidoreductase (PcyA). This enzyme mediates two subsequent electron reductions at both vinyl groups of BV (5). In this reaction, 181,182-dihydrobiliverdin is a visible semi-reduced intermediate. Because no metal or organic cofactors are detected in the PcyA family, a radical mechanism for PcyA was postulated (5). Evidence for the appearance of an intermediate substrate radical was recently demonstrated by absorbance and EPR spectroscopy (6). Structural information to this new family of enzymes has recently been added through the solved crystal structure of the Synechocystis sp. PCC 6803 PcyA (7).

In contrast to the PcyA-catalyzed reaction, the biosynthesis of PEB (which is an isomer of PCB) requires two independent enzymes, each catalyzing a two-electron reaction. 15,16-Dihydrobiliverdin:ferredoxin oxidoreductase (PebA) catalyzes the reduction of the C-15 methine bridge of BV and phycocerythrobilin:ferredoxin oxidoreductase (PebB) the A-ring diene structure of DHBV, respectively. The biosynthesis of phycourobilin still remains unknown, but it might proceed analogously to the PecE/F isomerase/lyase activity of Mastigocladus laminosus, which covalently attaches and isomerizes PCB to yield bound phycoviobilin (8).

Here we present the biochemical characterization of recombinant PebA and PebB from the filamentous freshwater cyanobacterium, F. diplosiphon, and the unicellular marine cyanobacterium Synechococcus sp. WH8020. From our results, the involvement of PebA and PebB in metabolic channeling is postulated.

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise specified, all chemical reagents were ACS grade or better. Spinacia oleracea ferredoxin, Clostridium pasteurianum ferredoxin, Porphyra umbilicalis ferredoxin, ferredoxin:NADP+ oxidoreductase, glucose-6-phosphate dehydrogenase, and NADP+ were purchased from Sigma-Aldrich. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs and Phusion™ DNA polymerase from Finzymes. HPLC grade acetone, chloroform, and formic acid were purchased from Acros, Sigma-Aldrich, and J. T. Baker, respectively.

Glutathione-Sepharose™ 4FF, PreScission™ protease, and expression vector pGEX-6P-3 were obtained from GE Healthcare. Expression vector pASK-IBA45+ and Strep-Tactin-
Production and Purification of Recombinant PebA and PebB—For production of recombinant PebAFredi, PebASyny, and PebBSyny, 2 liters of LB medium containing 100 μg/ml ampicillin was inoculated at 1:100 from an overnight culture of BL21(DE3) carrying the respective plasmid construct and cultivated at 37 °C to an A578nm ~ 0.6–0.8. After a temperature shift to 17 °C, protein expression was induced by adding 100 μM isopropyl-β-D-thiogalactopyranoside, and cells were cultured for a further 15 h at 180 rpm. Cells were harvested by centrifugation and stored at −20 °C. Frozen cells were thawed, resuspended in 20 ml of lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.05% Triton X-100), and disrupted by passage through a French press cell at 20,000 p.s.i. After ultracentrifugation for 50 min at 170,000 × g the supernatant was loaded on a glutathione-Sepharose™ 4FF column. Washing and elution were done according to instructions supplied by the manufacturer. Protein-containing fractions were cleaved with thrombin, collected by passing the elution through a 1–5 ml N-terminal fusion with Strep-tag® II and transformed in E. coli DH10B cells. The integrity of the plasmid constructs was confirmed by DNA sequencing. Cloning strategies for the Synechococcus sp. WH8020 (Synpy) pebA and pebB were described previously (10).

Purification of Recombinant Reductants—The DNA sequence of Synechococcus sp. PCC 7002 ferredoxin (petF) was amplified from the plasmid pSe280fd (obtained from D. Bryant) using the following forward and reverse primers: 5′-GGAAATTC-GATGCTCATAATAGGTACC-3′ and 5′-CCGCTCGAG-CCTAGTAGGTTTCCTCCCTTTT-3′ (the underlined sequences indicate the EcoRI or XhoI sites). The PCR product was ligated to pGEX-6P-3. Expression was done in NZCYM medium as described elsewhere (11), and protein production was induced using 1 mM isopropyl-β-D-thiogalactopyranoside at an A578nm of 0.6–0.8. Cells were harvested 4 h after induction. Purification was done with two sequential glutathione-Sepharose columns as described for the bilin reductases. The employed cleavage buffer was free of dithiothreitol and EDTA.

Determination of Protein and Bilin Concentrations—Concentrations of the bilin reductases were determined using the calculated molar extinction coefficient (ε380 nm) (12). The concentration of recombinantly produced ferredoxin from Synechococcus sp. PCC 7002 was determined using an ε420 nm of 9.7 mM −1 cm −1 (13). Concentration of BX IXα was calculated using an ε376 nm of 68.6 mM −1 cm −1 and an ε698 nm of 32.6 mM −1 cm −1 in 2.5% HCl-MeOH (14). The concentration of 3E-PEB was determined using ε526 nm of 15.8 mm −1 cm −1 and ε591 nm of 25.2 mm −1 cm −1 in 5% HCl-MeOH (15). The concentration of DHBV was determined by measuring the absorbance at the 561-nm maximum in 5% HCl-MeOH and using the long wavelength extinction coefficient of 3E-PEB for calculation. All concentrations were determined using a Uitrospec 2000 UV-visible spectrophotometer (GE Healthcare).

Bilin Reductase Activity Assay—Assays for bilin reductase activity were done as described previously with small modifications (10). The standard assays contained 1.5 μM bilin reductase, 5 μM bilin substrate, and 4.8 μM recombinantly produced Synechococcus sp. PCC 7002 ferredoxin or the alternative ferredoxins in reaction buffer. The assays were incubated for 15–30 min at 30 °C in the dark. Bilins were isolated using C18 Sep-Pak columns (Waters) and evaporated to dryness in vacuo. For spectrometric detection of electron transfer activity, the assay was performed using 10 μM PebAFredi, 10 μM BV, 40 μM NADP⁺, and 0.0125 units/ml ferredoxin: NADP⁺ oxidoreductase.

Preparative Production of 15,16-Dihydrobiliverdin—Larger quantities of DHBV were produced enzymatically by setting up a 10-ml bilin reductase activity assay containing 20 μM BV, 5 μM PebAFredi, and 4.8 μM Synechococcus sp. PCC 7002 ferredoxin in reaction buffer at 30 °C. The reaction progress was monitored by measuring absorbance spectra at different time points during the reaction. If no further substrate conversion was observed, the reaction was stopped immediately by adding 40 ml of 0.1% (v/v) trifluoroacetic acid and cooling on ice. A C18 Sep-Pak light column was preconditioned with sequential washes of CH₃CN, H₂O, 0.1% (v/v) trifluoroacetic acid, and 10% (v/v) MeOH in 0.1% trifluoroacetic acid. The bilin was loaded on the column washed with 6 ml 0.1% (v/v) trifluoroacetic acid and 6 ml of 20% MeOH in trifluoroacetic acid, eluted with CH₃CN, and dried in vacuo. The purity of produced DHBV was controlled by HPLC for contamination by other bilins.
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Absorption and Fluorescence Spectroscopic Analysis—All protein solutions used for binding site saturation experiments were checked for homogeneity with analytical gel permeation chromatography on a Superdex 75 HR10/30 column. Protein solutions were adjusted to concentrations ranging from 0.5 to 18 μM; substrate and product complexes were formed by incubating the protein solution with a 4 μM final concentration of the bilin (5–10 μl of stock solution) for 20 min on ice in the dark. All fluorescence measurements were performed under physiological conditions in reaction buffer using an Aminco Bowman AB2 spectrofluorimeter at a constant temperature of 20 °C. The excitation/emission wavelengths used were 590 nm/610 nm for PebA-DHBV, 605 nm/645 nm for PebB-DHBV, and 545 nm/630 nm for PebB-3E-PEB. Both excitation and emission slit widths were set at 4 nm, and the scan speed was 2.5 nm/s. Determination of binding constants of substrate/product complexes was done according to Clarke (16). Binding curves were measured by the increase of fluorescence intensities, as the bilin/enzyme complex is formed at equilibrium. Obtained data were analyzed using Sigma Plot 9.0 (Systat Software Inc.), and data were fitted against Equation 1.

\[
F = \frac{F_{\text{max}} \times [\text{enzyme}]}{K_d + [\text{enzyme}]} 
\]

(Eq. 1)

Time-dependent absorbance measurements (Fig. 4) were performed in a stirred cell tempered to 30 °C on an Agilent Technologies 8453 spectrophotometer with ChemStation biochemical analysis software. Absorbance spectra (Fig. 5) were measured using a Lambda 2 UV-visible spectrophotometer (PerkinElmer Life Sciences).

HPLC-Analysis—Bilin reaction products were analyzed as described previously (5).

Gel Permeation Chromatography of Enzyme-Bilin Complexes—Enzyme-bilin complexes were formed by incubating protein solution with approximately double the molar concentration of bilin for 3 min at 30 °C. The complex was purified by passing it through a NAP™-5 column (GE Healthcare) prior to analytical gel permeation chromatography on a Superdex 75 HR10/30 column. During isocratic elution, absorbance was simultaneously detected at 280, 585, and 605 nm using the UV-900 detector of the ÄKTApurifier system (GE Healthcare).

**RESULTS**

Recombinant Production and Purification of FDBRs—PebA of the filamentous freshwater cyanobacterium *F. diplosiphon* and also PebA and PebB of the coccolid marine cyanobacterium *Synechococcus sp.* WH8020 were expressed using a tac promoter–driven N-terminal GST fusion protein. A protocol using overnight proteolytic cleavage of the affinity-purified GST fusion protein followed by a second affinity chromatography to remove GST tag and protease led to the best results. This purification strategy led to less than 10% impurity (Fig. 2). Prior to all quantitative experiments, a third purification step using gel permeation chromatography was performed to remove possibly aggregated enzyme. N-terminal sequencing of PebAFredi by Edman degradation revealed no GST or other protein contamination after this purification step, and the yields of this method varied, depending on the enzyme, between 3 and 7 mg/liter cell culture. The best results for PebAFredi purification were achieved with tet promoter–driven expression followed by single-step purification of the Strep-tagged enzyme (Fig. 2). This procedure yielded about 1 mg/liter cell culture.

Activity of the Recombinant Enzymes—To verify the activity of the purified bilin reductases, we used an *in vitro* assay system as described previously with an excess of reductant (10). The optimal pH value for PebAFredi activity was determined to be pH 7.5, and therefore all further assays were performed at this pH. As expected, both PebAs were found to convert BV to DHBV (Fig. 3). The reduction of the 15,16-double bond of BV is accompanied by obvious blue-shifts of the absorption that enabled us to monitor the *in vitro* reaction progress spectroscopically (shown in Fig. 4). The analyzed PebBs catalyzed the reduction of the A-ring diene system of DHBV to PEB, which may likely be a 2,3-reduction, followed by isomerization to 3Z-PEB, which is supposed to be the natural chromophore of PE. The overall reaction can be followed by HPLC in an assay mixture containing BV, PebA, and PebB at once (Fig. 3) or individually using BV as a substrate for PebA or purified DHBV as substrate for PebB (data not shown) to confirm the specific catalytic activity for both enzymes. The appearance of the energetically stable 3E-PEB may be a result of the bilin extraction procedure as described previously (10). In our assay system both reactions were found to be most efficient using plant type *2Fe-2S* ferredoxins of *Synechococcus sp.* PCC7002 or *S. oleacea* as redox partners followed by the *2Fe-2S* ferredoxin from *P. umbilicalis*; only marginal activity could be detected using [4Fe-4S] ferredoxin from *C. pasteurianum*. These results are in good agreement with results obtained for PcY of *Anabaena* sp. PCC7120 (5) and for phytochromobilin synthase of *Avena sativa* (17). Consistent with PcY is the insensitivity of PebAFredi toward metal chelators like EDTA (10 μM), o-phenanthroline (5 μM), or 2,2′-dipyridyl (5 μM), indicating no involvement of protein-associated metal ion cofactors during catalysis (data not shown) (5).
the dark led to a visible transformation of the color from pink to green, the result of a back-oxidation of DHBV to BV, which was confirmed by HPLC (data not shown). This back-oxidation was slower under low oxygen conditions; a control experiment with carbonic anhydrase instead of PebA in the solution resulted in much lower DHBV reoxidation, indicating that this reaction is accelerated in the presence of PebA.

**Substrate Specificity of FDBRs**—To analyze the substrate specificities of the various FDBRs we examined different natural bilins (Table 1). In our standard HPLC assay system, we did not find 3E-PCB to be converted by PebA, indicating that a lack of A- and D-ring vinyl moieties, together with a changed geometry of the A-ring ethyldiene group, prevents recognition of 3E-PCB as substrate. DHBV was not converted by PcyA, demonstrating that the reduction of the 15,16-double bond causes structural difference in the bilin, which likely prevents proper placement of the bilin in the active site pocket. Interestingly, we found that PebA was able to reduce the plant bilin PΦB to PEB, indicating that an A-ring ethyldiene group instead of an A-ring vinyl group is not critical for substrate recognition by PebA.

**Spectroscopic Properties of FDBR Complexes with Their Substrates or Products**—All tested bilins incubated with PebA or PebB displayed distinct spectroscopic properties differing from those of the free pigments (Fig. 5). Not only were the absorbance maxima shifted, but also the peak intensities and the ratio of the long wavelength absorbance peak and the near UV absorbance peak intensities (λmax2/λmax1) changed (Table 2). Interestingly, the protein environment of PebA and PebB influences the spectral properties of one and the same bilin, indicating differences in the bilin binding pocket of both FDBRs. BV binding to PebA causes an increase in absorbance compared with free BV, with a shifted long wavelength absorbance maximum from 681 to 691 nm. Bound to PebB, the absorbance maximum is less intensely increased, but the long wavelength absorbance maximum is shifted from 681 to 706 nm. Consequently, the ratio of λmax2/λmax1 did not change notably, and lies between 0.3 for BV and 0.52 for PebA-BV (Fig. 5A and Table 2). Spectral analyses of complexes of the semi-reduced intermediate DHBV with PebA (enzyme-product complex) and PebB (enzyme-substrate complex) revealed noticeable differences. DHBV binding to PebA led to an absorbance increase at both maxima and to a shift from 565 to 590 nm for λmax2. Binding to PebB shifted the λmax2 to 605 nm and decreased the λmax1 absorbance, thereby changing the color of the complex from pink to blue. The λmax2/λmax1 ratio was changed from 0.63 (free DHBV) to 0.97 (PebA-DHBV) and 1.36 (PebB-DHBV) (Fig. 5B)

**TABLE 1**

| Bilin substrate | FDBR     |
|----------------|----------|
|                | PebA  | PebB  | PcyA  |
| BV IXα         | DHBV  | NM*   | 3E-/3Z-PCB |
| DHBV           | NM    | 3E-/3Z-PEB | NM    |
| 3E-PCB         | NM    | NM    | NM    |
| 3E-/3Z-PΦB     | 3E-/3Z-PEB* | NM    | NM/3E-/3Z-PCB |
| 18′18′-DHBV    | ND°   | NM    | 3E-/3Z-PCB |

* NM, not metabolized.
° The substrate used in the assay was the product of the HY2 reaction, which yields the 3E- and 3Z-isomers of PΦB.
° Only 3Z-PΦB is metabolized (5).
° ND, not determined.
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Comparative Binding Affinities of Bilin Substrates and Products—The specific fluorescent properties of enzyme-bilin complexes provide the opportunity to perform steady state binding analyses to determine binding constants for the respective enzyme-bilin complexes that occur during PEB biosynthesis in the absence of reduced ferredoxin. Because fluorescence signals of enzyme-bound bilins were intensely increased, we were able to determine binding constants by saturation titration of the bilin with increasing amounts of protein (16). Binding could only be determined at equilibrium because the binding was so rapid (probably in the ms range) that the kinetics were not measurable with our instruments (data not shown). At equilibrium we observed hyperbolic saturation for all tested enzyme complexes, indicating one binding site per enzyme. Using the fluorescent properties of enzyme-bound bilins we observed hyperbolic saturation for all tested enzyme complexes, indicating one binding site per enzyme.

Absorbance and fluorescence spectroscopic properties of bilins and enzyme-bilin complexes

| Bilin/enzyme-bilin complex | $\lambda_{\text{max} 1}$ | $\lambda_{\text{max} 2}$ | $\lambda_{\text{max} 1}/\lambda_{\text{max} 2}$ | $\lambda_{\text{max} \text{em}}^{\text{a}}$ |
|---------------------------|----------------------|----------------------|----------------------|----------------------|
| BV                        | 382                   | 618                   | 0.30                  | 581                   |
| PebA-BV                   | 382                   | 690                   | 0.52                  | 581                   |
| PebB-BV                   | 382                   | 706                   | 0.40                  | 581                   |
| DHBV                      | 335                   | 565                   | 0.63                  | 581                   |
| PebA-DHBV                 | 335                   | 590                   | 0.97                  | 581                   |
| PebB-DHBV                 | 342                   | 605                   | 1.36                  | 581                   |
| 3E-PEB                    | 370                   | 535                   | 0.64                  | 581                   |
| PebA-3E-PEB               | 372                   | 586                   | 0.86                  | 581                   |
| PebB-3E-PEB               | 372                   | 545                   | 0.63                  | 581                   |

$^{\text{a}}\lambda_{\text{max} \text{em}}$ emission wavelength.

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FIGURE 6. Fluorescence emission spectra of enzyme-substrate/product complexes. Fluorescence emission of enzyme-bilin complexes excited at their long wavelength absorbance maxima. Enzyme-bilin complexes were formed by incubating 8 $\mu$M FDBR with 4 $\mu$M bilin for 20 min on ice. Complexes of bilins with reduced 15,16-double bonds, like DHBV with PebA (solid line) or PebB (dashed line), as well as PebB with 3E-PEB (dash-dotted line), exhibit intensive fluorescence in the same intensity range. In contrast, the free bilins or the PebA-BV complex show only very weak or no fluorescence (data not shown).

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TABLE 2

Absorbance and fluorescence spectroscopic properties of bilins and enzyme-bilin complexes

| Bilin/enzyme-bilin complex | $\lambda_{\text{max} 1}$ | $\lambda_{\text{max} 2}$ | $\lambda_{\text{max} 1}/\lambda_{\text{max} 2}$ | $\lambda_{\text{max} \text{em}}^{\text{a}}$ |
|---------------------------|----------------------|----------------------|----------------------|----------------------|
| BV                        | 382                   | 618                   | 0.30                  | 581                   |
| PebA-BV                   | 382                   | 690                   | 0.52                  | 581                   |
| PebB-BV                   | 382                   | 706                   | 0.40                  | 581                   |
| DHBV                      | 335                   | 565                   | 0.63                  | 581                   |
| PebA-DHBV                 | 335                   | 590                   | 0.97                  | 581                   |
| PebB-DHBV                 | 342                   | 605                   | 1.36                  | 581                   |
| 3E-PEB                    | 370                   | 535                   | 0.64                  | 581                   |
| PebA-3E-PEB               | 372                   | 586                   | 0.86                  | 581                   |
| PebB-3E-PEB               | 372                   | 545                   | 0.63                  | 581                   |

$^{\text{a}}\lambda_{\text{max} \text{em}}$ emission wavelength.

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FIGURE 5. Spectroscopic properties of bilins and enzyme-bilin complexes. Enzyme-bilin complexes were formed by incubating 8 $\mu$M FDBR with 4 $\mu$M bilin for 20 min on ice. A, absorbance spectra of BV (solid line), PebA-BV (dashed line), and PebB-BV (dashed-dotted line). B, absorbance spectra of DHBV (solid line), PebA-DHBV (dashed line), and PebB-DHBV (dashed-dotted line). C, absorbance spectra of 3E-PEB (solid line), PebA-3E-PEB (dashed line), and PebB-3E-PEB (dashed-dotted line). Formation of enzyme-bilin complexes caused intensive changes in absorbance properties of the bilins. The shoulder at ~430 nm in the DHBV spectra (B) is possibly an indication of non-enzymatic rubin-like degradation products.

and Table 2). The binding of 3E-PEB to PebB led to an increased absorbance at the long wavelength absorbance maximum of 535 nm, and shifted the absorbance maximum to 545 nm. Binding to PebA shifted the absorbance maximum to 586 nm (Fig. 5C and Table 2).

Fluorescent Properties of FDBR-Bilin Complexes—During the bilin binding experiments with PebA and PebB, we observed that the intermediate enzyme-bilin complexes in PEB biosynthesis are fluorescent, although their attachment to the protein is not covalent. Excitation at their $\lambda_{\text{max} 2}$ absorbance maxima led to intense fluorescence emission, and the respective excitation/emission pairs were determined to be 590/610 nm for PebA-DHBV, 605/645 nm for PebB-DHBV, and 545/630 nm for PebB-3E-PEB (Fig. 6).
same range as the substrate binding to PebA. The $K_d$ for binding of PebB to the product $3E$-PEB was determined to be about 5-fold higher ($K_d = 5.8 \mu M$).

Transfer of DHBV Intermediate from PebA to PebB—The binding of DHBV to PebA was tight enough to enable the purification of a preformed complex by passing it through a NAP\textsuperscript{TM} 5 column. Because of the high affinity of the Sephadex G25 material toward the free bilins, we were able to generate enzyme solutions that contained only marginal concentrations of unbound bilin. The purified complexes were subjected to gel permeation chromatography analysis, and we are able to detect protein-specific absorbance as well as specific absorbance for the PebA-DHBV complex (Fig. 8A). All analyzed FDBRs were determined to elute as monomers under the employed conditions, with a relative molecular weight of about 30,000. In contrast, the GST fusion protein of PebB eluted as a dimer of about 115,000 (Fig. 8B). The latter result is not surprising because GST is known to form dimers (18). The spectroscopic properties of the GST fusion proteins do not differ from those of GST-free protein (data not shown), indicating that the N-terminal GST fusion does not alter the substrate binding pocket that is structurally located between the central $\beta$-sheet and the C-terminal $\alpha$-helices (7).

Gel permeation chromatography analysis of a molar 1:1 mixture of the purified PebA-DHBV complex and the GST-PebB fusion revealed approximately equal quantities of DHBV bound to PebA and PebB (Fig. 8C).

DISCUSSION

PebA and PebB Belong to the FDBR Family of Radical Enzymes—In this current work we have presented the first detailed biochemical characterization of the two enzymes involved in PEB biosynthesis. They belong to the newly defined family of FDBRs. The best described member of this family is PcyA, which catalyzes the four-electron reduction of BV IX to PCB. Because of the lack of bound metal cofactors and the detection of a bilin radical intermediate, the family of FDBR constitutes a novel family of radical enzymes (6). Although we have not presented any EPR data, PebA lacks metal ion cofactors, and the reaction most likely proceeds via radical intermediates. Interestingly, we observed that DHBV bound to PebA can be reoxidized to BV by molecular oxygen. Reactive oxygen species such as peroxyl radicals are known to reoxidize albumin bound bilirubin (BR) to BV (19). Although it seems unlikely that reactive oxygen species are produced under our experimental conditions, reactive oxygen species or molecular oxygen itself could serve as the oxidant. These observations fit the results in which a higher yield of a recombinant, in vivo produced PEB adduct of phytochrome (Cph1) was achieved under low aeration as reported recently (20). In this regard, PcyA has been described to reduce BV much more efficiently under anaerobic conditions (6). However, in our assay system removal of oxygen did not significantly enhance the rate of BV
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reduction, but the intermediate DHBV is more stable under anaerobic conditions (data not shown).

All Members of the FDBR Family Have Distinct Substrate Specificities—All tested bilins that occur in one organism cannot be converted by the other bilin reductases. But we find that PebA can convert PFB to PEB indicating that an A-ring ethyl group instead of a vinyl group is not critical for substrate recognition by PebA (Table 1). Because we used a mixture of the 3E- and 3Z-isomer, of PFB we cannot precisely claim which isomer is a substrate for PebA. Results from PcA would infer that only the 3Z-isomer can be turned over. This is an interesting result insofar as PFB had originally been reported to be an intermediate in PEB biosynthesis in Cyanidium caldarium (21). The inability of PcA to convert DHBV and of PebB to convert 181,182-DHBV is a result of the changed planarity in DHBV. These high substrate specificities of the FDBRs demonstrate that bilin biosynthetic pathways are evolutionary designed to enable strict regulation and to avoid cross-reactions between different bilins occurring in the same organism.

PebA and PebB Form Distinct Complexes—Binding of substrate or product to the enzymes leads in all cases to a red shift of the $\lambda_{max}$. Red shifts like that were reported previously to occur upon protonation of the basic pyrrolic nitrogen atom within the tetrapyrrole ring structure (22, 23) or as a result of an enhanced conjugated $\pi$-electron system of the bilin (23, 24). Because protonation is presumably the first step in the catalytic bilin reduction by PcA (6), it is quite possible that during enzyme binding the positioning of the bilin close to a proton donating amino acid or the coordination by an electropositive ligand causes these red shifts. Interestingly, DHBV in acidic methanol displays a rather blue-shifted spectrum (data not shown). Therefore, the observed spectral effects cannot easily be assigned as due to protonation or geometrical changes. The massive changes in the $\lambda_{max}$/$\lambda_{max}$ ratio that occur during binding of DHBV to PebA or PebB suggest a more linearly stretched conformation of the DHBV bound to the enzyme compared with free DHBV (22). This fact is interesting in regard to the conformation of BV in the PcA crystal structure, which is cyclic (7). The spectroscopic differences between PebA-DHBV and PebB-DHBV indicate that the protein environment, possibly the conformation or orientation of the tetrapyrrole, is different in both complexes.

Fluorescent Properties of PebA-Bilin and PebB-Bilin Complexes—Free bilins exhibit very low fluorescence, but it has long been known that the covalent adducts of bilins to proteins lead to fluorescent proteins with high fluorescence quantum yields (25, 26). Because of their fluorescent properties, phycobiliproteins, especially PEs, are frequently used for fluorescence labeling (25, 26). Because of their fluorescent properties, phycobiliproteins, especially PEs, are frequently used for fluorescence labeling (25, 26). Because of their fluorescent properties, phycobiliproteins, especially PEs, are frequently used for fluorescence labeling (25, 26). Because of their fluorescent properties, phycobiliproteins, especially PEs, are frequently used for fluorescence labeling (25, 26). Because of their fluorescent properties, phycobiliproteins, especially PEs, are frequently used for fluorescence labeling (25, 26).

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