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Phycocyanobilin:Ferredoxin Oxidoreductase of *Anabaena* sp. PCC 7120

BIOCHEMICAL AND SPECTROSCOPIC CHARACTERIZATION*

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In cyanobacteria, the biosynthesis of the phycobiliprotein and phytochrome chromophore precursor phycocyanobilin is catalyzed by the ferredoxin-dependent enzyme phycocyanobilin:ferredoxin oxidoreductase (PcyA), which mediates an atypical four-electron reduction of biliverdin IXα. Here we describe the expression, affinity purification, and biochemical characterization of recombinant PcyA from *Anabaena* sp. PCC 7120. A monomeric protein with a native M_r of 30,400 ± 5,000, recombinant PcyA forms a tight and stable stoichiometric complex with its substrate biliverdin IXα. The enzyme exhibits a strong preference for plant type [2Fe-2S] ferredoxins; however, flavodoxin can also serve as an electron donor. HPLC analyses establish that catalysis proceeds via the two electron-reduced intermediate 18^1,18^2-dihydrobiliverdin, indicating that exovinyl reduction precedes A-ring (endovinyl) reduction. Substrate specificity studies indicate that the arrangement of the A- and D-ring substituents alters the positioning of the bilin substrate within the enzyme, profoundly influencing the course of catalysis. Based on these observations and the apparent lack of a metal or small molecule cofactor, a radical mechanism for biliverdin IXα reduction by phycocyanobilin:ferredoxin oxidoreductase is envisaged.

Phycocyanobilin (PCB)[1] is a linear tetrapyrrole (bilin) found in cyanobacteria, algae, and cryptomonads that functions as the direct precursor of the chromophores of the light-harvesting phycobiliproteins and cyanobacterial/algal phytochromes (1, 2). The PCB biosynthetic pathway shares common intermediates with those of heme and chlorophyll to the level of protoporphyrin IX, whereupon the pathways diverge upon metalation with either iron or magnesium (1). All phycobilins share the common intermediacy of biliverdin IXα (BV IXα), which is derived from heme (1, 3). BV IXα is the product of heme oxygenase-mediated cleavage of the heme macrocycle, which yields equimolar quantities of BV IXα, CO, and iron. The metabolic fate of BV IXα differs in mammals, cyanobacteria, and plants, where BV IXα is metabolized by different reductases with unique double bond specificities. In contrast with the mammalian NAD(P)H-dependent BV IXα reductase, cyanobacteria and red algae possess ferredoxin-dependent bilin reductases primarily for the synthesis of the linear tetrapyrrole precursors of their phycobiliprotein light-harvesting antennae complexes, whereas evolutionarily related ferredoxin-dependent bilin reductases are found in higher plants for the synthesis of the phytochrome chromophore precursor phytochromobilin (PΦB) (3, 4).

We recently documented that *pcyA* genes from cyanobacteria and oxyphotobacteria encode bilin reductases, which catalyze the ferredoxin-dependent reduction of BV IXα to (3*Z*)-PCB, the bilin precursor of their phycobiliprotein and phytochrome chromophores. Designated phycocyanobilin:ferredoxin oxidoreductases (EC 1.3.7.5), PcyA enzymes are atypical bilin reductases, because they catalyze a four-electron reduction; all others catalyze two-electron reductions (4). Formally two-electron reductions of vinyl substituents on the pyrrole A- and D-rings of BV IXα, the sequence of reductions mediated by PcyA, is presently unknown (see Fig. 1). Neither of the two likely dihydrobiliverdin (DHBV) intermediates, (3*Z*)-PΦB or 18^1,18^2-DHBV IXα, have yet been detected (4). Since (3*Z*)-PΦB is an intermediate in the biosynthesis of PCB, the precursor of the phytochrome chromophore in the green alga *Mesotaenium caldariorum* (5), its intermediacy in the PcyA-mediated biosynthesis of PCB in cyanobacteria is therefore a reasonable possibility. Previous studies have shown that the biosynthesis of PCB in the red alga *Cyanidium caldarium* proceeds via phycoerythrobilin as an intermediate (Fig. 1) (1). The enzyme(s) that catalyze the conversion of phycoerythrobilin to PCB have not yet been identified; hence, it is conceivable that PcyA might also mediate this conversion.

This study was undertaken to characterize the biochemical properties of a representative PcyA enzyme from the filamentous cyanobacterium *Anabaena* sp. PCC 7120. The specific objectives of these experiments were to identify the semireduced intermediate produced during the catalysis of BV and to probe the bilin substrate specificity of this unusual ferredoxin-dependent four-electron reductase. Based on these investigations, a chemical mechanism for PcyA-mediated bilin reduction is proposed.

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise specified, all chemical reagents were American Chemical Society grade or better. Glutathione-agarose, spinach ferredoxin, *Clostridium pasteurianum* ferredoxin, ferredoxin:NADP$^+$ oxidoreductase (FNR), and size exclusion molecular weight markers (MW-GF-200) were purchased from Sigma. Restriction enzymes and *Taq* polymerase were obtained from Invitrogen. HPLC grade

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[1] The abbreviations used are: PCB, phycocyanobilin; PΦB, phytochromobilin; BR, bilirubin; BV, biliverdin; Cph1, cyanobacterial phytochrome 1; DHBV, dihydrobiliverdin; Fd, ferredoxin; Fldx, flavodoxin; FNR, ferredoxin-NADP$^+$-oxidoreductase; PcyA, phycocyanobilin:ferredoxin oxidoreductase; HPLC, high pressure liquid chromatography; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

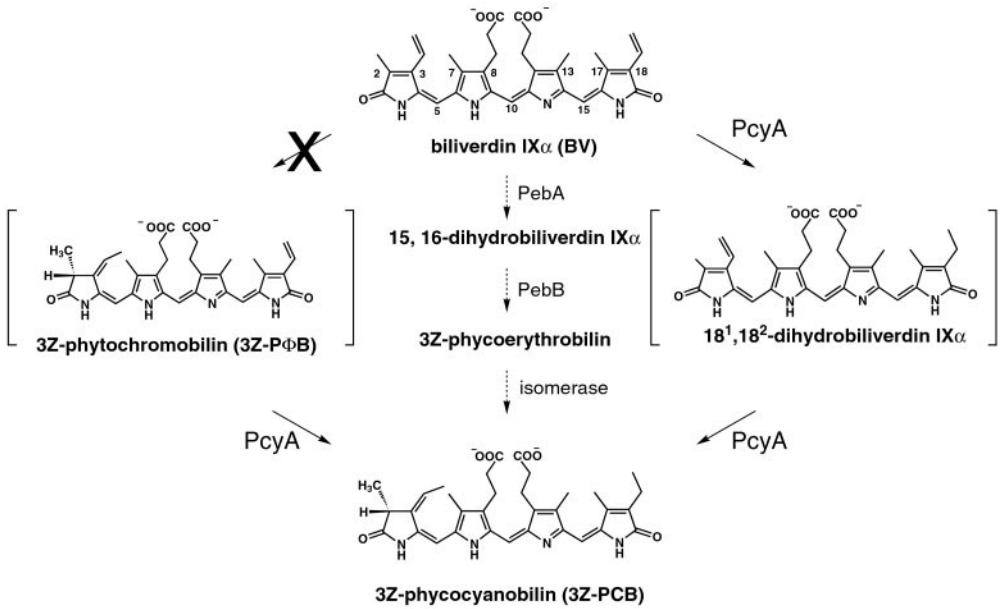

FIG. 1. **The reduction of biliverdin IXα by PcyA can proceed via two possible intermediates.** PcyA-mediated reduction of BV IXα could result in the formation of (3*Z*)-PΦB or 18^1,18^2-dihydrobiliverdin IXα as an intermediate. Results presented here support the intermediacy of 18^1,18^2-dihydrobiliverdin IXα; however, (3*Z*)-PΦB is also metabolized by PcyA (see "Results" for details). An alternative pathway for the formation of PCB in the red alga *C. caldarium* via the intermediacy of 15,16-dihydrobiliverdin IXα and phycoerythrobilin is also shown in the center. This conversion is catalyzed by the bilin reductases, PebA and PebB, and an as yet unidentified isomerase (1, 4).

acetone, chloroform, and 80% formic acid were purchased from Fisher. The expression vector pGEX-6P-1 and PreScission™ protease were obtained from Amersham Biosciences. Centricon-10 concentrator devices were purchased from Amicon (Beverly, MA).

Bilin Preparations—BV IXα, BV XIIIα, BV IIIα, PCB, and PΦB preparations used as substrate and/or HPLC standards were obtained as described previously (6, 7). 18^1,18^2-DHBV IXα was synthesized by acid scrambling of a mixture of meso-BR IIIα and BR XIIIα followed by oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (8). Meso-BR IIIα was kindly provided by Dr. D. A. Lightner (University of Nevada, Reno, NV). BR XIIIα was prepared by acid scrambling of commercially obtained BR (7, 9).

Expression and Purification of PcyA—*Anabaena* sp. PCC 7120 *pcyA* was cloned into the *Escherichia coli* expression vector pGEX-6-P1 (Amersham Biosciences) to produce pGEX*pcyA* (4). *E. coli* strain DH5α containing pGEX*pcyA* was induced to express glutathione *S*-transferase-PcyA, which was purified according to instructions supplied by the manufacturer and protocols described earlier (10). Proteolytic cleavage with the PreScission™ protease yielded the native protein with the N-terminal amino acid extension GPLGSPEF and with the initiator methionine residue changed to isoleucine. Purified PcyA protein concentration was estimated from the absorbance at 280 nm using the calculated $\epsilon_{280\,nm}$ of 29,726 M^{-1} cm^{-1} (11).

Purification of Recombinant Reductants—*Synechococcus* sp. PCC 7002 ferredoxin and flavodoxin clones, obtained from Dr. D. A. Bryant, were expressed and purified as described previously (12, 13). Expression and purification of putidaredoxin and putidaredoxin reductase, whose clones were kindly provided by Dr. Paul Ortiz de Montellano (University of California, San Francisco), were performed as described.[2] Flavodoxin was quantified by absorption at 467 nm and an absorption coefficient of $\epsilon_{467\,nm}$ 9,500 M^{-1} cm^{-1} (13), whereas putidaredoxin and putidaredoxin reductase were quantified by absorption at 454/415 nm, respectively, using the absorption coefficients of $\epsilon_{454\,nm}$ 10,000 M^{-1} cm^{-1} and $\epsilon_{415\,nm}$ 11,100 M^{-1} cm^{-1}.[2]

Standard Bilin Reductase Activity Assay—Assays for bilin reductase activity were performed as described previously (10, 14). Standard assays contained 1.5 μM PcyA, 4.8 μM ferredoxin, and 5 μM BV IXα in 25 mM TES-KOH, pH 7.5 (assay buffer), and were incubated for 30 min at 28 °C under green safe light unless otherwise specified. Following catalysis, bilins were isolated using a C18 Sep-Pak column (Waters) and evaporated to dryness *in vacuo* (4).

Direct HPLC Analysis—Bilin reaction products were dissolved in 10 μl of Me_2SO and diluted with 200 μl of the HPLC mobile phase. Following brief centrifugation and filtration through a 0.45-μm polytetrafluoroethylene syringe filter, bilins were resolved by reversed phase chromatography using an Agilent Technologies 1100 Liquid Chromatograph. The HPLC column used for all of the analyses was a 4.6 × 250-mm Phenomenex Ultracarb 5-μm ODS(20) analytical column with a 4.6 × 30-mm guard column of the same material. The mobile phase consisted of acetone, 20 mM formic acid (50:50 by volume), and the flow rate was 0.6 ml/min. Eluates were monitored at 650, 560, and 380 nm using an Agilent Technologies 1100 series diode array detector. As needed, complete spectra were obtained for the peaks desired. Peak areas were quantitated using Agilent Technologies Chemstation software.

Size Exclusion Chromatography—An Amersham Biosciences Superdex 200 HR10/30 size exclusion column was equilibrated in 50 mM TES-KOH buffer, pH 7.5, containing 100 mM KCl and 10% (v/v) glycerol (size exclusion chromatography buffer) at a flow rate of 0.4 ml/min. Standards with known M_r (*i.e.* β-amylase, 200,000; alcohol dehydrogenase, 150,000; bovine serum albumin, 66,000; carbonic anhydrase, 29,000; cytochrome *c*, 12,600) were applied to the column (100 μg), and their elution volumes were determined spectroscopically. *Anabaena* sp. PcyA, PcyA/BV (1:1, mol/mol), Fd/PcyA (2:1, mol/mol), and Fd/PcyA/BV (2:1:1, mol/mol/mol) were chromatographed under identical conditions.

Glycerol Gradient Centrifugation—PcyA preparations (40 μg) were sedimented through a 2.5-ml continuous 10–25% glycerol gradient in size exclusion chromatography buffer. A detailed experimental procedure described previously was used for sedimentation coefficient determination (15).

Spectroscopic Analysis of Biliverdin Binding—Increasing amounts of PcyA were added to 5 μM (final concentration) BV IXα, BV XIIIα, or BV IIIα solutions in a final volume of 500 μl of 25 mM TES-KOH, pH 7.5, buffer under green safe light. After incubation for 30 min at room temperature, absorbance spectra were recorded using an HP 8453 spectrophotometer. Normalization of the spectra and spectral deconvolution were performed using Microsoft Excel. To obtain bilin-PcyA dissociation constants, absorbance differences (ΔA) at the λ_{max} of each bilin-PcyA complex were plotted as a function of PcyA concentration. Dissociation constants were obtained by fitting this data to the hyperbolic equation $\Delta A = \Delta A_{max} \times [PcyA]/(K_{app} + [PcyA])$ using DeltaGraph® Pro version 3.5 (DeltaPoint, Monterey, CA), where $K_d = K_{app} - 2.5$ μM.

RESULTS

Expression and Purification of Recombinant Phycocyanobilin:Ferredoxin Oxidoreductase—The *Anabaena* sp. PCC 7120 *pcyA* gene was expressed using a *tac* promoter-driven N-terminal glutathione *S*-transferase fusion expression system. Re-

[2] C. Nishida and P. Ortiz de Montellano, personal communication.

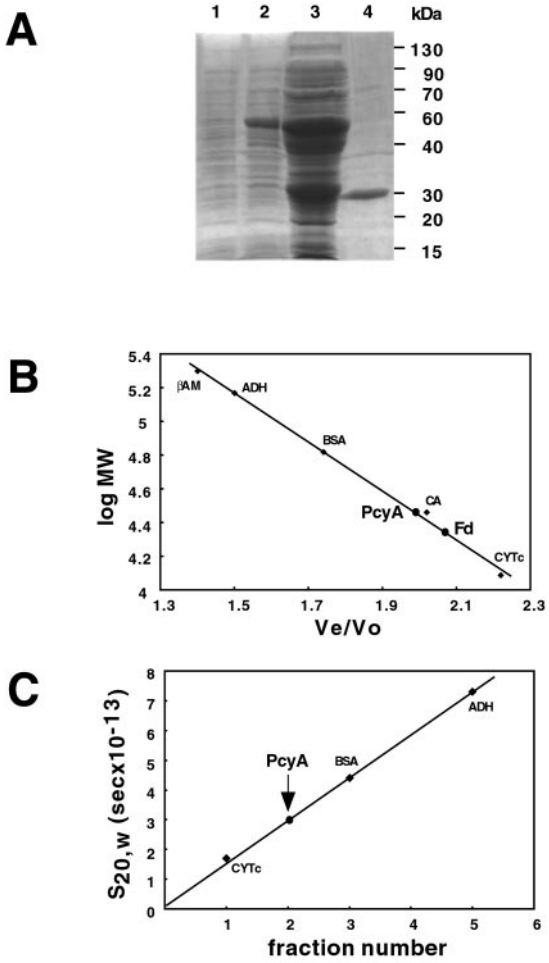

FIG. 2. **Affinity purification of recombinant PcyA and determination of the molecular mass.** *A*, SDS-PAGE analysis of whole cell protein extracts before (*lane 1*) and after (*lane 2*) induction with isopropyl-1-thio-β-D-galactopyranoside. *Lane 3*, the soluble fraction after induction; *lane 4*, recombinant PcyA after on-column cleavage and elution from glutathione-agarose. The *numbers* on the *right* indicate positions of molecular weight markers. *B*, size exclusion chromatography using a Superdex 200 HR10/30 column that had been calibrated with the following marker proteins: β-amylase (*βAM*; 200 kDa), alcohol dehydrogenase (*ADH*; 150 kDa), bovine serum albumin (*BSA*; 66 kDa), carbonic anhydrase (*CA*; 29 kDa), and cytochrome *c* (*CYTc*; 12.4 kDa). The elution positions of PcyA and ferredoxin are shown. *C*, molecular mass determination by glycerol gradient sedimentation. Sedimentation positions of marker protein are plotted against known sedimentation coefficients. The sedimentation position of PcyA is indicated with an *arrow*.

combinant PcyA, obtained by "on-column" proteolytic cleavage of the glutathione *S*-transferase fusion protein, was purified to ~90% homogeneity as shown in Fig. 2*A*. On-column cleavage was preferable to "in-solution" proteolysis of the glutathione *S*-transferase-PcyA fusion protein, which led to extensive protein precipitation and poor protein recovery. One-liter bacterial cultures typically yielded 3 mg of on-column cleaved PcyA. All results presented here correspond to PcyA. However, glutathione *S*-transferase-PcyA fusion protein preparations showed nearly identical catalytic properties (data not shown).

Determination of the Native Molecular Mass of PcyA—The native molecular mass of PcyA was determined using size exclusion chromatography and glycerol gradient sedimentation (Fig. 2, *B* and *C*). A relative molecular weight of 30,400 ± 5,000 for PcyA was deduced with both methods, which is in good agreement with the calculated molecular mass of 28,726 daltons. Thus, recombinant PcyA appears to be a monomeric enzyme. In order to determine whether PcyA can form a stable

TABLE I
Reductant dependency of Anabaena sp. PcyA

Reductant	Concentration	PcyA activity[a]
	μM	%
Spinach [2Fe-2S] ferredoxin	4.8	100
Synechococcus [2Fe-2S] ferredoxin	4.8	100
Synechococcus flavodoxin	3	13.2 (31.5)[b]
Clostridium [4Fe-4S] ferredoxin	4.8	1.9 (13.4)[b]
P. putida putidaredoxin (+ putidaredoxin reductase)	170 (70)	0.8

[a] PcyA assays were performed as described under "Experimental Procedures" for 20 min using the HPLC method. Since the use of spinach ferredoxin represents standard assay conditions, this activity was set to 100% based on the production of PCB (peak absorbance area).
[b] 13.2% represents the percentage of PCB produced; the value in parenthesis includes the amount of produced intermediate.

complex with spinach ferredoxin, PcyA was incubated with a 2-fold molar excess of spinach Fd and evaluated by both methods. Higher order complex formation between PcyA and Fd was not observed with either method under the conditions examined; nor did the addition of a 2-fold molar excess of BV IXα influence the result (data not shown).

PcyA Lacks Metal or Small Molecule Cofactors—Purified recombinant PcyA was analyzed using absorption spectroscopy for the presence of light-absorbing cofactors such as hemes, flavins, iron-sulfur clusters, *etc*. Spectroscopic evidence for any of these cofactors was not obtained for PcyA at concentrations as high as 5 mg/ml. In order to understand whether solvent-accessible metal ions are critical for activity (directly or indirectly as structural components), purified PcyA was incubated with the metal chelators EDTA (10 mM), 1,10-phenanthroline, and 2,2′-dipyridyl (5 mM each). After removal of the chelator from the protein by passing the mixture through a G-25 desalting column (Amersham Biosciences), enzyme activity was determined. None of the chelators had any inhibitory effect on the activity of PcyA (data not shown).

Reductant Specificity of PcyA—The enzymes that mediate the reductive conversion of BV IXα to phycobilins are all dependent on plant type [2Fe-2S] ferredoxins (4, 14). For this reason, all PcyA assays were performed in the presence of saturating levels of spinach ferredoxin and the ferredoxin-reducing system, consisting of spinach FNR and NADPH. Omission of any of these components led to no PcyA activity (data not shown) (4). To test whether other reductants can serve as electron donors to PcyA, we tested recombinant *Synechococcus* sp. PCC 7002 ferredoxin and flavodoxin also in combination with spinach FNR. Other reductants tested included *C. pasteurianum* ferredoxin, a 2[4Fe-4S] ferredoxin, and a putidaredoxin/putidaredoxin reductase system from *Pseudomonas putida*. As shown in Table I, maximum PcyA activity was obtained with plant type ferredoxin either from spinach or *Synechococcus*, whereas the FMN-containing redox protein flavodoxin less effectively supported PcyA catalysis. *C. pasteurianum* ferredoxin and the putidaredoxin/putidaredoxin reductase system from *P. putida* were considerably less active.

Bilin Binding to PcyA—Bilin binding experiments demonstrated that PcyA forms a complex with its bilin substrate that is stable through ultrafiltration, size exclusion chromatography, and dialysis. Spectrophotometric titration experiments with its natural substrate BV IXα and the two analogs, BV XIIIα and BV IIIα, are shown in Fig. 3*A*. Upon binding to PcyA, significant blue shifts of the long wavelength absorption maxima were detected for all of the bilin analogs along with an increase in molar absorption coefficient and the appearance of a shoulder at longer wavelengths. The appearance of the shoul-

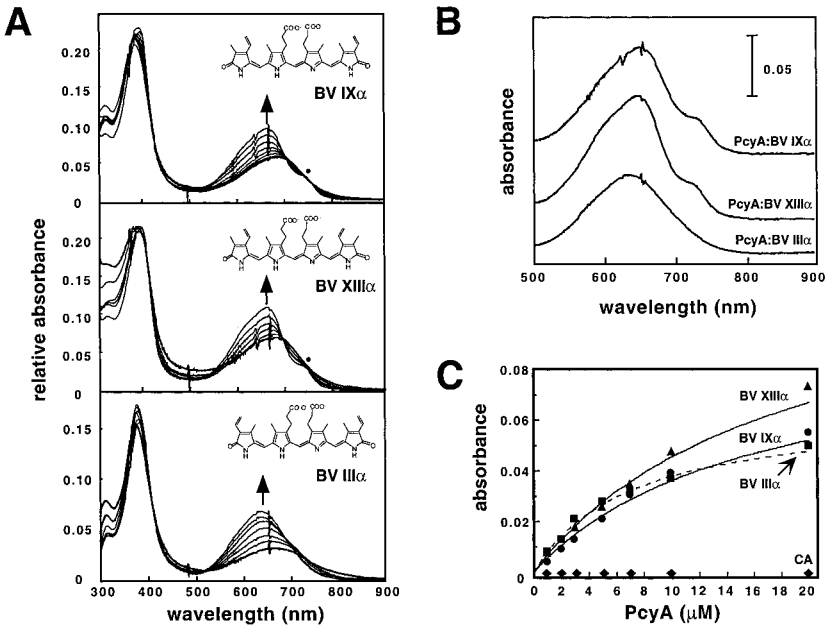

FIG. 3. **Binding of PcyA to BV measured by absorbance spectroscopy.** *A*, equilibrium binding experiments of BV IXα (*top panel*), BV XIIIα (*middle panel*), and BV IIIα (*bottom panel*) with increasing amounts of PcyA were performed as described under "Experimental Procedures." The direction of the spectral changes with increasing PcyA concentrations is indicated by *arrows*. For the IXα and XIIIα isomers, PcyA-BV complex formation resulted in a shoulder at longer wavelength (indicated by a *black dot*). *B*, long wavelength absorption spectra of 5 μM PcyA-BV complexes at saturating levels of PcyA (*i.e.* 20 μM PcyA). *C*, absorbance changes at the wavelength maxima of the PcyA-BV complexes (λ_{max}) were plotted as a function of PcyA concentration. Peak positions were 655 nm (BV IXα), 640 nm (BV IIIα), and 660 nm (BV XIIIα). A control experiment using bovine carbonic anhydrase instead of PcyA is shown. All data were fitted to a hyperbolic equation as described under "Experimental Procedures." R^2 values were determined to be 0.995, 0.996, and 0.992 for the IIIα, IXα, and XIIIα isomers, respectively.

der was evident only for BV IXα and BV XIIIα, but not BV IIIα (Fig. 3*B*), a result that was consistent with the ability of PcyA to metabolize these two bilins (see below). Fig. 3*C* depicts replots of these absorption changes as a function of increasing PcyA concentration. From hyperbolic curve fitting of this data, the equilibrium dissociation constants were estimated to be 12.5, 14.5, and 4.5 μM for the respective IXα, XIIIα, and IIIα isomers under these experimental conditions. As a control, BV IXα binding experiments were also performed using bovine carbonic anhydrase from the Sigma MW-GF-200 molecular weight kit. No spectral changes were observed with increasing carbonic anhydrase concentration, indicating that the altered spectra reflected the formation of bilin-PcyA complexes (Fig. 3*C*).

The PcyA Two-electron Reduced Intermediate—The conversion of BV to PCB is a four-electron reduction that formally consists of sequential two-electron reductions with the intermediacy of a DHBV. As shown in Fig. 1, the most likely candidates for this intermediate are (3*Z*)-PΦB or 18^1,18^2-DHBV IXα, in which initial reduction occurs at the A- or D-rings of BV, respectively. To identify the putative DHBV intermediate, time course experiments were performed. HPLC analyses revealed the transient appearance of a new pigment during the course of catalysis (Fig. 4 and Table II). This new pigment, which eluted earlier than (3*Z*)-PCB (labeled *I* in Fig. 4*A*), reached a maximum level within 10 min and disappeared after 30 min (Fig. 4*B*). Although pigment I eluted at the same retention time as (3*E*)-PΦB, its absorption spectrum differed from that of (3*E*)-PΦB (Table II). The time course of the disappearance of BV IXα, the appearance/disappearance of pigment I, and the appearance of the two isomers of PCB, shown in Fig. 4*B*, supports the intermediacy of pigment I in the PcyA-mediated conversion of BV IXα to PCB.

To verify that pigment I was a bona fide intermediate in the formation of PCB, it was collected and tested for its ability to bind to apo-Cph1 using a coupled phytochrome assembly assay (10, 14) and for its ability to be further metabolized by PcyA to PCB using HPLC. As shown in Fig. 4*C*, isolated pigment I failed to produce a photoactive phytochrome upon incubation with apo-Cph1, indicating that pigment I was not (3*E*)-PΦB or (3*Z*)/(3*E*)-PCB. Further incubation of pigment I with PcyA yielded products that could assemble with apo-Cph1 to produce a photoactive bilin adduct. Fig. 4*C* shows that the phytochrome difference spectrum of this adduct was identical to that of the authentic PCB adduct, both of which were blue-shifted from that of the Cph1:PΦB adduct. Together with the ability of PcyA to convert pigment I to a mixture of pigments that co-elute with (3*Z*)- and (3*E*)-PCB (data not shown), these studies support the conclusion that pigment I is a *bona fide* intermediate in the PcyA-mediated conversion of BV to PCB.

18^1,18^2-DHBV Is the Intermediate in the PcyA-mediated Reduction of BV—Since the intermediate failed to form a photoactive bilin-adduct with apo-Cph1 but had the same retention time as (3*E*)-PΦB, we tested both (3*E*)- and (3*Z*)-PΦB as substrates for PcyA. These studies showed that (3*Z*)-PΦB, but not (3*E*)-PΦB, was metabolized by PcyA. (3*Z*)-PΦB was converted to a mixture of (3*Z*)- and (3*E*)-isomers of PCB, a result that was confirmed by assembly with apo-Cph1 (data not shown). Since (3*Z*)-PΦB elutes at a different retention time from the intermediate on the HPLC (Fig. 4*A*, Table II), these studies confirm that neither PΦB isomer is the semireduced intermediate in the PcyA-mediated reduction of BV. According to Fig. 1, the other likely intermediate is 18^1,18^2-DHBV IXα. For this reason, 18^1,18^2-DHBV IXα was synthesized by acid scrambling of BR XIIIα and mBRIIIα followed by oxidation as described under "Experimental Procedures." Fig. 4*A* shows that 18^1,18^2-DHBV IXα elutes at the same retention time as pigment I. These studies also show that PcyA converts 18^1,18^2-DHBV IXα to a mixture of (3*E*)- and (3*Z*)-PCB, thereby confirming the

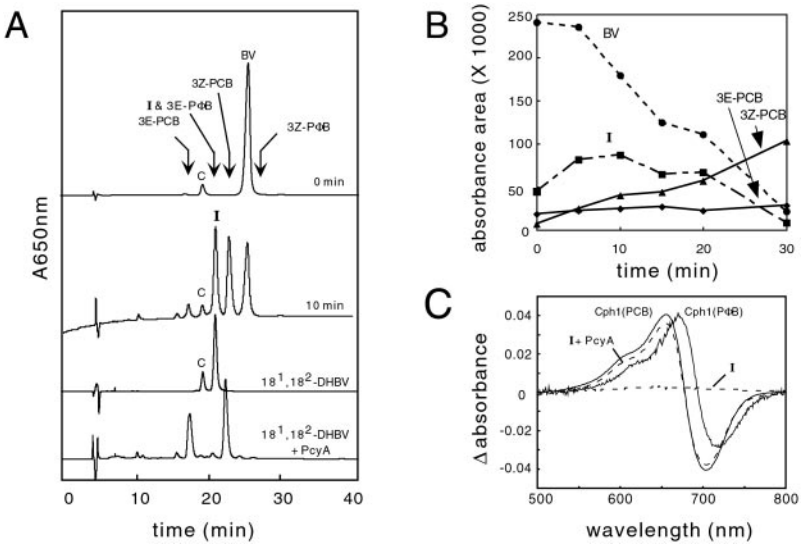

FIG. 4. **Identification of an intermediate in the PcyA-catalyzed reaction.** *A*, HPLC profiles of reaction products monitored at 650 nm were determined following the PcyA-mediated reduction of BV IXα for 0 and 10 min (*upper two profiles*). In addition to (3*Z*)- and (3*E*)-PCB products, an unknown pigment, labeled *I*, which co-elutes with the (3*E*)-PΦB standard, was detected. Synthetic 18^1,18^2-dihydrobiliverdin IXα co-elutes with pigment I and can be converted to a mixture of (3*Z*)-and (3*E*)-PCB (*two bottom elution profiles*). The peak labeled *C* corresponds to a contaminant. *B*, the course of the reaction is plotted as peak areas as a function of reaction time. ●, BV; ■, intermediate; ◆, (3*E*)-PCB; ▲, (3*Z*)-PCB. *C*, phytochrome difference spectra were obtained following incubation of apo-Cph1 with pigment I before or after metabolism with PcyA (labeled *I* and *I* + *PcyA*, respectively). Phytochrome difference spectra of PCB and PΦB adducts of apo-Cph1 (*i.e.* Cph1(PCB) and Cph1(PΦB)) are shown for comparison.

TABLE II
Reversed phase HPLC retention times and absorption spectra properties of bilin substrates and products

Absorption maxima in HPLC mobile phase buffer (acetone: 20 mM formic acid; 50:50; v/v) were determined with an Agilent Technologies 1100 Series diode array flow-through detector.

	Retention time[a]	λ_{max1}	λ_{max2}	Ratio ($\lambda_{max2}/\lambda_{max1}$)
	min	*nm*	*nm*	
BV IXα	23.2	376	668	0.35
BV XIIIα	18.3	374	656	0.46
BV IIIα	27.7	380	676	0.29
(3*Z*)-PΦB	24.1	372	646	0.4
(3*E*)-PΦB	19.0	380	654	0.5
Pigment I[b]	19.0	368	656	0.44
18^1,18^2-DHBV	19.0	368	656	0.45
(3*Z*)-iso-PΦB	19.3	368	638	0.52
(3*E*)-iso-PΦB	14.7	374	648	0.56
(3*Z*)-PCB	20.9	362	636	0.46
(3*E*)-PCB	15.5	368	650	0.53

[a] Retention times ± 0.5 min were determined using a C18 reversed phase HPLC system as described under "Experimental Procedures."
[b] Pigment I is the transient intermediate produced during metabolism of BV IXα by PcyA.

identity of the semireduced intermediate to be 18^1,18^2-DHBV IXα.

Bilin Substrate Specificity Studies—Since the substrate analogs BV XIIIα and BV IIIα bind to PcyA (see Fig. 3), their ability to be metabolized by PcyA was also examined. As shown in Fig. 5*A*, BV XIIIα could be metabolized by PcyA to yield two products. Based on the relative retention time of known bilins in our HPLC system and the absorbance spectra of the two products (Fig. 5*B*), we propose that BV XIIIα is converted by PcyA to the (3*E*)- and (3*Z*)-isomers of iso-PΦB (16). This hypothesis is also supported by the observation that both products yield identical difference spectra upon incubation with apo-Cph1 (Fig. 5*C*). By contrast with the other two BV isomers, BV IIIα was not metabolized by PcyA. This result is interesting in view of the observation that BV IIIα has the highest binding affinity for PcyA of the three BV isomers (Fig. 3*C*). The results of the bilin substrate specificity experiments are summarized in Table III.

DISCUSSION

PcyA Is a Monomeric Enzyme That Forms a Stable Porphyrin-like Complex with Bilins—Among the family of ferredoxin-dependent bilin reductases, PcyA is unique in its ability to catalyze the four-electron reduction of BV IXα (4). Like oat phytochromobilin synthase, a ferredoxin-dependent bilin reductase that converts BV IXα to PΦB in plants (14), PcyA is a monomeric enzyme. BV binding neither promoted PcyA dimerization nor oligomerization, suggesting that the distinct spectral properties of the three PcyA-BV complexes studied here reflect the unique protein environment and conformation of the bound bilin. The observed spectral features (*i.e.* long to short wavelength absorption ratio <1) indicate that bilins bind to PcyA in a cyclic, porphyrin-like configuration, as opposed to the more extended configurations found in phytochromes and phycobiliproteins (17). This cyclic configuration precludes simultaneous protonation of both B- and C-ring nitrogen atoms of the bilin prosthetic group due to steric crowding (see Fig. 6). This conclusion is further supported by the observed lack of fluorescence of the PcyA-BV complex as efficient proton transfer between hydrogen-bonded pyrrole rings would be expected to quench the excited state of the PcyA-BV complex.

Interestingly, a long wavelength shoulder was detected in the spectra of the PcyA complexes of BV IXα and BV XIIIα. Since this shoulder was not observed for BV IIIα, a nonmetabolized PcyA substrate analog, we speculate that this new absorption band corresponds to a distinct bilin-PcyA interaction, which reflects the ability of bilin to be reduced (*i.e.* hydrogen bonding, protonation, or aromatic π-π interaction).

PcyA Prefers Plant Type [2Fe-2S] Ferredoxins—Pioneering work by Beale and Cornejo (18) has established that reduction of BV in the rhodophyte *C. caldarium* is Fd-mediated. This result was later confirmed with the cloning of the bilin reductase family and the demonstration that all bilin reductases are Fd-dependent enzymes (4, 10). The present studies revealed that PcyA exhibits a preference for plant-type [2Fe-2S] Fds. Fldx, a two-electron acceptor that also can undergo two succes-

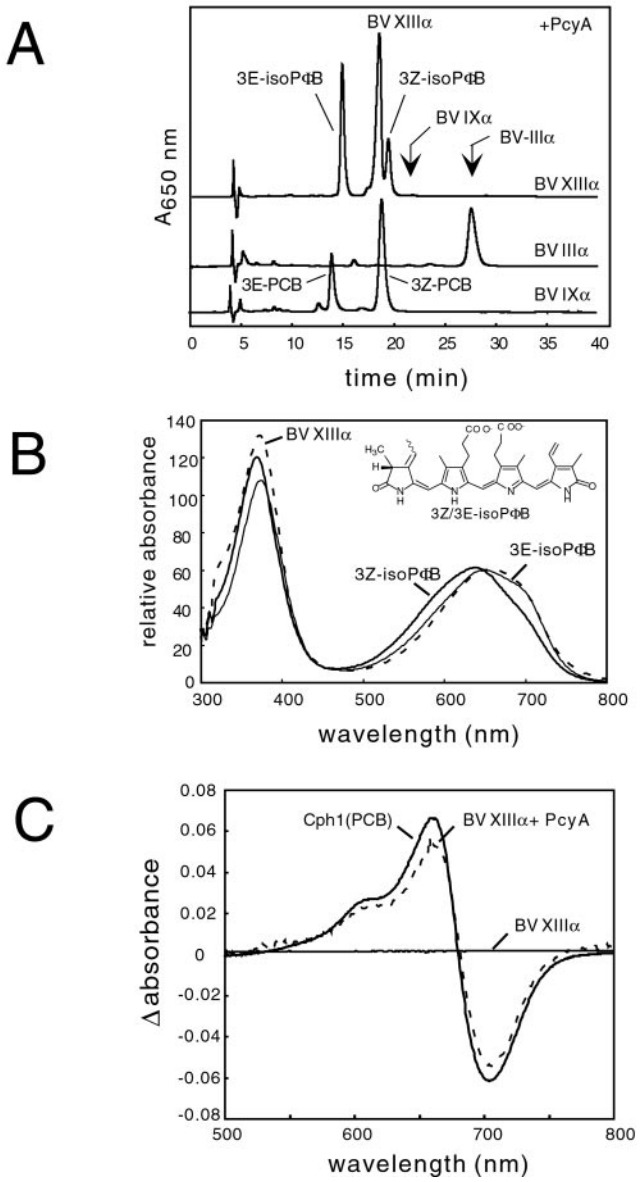

FIG. 5. **PcyA-mediated metabolism of BV-analogs.** *A*, HPLC product profiles for BV analogs after metabolism by PcyA for 30 min as described under "Experimental Procedures." Elution position of BV IXα, XIIIα, and IIIα are indicated by *arrows*. *B*, absorbance spectra of BV XIIIα (*dashed line*) and the two iso-PΦB reaction products (*solid lines*) are shown. *C*, phytochrome difference spectra assay following assembly of apo-Cph1 with BV XIIIα before (*solid line*) and after PcyA-mediated catalysis (*dashed line*) are shown. The difference spectra of the PCB adduct of apo-Cph1 (*i.e.* Cph1(PCB)) is shown for comparison.

sive low potential single electron reductions (*i.e.* −413 mV at pH 7, 25 °C) (19), also supported PcyA activity. Under the conditions tested here, the Fldx-dependent activity was ~13% of that of Fd. More recently, we have been able to increase the Fldx-mediated PcyA activity up to 50% of that of Fd by increasing the pH of the assay buffer to 8.5 and adding 100 mM KCl.[3] Based on these results, it is conceivable that Fldx can functionally substitute for Fd to drive PcyA activity under iron-limiting conditions *in vivo* (20).

The observation that the 2[4Fe-4S] ferredoxin from *C. pasteurianum* poorly supports PcyA-mediated BV reduction needs to be interpreted with the following caveats. The PcyA assay requires a spinach FNR-containing ferredoxin reducing system, which may be unable to effectively reduce the *C. pasteurianum* Fd, thereby limiting PcyA activity. Since spinach Fd and *C. pasteurianum* Fd have the same redox potential around −420 mV, *C. pasteurianum* Fd conceivably could support PcyA activity, assuming the right reducing system is present. In this regard, *C. pasteurianum* Fd serves as an electron acceptor in the anaerobic oxidation of pyruvate (21), the components of which may be able to drive *C. pasteurianum* Fd-dependent PcyA activity.

[3] S.-L. Tu, unpublished data.

TABLE III
Bilin substrate specificity of PcyA

Substrate	Product(s)
BV IXα	(3*E*)-/(3*Z*)-PCB
BV XIIIα	(3*E*)-/(3*Z*)-iso-PΦB[a]
BV IIIα	Not metabolized
Intermediate	(3*E*)-/(3*Z*)-PCB
18^1,18^2-DHBV	(3*E*)-/(3*Z*)-PCB
(3*Z*)-PΦB	(3*E*)-/(3*Z*)-PCB
(3*E*)-PΦB	Not metabolized

[a] The identity of this product was ascertained by analogy (relative retention times on the HPLC) and in comparison with published spectra (16).

The Endogenous Reductant for PcyA in E. coli May Be Flavodoxin—Recent studies reporting the assembly of holophytochrome and holophycobiliproteins in *E. coli* (22–25) indicate that PcyA can use naturally occurring reductants in living cells. *E. coli* cells possess several possible reductants. *E. coli* Fd is an adrenodoxin-type [2Fe-2S] ferredoxin, which genetic analyses have shown performs an essential role in the maturation of various iron-sulfur proteins (26). Indeed, *E. coli* Fd is more structurally related to the adrenodoxin-type ferredoxins (*i.e.* bovine adrenodoxin and *P. putida* putidaredoxin) than to plant-type Fds (27). As such, *E. coli* Fd probably functions as a component of the complex machinery responsible for the biogenesis of Fe-S clusters. Based on the observation that the PcyA-mediated catalysis is poorly supported by the putidaredoxin system (see Table I), we hypothesize that engineered PCB biosynthesis in *E. coli* uses a different reducing system. Other than this adrenodoxin-type ferredoxin, the *E. coli* genome possesses two Fldx genes and a flavorubredoxin gene (28). In light of the data presented here, we propose that the biosynthesis of PCB in *E. coli* is driven by one of the two Fldxs.

PcyA-mediated 18-Vinyl Reduction Precedes A-ring Reduction—The identification of 18^1,18^2-DHBV IXα as an intermediate in the conversion of BV to PCB has established that D-ring exovinyl reduction precedes A-ring reduction. PcyA is therefore composed of two separate activities mediated by a 18^1,18^2-DHBV:ferredoxin oxidoreductase and a PCB:ferredoxin oxidoreductase. This double bond specificity of PcyA presumably ensures that PΦBs are never produced in PcyA-containing cyanobacteria or red algae, the production of which might lead to misincorporation of PΦBs into their phycobiliproteins. We speculate that PΦB-containing phycobiliproteins would be more susceptible to photooxidative damage than the natural PCB-containing antennae of these organisms due to the presence of the reactive exovinyl group on the former. Evolution of PΦB-producing bilin reductases, such as HY2, would therefore prove a selective disadvantage to these organisms, a selection pressure that would not apply to terrestrial plants that lack phycobiliproteins. In this regard, it will be of interest to clone the genes for these enzymes from the green alga *M. caldariorum*, which mediate the conversion of BV to PCB via the intermediacy of PΦB (5).

Through examination of substrate analogs, which include the unnatural XIIIα and IIIα isomers of BV and the A-ring reduced phytochromobilin isomers, (3*Z*)- and (3*E*)-PΦB, our studies have provided insight into the catalytic specificity of

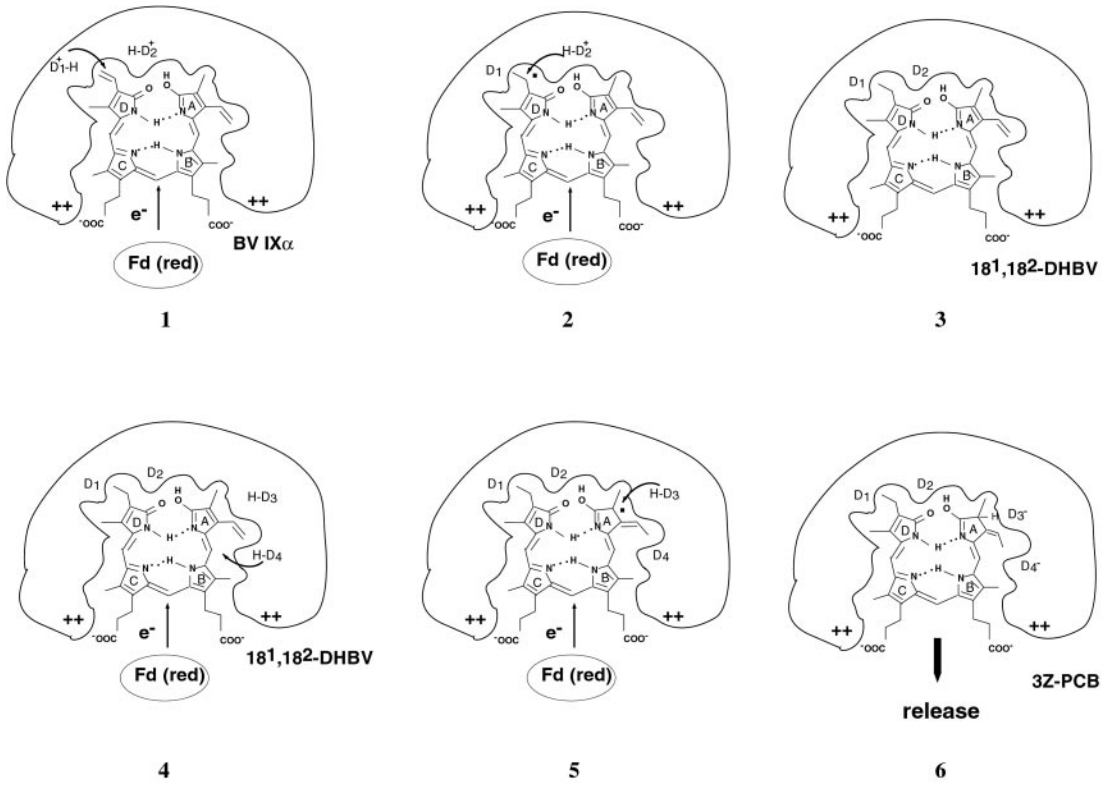

FIG. 6. **Proposed radical mechanism for the PcyA-catalyzed reduction of BV IXα.**

PcyA. Of the two unnatural BV analogs, only BV XIIIα was metabolized by PcyA, yielding the two-electron reduced iso-PΦB product (both (3*Z*)- and (3*E*)-isomers). Since BV XIIIα is symmetrical and lacks the exovinyl group found on BV IXα, this result indicated that PcyA-mediated A-ring reduction can occur in the absence of exovinyl reduction. The apparent lack of the reduction of the second endovinyl group of BV XIIIα also indicated that iso-PΦB is a poor PcyA substrate. By contrast to the IXα and XIIIα isomers, BV IIIα was not metabolized by PcyA. This result was unexpected, given the sequence of PcyA-mediated vinyl reductions of BV IXα. In this regard, BV IIIα is symmetrical, possessing two exovinyl groups, one or both of which should have been reduced by PcyA. Moreover, our results indicate that BV IIIα binds to PcyA with the highest affinity of the three BV isomers tested. These data indicate that bound BV IIIα is not properly oriented within the enzyme's bilin binding site for catalysis. Our studies show that (3*Z*)-PΦB can be metabolized by PcyA, yielding a mixture of PCB isomer products, whereas (3*E*)-PΦB is not a substrate for PcyA. These data indicate a strong influence of the geometry of the 3-ethylidene moiety on catalysis. Whether this is due to a positioning defect or to a lack of binding of (3*E*)-PΦB to PcyA remains to be determined. Taken together, these studies suggest that proper substrate positioning/activation within the enzyme is a prerequisite for catalysis.

A Radical Mechanism for Bilin Reduction by PcyA—Four major Fd-dependent enzymes have been characterized to date: FNR, Fd:nitrite oxidoreductase, glutamate synthase, and Fd:thioredoxin reductase (29). All of these enzymes contain redox-active cofactors including FAD (FNR), iron-sulfur clusters (glutamate synthase, nitrite reductase, and sulfite reductase), and siroheme (nitrite reductase). By contrast with these Fd-dependent enzymes, PcyA appears to lack a metal or flavin cofactor that can mediate single electron transfers. For these reasons, we propose that the PcyA-mediated reduction of BV proceeds via bilin radical intermediates as depicted in Fig. 6.

Based upon the absorption spectrum of the PcyA·BV complex, the bilin substrate is depicted in a cyclic conformation within the protein cavity (see Fig. 6). The lack of photochromism of the PcyA·BV complex can be rationalized by the binding of the terminal pyrrolinone A- and D-rings into the protein matrix with its propionate side chains extending toward the solvent. This porphyrin-like configuration not only would sterically prevent photoisomerization of the C5 and C15 double bonds but would also bury reactive radical intermediates within the protein matrix, thus minimizing side reactions with molecular oxygen. This substrate binding model is consistent with the broad substrate specificity of the extended bilin reductase family, which includes the enzyme RCCR that metabolizes a chlorophyll catabolite with monomethyl ester and isocyclic ring substituents (30). In this regard, the hypothesis that the bilin reductase family may have evolved from ancestors that metabolized (Mg)-porphyrins remains a intriguing possibility.

As shown in Fig. 6 (*step 1*), we envisage that bilin reduction occurs by binding of reduced Fd to the PcyA-BV complex, followed by electron transfer to the bound bilin and proton transfer from a protein residue labeled D_1-H to generate a neutral radical shown in *step 2*. The benzylic position would help to stabilize this radical by resonance within the extended tetrapyrrole π-system, until a second electron and proton transfer, shown in *steps 2* and *3*, occurs to produce the intermediate $18^1,18^2$-DHBV IXα. The hypothetical proton donors, D_1-H and D_2-H, could either be carboxylic acids (*i.e.* Asp or Glu), sulfhydryls (*i.e.* Cys), phenolics (*i.e.* Tyr), or even protonated nitrogen residues such as histidine or lysine. It is also possible that protons are derived from bound water molecules that are protonated by appropriate protein residues. For all of these protein residues except for histidine or lysine, proton transfer would be accompanied by an increase in negative charge, which would be a reasonable "driving force" for the release of product. Since PcyA kinetically reduces the intermediate $18^1,18^2$-DHBV IXα

without its release, we hypothesize that D1 and D2 are protonated histidine and/or lysine residues.

We propose that the subsequent reduction of the A-ring of 18^1,18^2-DHBV IXα proceeds in a similar fashion, generating another resonance-stabilized bilin radical intermediate shown in Fig. 6 (*steps 4–6*). For this transformation, we hypothesize that the proton-donating residues are carboxylic acids, sulfhydryls, and/or phenolics, which would generate negative charge within the bilin pocket, thereby promoting release of the PCB product (Fig. 6, *step 6*). Experiments to detect potential radical intermediates by electron spin resonance spectroscopy and to identify putative proton-donating residues within the PcyA polypeptide by site-directed mutagenesis are in progress. With this experimental approach, we hope to elucidate the molecular basis for the unique double bond reduction specificities of the different members of the extended bilin reductase family (4) and ultimately to engineer novel specificity of this important family of enzymes.

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