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STRUCTURAL AND MECHANISTIC INSIGHT INTO THE FERREDOXIN-MEDIATED TWO ELECTRON REDUCTION OF BILINS

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¹**Abbreviations:** BV, biliverdin IXα; DHBV, dihydrobiliverdin; EPR, electron paramagnetic resonance; FDBR, ferredoxin-dependent bilin reductase; FNR, ferredoxin:NADP⁺ oxidoreductase; PCB, phycocyanobilin; PEB, phycoerythrobilin; PebS, PEB synthase; PΦB, phytochromobilin; SeMet, selenomethionine; WT, wild-type.

SYNOPSIS

Phycoerythrobilin (PEB) is one of the major open-chain tetrapyrrole molecules found in cyanobacterial light-harvesting phycobiliproteins. In these organisms, two enzymes of the ferredoxin-dependent bilin reductase family work in tandem to reduce biliverdin IX α to PEB. In contrast, a single cyanophage-encoded enzyme of the same family has been identified to catalyze the identical reaction. Using UV-Vis and EPR spectroscopy we investigated the two individual cyanobacterial enzymes PebA and PebB showing that the two subsequent reactions catalyzed by the phage enzyme PebS are clearly dissected in the cyanobacterial versions. While a highly conserved aspartate residue is critical for both reductions, a second conserved aspartate is only involved in the A-ring reduction of the tetrapyrrole in PebB and PebS. The crystal structure of PebA from *Synechococcus* sp. WH8020 in complex with its substrate BV at 1.55 Å resolution revealed further insight into the understanding of enzyme evolution and function. Based on the structure it becomes obvious that in addition to the importance of certain catalytic residues, the shape of the active site and consequently the binding of the substrate highly determines the catalytic properties.

INTRODUCTION

Ferredoxin-dependent bilin reductases (FDBRs¹) are a class of enzymes involved in reducing the heme metabolite biliverdin IX α (BV) to form several individual open-chain tetrapyrroles (phycobilins) used for light-perception or light-harvesting in plants and cyanobacteria [1]. FDBRs are distinct from BV reductases in mammals or cyanobacteria which are mainly involved in catabolic degradation of BV to bilirubin [2, 3]. Currently several members of the FDBR family are known. The first cloned member was phytochromobilin (PΦB) synthase (HY2) from Arabidopsis thaliana, producing PΦB, the chromophore of the photoreceptor phytochrome [4]. Based on the amino acid sequence of $P\Phi B$ synthase, additional members of the FDBR family were identified [5, 6]. Among those are enzymes, which likewise catalyze a two-electron reduction but also two members that catalyze a formal four-electron reduction. The most common target for reduction within the FDBR family is the $2,3,3^{1},3^{2}$ -diene system of the A-ring (Figure 1). However, only PΦB synthase acts on the substrate BV directly thereby producing PΦB. All other Aring reductions target intermediates of four-electron reductions leading to the cyanobacterial pigments phycocyanobilin (PCB) and phycoerythrobilin (PEB). Specifically, the second reduction performed by PcyA (PCB:ferredoxin oxidoreductase) targets the A-ring of 18¹,18²-dihydrobiliverdin (DHBV), an isolatable intermediate in the reduction of BV to PCB [7]. The recently identified cyanophage PEB synthase (PebS) on the other hand utilizes the intermediate 15,16-DHBV to produce PEB [6, 8]. The identical A-ring reduction is also performed by the two-electron reducing PebB (PEB:ferredoxin oxidoreductase). This enzyme acts in tandem with PebA (15,16-DHBV:ferredoxin oxidoreductase), which reduces BV at the C15-C16 double bond to produce 15,16-DHBV [5, 9]. Both enzymes are proposed to function in close contact and metabolic channeling of 15,16-DHBV has been postulated [9]. In contrast to that, PebS realizes a perfect metabolic channeling of the same intermediate by combining the two activities of PebA and PebB in one enzyme. While the sequence identity between PebS and PebA is rather low (27 %), they do however serve as a great paradigm of enzyme evolution and function. We previously presented the crystal structure and biochemical analysis of cyanophage PebS [8, 10]. Like PcvA, the first crystallized member of the FDBR family [11, 12], PebS shows an $\alpha/\beta/\alpha$ -sandwich fold, with a central substrate binding site parallel to the plane of the sheet [8]. The binding of the substrate BV is rather flexible which was reflected in the different binding modes observed. Concurrent with these different binding modes is also the flexibility of aspartate 206, one of two aspartate residues (D105 and D206) shown to be critical for the reaction. While D206 seems to be important for the A-ring reduction activity of PebS, D105 is proposed to be involved in both enzymatic steps which both proceed via a

tetrapyrrole radical intermediate [10]. Interestingly, both residues are highly conserved within the whole FDBR family (Figure S1) and a common function has been discussed [5, 10, 13].

This current study has been undertaken to understand the structural and mechanistic details discriminating between the two cyanobacterial and the phage enzyme. While PebS is able to reduce the intermediate 15,16-DHBV further to the final product, the PebA reaction is terminated at this point. PebB on the other hand has a distinct substrate specificity for the intermediate 15,16-DHBV. In an interdisciplinary approach we combined X-ray crystallography with biochemical and biophysical characterization of wild type and mutant proteins to gain a deeper understanding of the evolution of these three enzymes.

EXPERIMENTAL

Reagents – Unless otherwise specified, all chemical reagents were ACS grade or better. Glucose-6-phosphate dehydrogenase, NADP⁺, catalase, glucose, glucose-6-phosphate, spectrophotometric grade glycerol, trifluoro acetic acid, 4-methylmorpholine and aminoacids were purchased from Sigma-Aldrich. HPLC grade formic acid, acetone and acetonitrile were purchased from J.T. Baker. Biliverdin IX α (BV) was obtained from Frontier Scientific.

Production and purification of recombinant proteins – PebA and PebB from *Synechococcus* sp. WH8020 were expressed, purified and the concentration determined as described previously [9]. The purified proteins contained eight additional N-terminal residues from the expression vector. Selenomethionine (SeMet) labeled PebA (SePebA) was produced using M9 minimal medium. Fifteen minutes prior to induction with isopropyl-β-thiogalactoside 100 mg/L lysine, phenylalanine, threonine, 50 mg/L isoleucine, valine, leucine and 60 mg/L selenomethionine were added to the culture. Expression and purification for labeled protein followed the same procedure as for unlabeled PebA with the exception that all buffers contained 5 mM dithiothreitol. Ferredoxin from cyanophage P-SSM2 and ferredoxin:NADP+ oxidoreductase (FNR) from *Synechococcus sp.* WH 7002 were prepared as described earlier [10].

All protein variants were generated by site-directed mutagenesis from pGEX-6P-1_PebB_{Syn} and pGEX-6P-1_PebB_{Syn} [5] using the QuikChange[®] site-directed mutagenesis kit (Stratagene). The used primers are listed in Table S1.

Anaerobic bilin reductase assay, electron paramagnetic resonance and HPLC measurements – Bilin reductase activity assays, electron paramagnetic resonance (EPR) measurements, HPLC analyses and preparative production of 15,16-DHBV were performed under anaerobic conditions as described [10]. To start the reaction, an NADPH-regenerating system was used [5]. Only the reaction of the PebA_D84N variant was started with an excess of NADPH (10 electron equivalents, 50 μ M final concentration) instead of the NADPH-regenerating system to reduce unspecific reduction of accumulating radical species. For PebB, the following modifications were used: The assay was performed at 20 °C with 0.1 μ M FNR and 25 Units catalase. After 2 min 10 μ M of PebB_WT or PebB variant was added to the reaction mixture. The reaction was stopped after a total of 10 min.

EPR measurements were performed as described in [10]. The following modifications were applied only for the PebB reaction: The reaction was performed at 20 °C with 37.5 Units catalase, 0.032 μ M FNR and 4 μ M ferredoxin using PebA_WT and BV as the substrate. After 2 min PebB was added to the PebA-DHBV complex at a 1:1 ratio.

Crystallization – Crystallization conditions were screened by the sitting drop vapor diffusion method utilizing the Cryos, PEG and PACT Suites (Qiagen) applying 200/100 nL and 100/100 nL mixtures of the protein solution (10 - 20 mg/ml) / reservoir solution incubated at 18 °C in the dark. Conditions were further optimized with the hanging drop vapor diffusion method. Substrate BV was added in two-fold excess. PebA crystallized at protein concentrations between 12-20 mg/ml in 0.1 M HEPES, pH 7 and 28 % PEG 4000.

Structure determination and refinement – Oscillation data of SeMet-labelled protein crystals were collected at 100K at the Swiss Light Source (SLS) on beamline X10SA. Data were processed using XDS [14]. 5% of the data were randomly assigned as test set. Data statistics are given in Table 1. The Matthews coefficient was estimated to 2.1 Å³/Da for one molecule per asymmetric unit.

Phases were determined from a 2 Å dataset collected at the Se-K-edge. AutoSharp readily located the two Se-atoms in the asymmetric unit [15]. Arp/Warp was used to autotrace PebA in the resulting map [16]. This model was then used with Arp/Warp to rebuild the model with the second 1.55 Å PebA dataset. The resulting model was improved using alternating cycles of manual correction in COOT [17] and automatic refinement in PHENIX [18]. The first six residues from the expression vector, residues 128 and 129, and the last three residues were not modeled due to missing density. The model has been deposited at the Protein Data Bank under accession number 3X9O.

RESULTS AND DISCUSSION

All members of the FDBR family are radical enzymes

Cyanobacteria use the dual enzyme system PebA and PebB to produce the phycobilin PEB while a single enzyme PebS encoded by a cyanophage does the same job. We used comparative enzymology to understand the similarities and differences between PebA, PebB and PebS which are homologous to each other and which all belong to the FDBR family. This work was furthermore intended to gain insight into enzyme evolution. Although PebA and PebB have in parts already been studied biochemically [9], we reinvestigated their enzymatic properties under anaerobic conditions. This is essential to detect and stabilize possible tetrapyrrole radical intermediates as shown for other members of the FDBR family, including the recently investigated cyanophage PebS [10, 13, 19]. In contrast to the aerobic reduction of BV to 15,16-DHBV by PebA, which showed a decrease of absorption at 690 nm and a concomitant increase at 590 nm [9], two additional absorption maxima are observed under anaerobic conditions. In the course of the reaction an increase and further decrease at ~ 440 nm and ~ 750 nm was monitored (Fig. 2A, upper panel). Absorbance at these wavelengths has been attributed to bilin radical intermediates [10, 13, 19]. EPR studies confirmed this assumption. For PebA, the strongest EPR signal was observed 1 min after start of the reaction (Fig. 2 B), when no product was yet detectable via HPLC (data not shown). The appearance of this signal followed similar kinetics as the absorption at ~ 440 nm and ~ 750 nm. In addition, the decrease of radical signal was accompanied by an increase of product formation indicating that the observed radical is a BV radical.

For the first time we monitored the PebB reaction which uses 15,16-DHBV as a substrate. In order to facilitate proper 15,16-DHBV delivery, a completed PebA reaction was employed. This was preferred over external supply of 15,16-DHBV because metabolic channeling of 15,16-DHBV from PebA to PebB has been postulated and might be involved in proper delivery and subsequent binding of 15,16-DHBV [9]. This is underlined by the observation that 15,16-DHBV externally supplied to the PebS D206N variant binds differently than the intermediate produced by itself [10]. Due to the lack of an extinction coefficient and the instability of 15,16-DHBV, this approach provides an easy and accurate way to supply PebB with equimolar amounts of substrate. First, a regular PebA reaction employing a PebA-BV complex was used. After 2 min almost all BV was converted to 15,16-DHBV which remained bound to PebA (PebA-DHBV). Subsequently, PebB WT was added leading to an immediate transfer and binding of 15,16-DHBV to PebB (PebB-DHBV) as observed by a shift of 15,16-DHBV absorbance from 590 nm (PebA-DHBV) to ~ 605 nm (PebB-DHBV) (Fig. 3A, Fig. S2). Almost simultaneously a strong increase at 682 nm was observed, most likely representing the formation of radical intermediates which were again confirmed by EPR measurements (Fig. 3B). A small EPR signal was still detected at 2 min due to a not fully completed PebA reaction. Addition of PebB WT resulted in an increased radical signal, which disappeared with the completion of the reaction at ten minutes. At this time point the formation of PebBbound 3Z-PEB (PebB-PEB) was detected at 548 nm (Fig. S2). This is in agreement with the PebS

reaction where the final product is also *3Z*-PEB [10]. Herewith we prove that all different members of FDBRs act via radical intermediates.

Identification of critical residues for catalytic activity

Central to the proposed reaction mechanism of PebS are two aspartate residues D105 and D206, both involved in interactions with the pyrrole nitrogens upon substrate binding [8]. Both are shown to be essential for the complete reduction of BV to PEB by PebS and are highly conserved throughout the family of FDBRs (Fig. S1). To study the role of the corresponding residues in PebA and PebB (Table 1), several variants were characterized in anaerobic bilin reductase assays. The resulting products were analyzed by HPLC and UV-Vis spectroscopy.

When aspartate at position 84 in PebA is changed into an asparagine, no conversion of BV is observed. Interestingly, the D84N variant appears to stabilize a radical intermediate as indicated by a fast increase and very low decrease of an absorption maximum at 740 nm (Fig. 2A). When the carboxylic side chain is retained, but exchanged by a longer side chain which is expected to sterically interfere with substrate binding, the resultant PebA variant D84E is still able to convert BV to 15,16-DHBV at a similar efficiency (Fig. 2A). However, the produced 15,16-DHBV is significantly more unstable (data not shown) suggesting that D84 is also involved in stabilization of the enzyme-product complex. This catalytic behaviour is in agreement with data of the PebS_D105N/E variants which showed the same properties for the first reduction [10].

Similar results were obtained for the asparagine variant of the homologous amino acid residue in PebB (PebB_D107N). A stabilization of a radical intermediate was observed both in UV-Vis (increase of absorption at 670 nm) and EPR spectroscopy (Fig. 3). Consequently, no product formation was detected (data not shown). Interestingly, retaining the carboxylic side chain of this residue in a glutamate variant (PebB_D107E) did not rescue the activity indicating that space constraints might be crucial for reduction. However, this variant is still able to bind 15,16-DHBV in a manner similar to the WT protein (Fig. S2) and trace amounts of product PEB were monitored by HPLC (Table 1).

When D205 is changed into an asparagine, PebA retains its activity. The D205N variant converted its substrate BV to 15,16-DHBV (Fig. 2A). In contrast, the homologous variant of PebB (PebB_D231N) showed a complete loss of PEB formation. An increase of absorption at 666 nm with no further decay again suggested the stabilization of a radical intermediate (Fig. 3A). The observed EPR signal increased over time with highest signal intensity at 10 min (Fig. S3). PebB_D231E on the other hand showed only slightly decreased activity with significant product formation.

The presented data clearly demonstrate that the individual activities of PebA and PebB with their dependency on important catalytic residues are combined in the phage enzyme PebS. Specifically, D105 is important for both reductions while D206, although conserved, is only critical for A-ring reduction [10].

Overall structure of PebA

In order to determine the structural differences between PebS and PebA, which only catalyzes the first reduction of BV to 15,16-DHBV, the structure of selenomethionine labeled PebA with BV IX α was solved by the single wavelength anomalous dispersion method and was refined at 1.55 Å resolution (Table 2). Six residues of the linker peptide used in the expression construct and the last three residues of the mature protein were not modeled due to missing electron density. In addition, the two residues N128 and G129 could not be placed for the same reason. Clear density for the substrate allowed to unambiguously place BV into the binding site. Identification of the correct orientation was possible due to the asymmetric vinyl substituents of the A- and D-ring.

The structure of PebA represents the first structural view of an FDBR catalyzing a two-electron reduction. Together with the different structures available for the four-electron reduction enzymes PcyA and PebS we are now able to make a clearer discrimination between structural features preserved throughout the family and features with distinct differences, which might be key elements in controlling the stereoselective reactions. The overall structure consists of a central seven-stranded antiparallel β -sheet,

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which is from both sides flanked by a total of six α -helices (Fig. 4). All structurally solved FDBRs share the presence of this central seven-stranded β -sheet [8, 11] which forms a mostly hydrophobic basis of the substrate binding site. In addition, the binding pocket is always formed by two long helices (PebA:H5-H6, PebS:H3-H4, PcyA:H7-H8) connected by the "D-loop" (residues connecting H5 and H6). The position of these helices is similar in all structures, but variations of the linker geometry occur between enzymes and upon substrate binding. Indeed, PebA can be superimposed with low RMSDs of 2.02 Å and 2.39 Å to PebS (2VCK, chainC) and PcyA (2D1E), respectively. The largest structural variations are located in the "lid" (loop between helix H3 and strand S7 and by helices H5 and H6) in response to substrate binding in both PebS and PcyA (Fig. 4) [8, 20]. In addition, large differences in length and folding of the lid region exist between the different FDBRs. In PebA and PcyA helical elements are found, which are completely missing in PebS (Fig. S4). Both the changes upon substrate binding and differences in structure make this area the most promising target for interaction of PebA with both ferredoxin (as proposed by [21]) and PebB.

The most prominent feature of the binding pocket is the central polar centering "pin", formed by D84 (PebA numbering used throughout for simplicity reasons) and the adjacent N67 on strands S5 and S4, respectively (Fig. 5, 6, S5). Both residues are involved in coordination of the polar pyrrol nitrogens and the carbonyl oxygens (Fig. 5, 6). Only for HY2, D84 is replaced by asparagine, while at the same time the neighboring N67 is changed to aspartate, theirby restoring the placement of a functional carboxylic group at this position [13]. The importance of both residues for the HY2 catalytic mechanism is not yet fully understood and awaits detailed biophysical and structural analyses.

Inside the binding niche of PebA six water molecules are resolved. The BV propionate side chains are coordinated by salt bridges with three positively charged protein side chains (K93, R134, R150). In contrast to PcyA and PebS, BV in PebA is bound in a roofed conformation, with both A- and D-ring tilted 40° out of the plane and the A-ring buried deeper inside the pocket. The catalytic residue D84 is in an identical orientation in PebA as compared to D105 in PebS and PcyA and is therefore not the structural reason for the roofed BV binding. The other conserved aspartate D205 which is of catalytic importance for PebS but has been shown to adopt different conformations in PebS is rotated away from the active site in PebA. It is the first of three residues of the D-loop. This residue is not involved in direct ligand interaction which is consistent with its non-catalytic role in PebA.

Structural flexibility of the active site is required for two consecutive reductions within one FDBR

Based on the structures and on the sequence alignment (Fig. S1), the binding pockets of all FDBRs are lined with hydrophobic and aromatic residues, making van-der-Waals and π -stacking interactions the dominating factor for substrate binding. Very few residues in the active site are chemically able to participate in substrate protonation and are found to be highly conserved amongst FDBRs. Also the general binding mode with the A-ring buried deeper seems to be identical throughout the FDBR family, regardless of the site of reduction (Fig. S5).

Considering the similarities between PebS and PebA, the question arises, which structural features of PebA are responsible for the termination of the BV reduction at the intermediate 15,16-DHBV, while it is directly processed to PEB in PebS. The binding pocket of PebA forces BV into a strained, roofed conformation (Fig. 5). This conformation results from steric restraints imposed by size variation of apolar protein side chains. For example, substitution of methionine M212 by phenylalanine in combination with the substitution of isoleucine I86 by value in PebA forces the rotation of the D-ring of BV (Fig. 6 and S5). In contrast, in PebS the pocket is much larger, leaving room for the tilting of the D-ring after reduction of the C15-C16 double bond to form the nonplanar product 15,16-DHBV (Fig. 6). It has been proposed that rearrangement of 15,16-DHBV in PebS is required for stereospecific reduction of the A-ring. Even though a structure of PebS with the bound intermediate 15,16-DHBV is still missing, the observed binding flexibility for BV supports such a rearrangement [8]. In contrast, very little conformation for a variety of substrates has been observed in PcyA [11, 20, 22]. The intermediate 18^1 ,18²-DHBV of the PcyA reduction still retains the conjugated π -system of BV, which

extends over all four tetrapyrrole rings. Thereby no large structural changes have to be accomodated during the two successive reductions (Fig. 6 and S5).

D205 is critical for A-ring reduction

Based on our and other biochemical and structural data it is obvious that the conserved D84 residue is highly important for bilin reduction in PcyA, PebS, PebA and PebB. The only exception so far seems to be the above mentioned HY2 protein where double exchange of an Asp/Asn pair occurs. D84 is well positioned to facilitate stereospecific protonation to both C-2 and C-16 to generate the R configuration at these carbon atoms. In contrast to the general importance of D84, D205, although conserved, is only important for A-ring reduction of BV (in HY2) and 15,16-DHBV (in PebS and PebB). For PebS we previously postulated a role of D205 as a proton shuttle to supply the protons for the second reduction. In PebA this proton shuttle is not required since only a two-electron reduction is catalyzed, representing the first reduction in PebS. In both cases the active site seems to be able to supply the two protons necessary for the reaction either from D84 and/or water molecule(s). For the second reduction PebS requires additional delivery of protons from the surrounding medium as the intermediate stays tightly bound in the active site. D84 is proposed to fulfill this function. In contrast, it is hypothesized that PebB binds 15,16-DHBV into its protonated active site analogous to BV binding to PebA and PebS alleviating the need for an additional proton shuttle. We would expect at least residual function of the PebB D231N variant if this residue is only relevant for proton delivery in PebB. Therefore, an additional function of D205 in substrate coordination is required to fully explain our data. We believe that D205 will be involved in direct coordination of 15,16-DHBV, thereby affecting local reactivity of the substrate to support stereospecific protonation by D84. In turn, these data suggest a similar function of D205 also for PebS. As implicated by our biochemical data these two aspartate residues are similarly positioned in a PebB model (Fig. S5). Full understanding of these processes will require structures of PebA, PebB and PebS with bound 15,16-DHBV as well as high field EPR characterization of the substrate radicals. These experiments are currently underway in our laboratories.

FDBRs are a great paradigm to study enzyme evolution

Based on numerous biochemical and structural data on members of the FDBR family it becomes obvious that nature has retained one general fold to evolve several distinct enzymatic functions. Even key catalytic residues have been retained (i.e. D84 and D205) but still different reduction sites of the same or a similar substrate are targeted. This seems basically facilitated through the very specific positioning of the substrate molecule and substrate rearrangement after initial reduction in the four-electron reducing FDBRs. Based on our current knowledge we are quite confident that other FDBRs with different regiospecificities do exit in nature or can be engineered using directed evolution.

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AUTHOR CONTRIBUTION

AWUB and NFD designed the study; AWUB performed all biochemical experiments; AWUB and EJR performed the EPR experiment; AWUB, EJR and WL analysed the EPR data; EH solved the crystal structure; AWUB, EH and NFD analysed the biochemical data; AWUB, EH and NFD wrote the paper.

SUPPOPRTING INFORMATION

One Table and five supplemental Figures are available online.

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В

FIGURE LEGENDS

Figure 1. Reactions catalyzed by the FDBR family. Highlighted in the box is the most common reduction site within the family, the A-ring $2,3,3^1,3^2$ -diene system. The plant enzyme phytochromobilin synthase (HY2) catalyzes the reduction of biliverdin (BV) to 3Z-P Φ B; cyanobacterial PEB:ferredoxin oxidoreductase (PebB) and cyanophage phycoerythrobilin synthase (PebS) the reduction of 15,16dihydrobiliverdin (DHBV) to 3Z-phycoerythrobilin (3Z-PEB), and PcyA the reduction of $18^1,18^2$ dihydrobiliverdin to 3Z-phycocyanobilin (3Z-PCB). The reduction sites are highlighted by the circles.

Figure 2. Reaction of PebA_WT and variants monitored by UV-Vis and EPR spectroscopy.

A. Anaerobic reduction of BV by PebA and variants was monitored via UV-Vis spectroscopy for 10 min, except for variant D84E. Spectra were taken every 30 sec. Possible radical absorptions (~440 and ~750 nm) are indicated by an asterisk, development of the spectra over time is indicated by arrows. The WT reaction contained a four-fold excess of enzyme as described in [10] and represents the sample used for the EPR measurement shown in panel B. The reaction of the D84N variant employed NADPH instead of the regenerating system as described in the experimental procedures. Spectra shown in bold and grey are the starting spectra, the final spectra are presented in black. **B**. EPR measurements were performed as described previously with samples taken from the reaction at indicated time points. All spectra are on the same scale and were recorded at T=40 K, a microwave power of 20 μ W and 1 mT field modulation (see [10] for further experimental details).

Figure 3. Reaction of PebB_WT and variants monitored by UV-Vis and EPR spectroscopy.

A. Anaerobic reduction of BV by PebA was used to produce the substrate 15,16-DHBV (Dotted spectra represent 15,16-DHBV bound to PebA). At the end of the reaction when all BV was reduced to 15,16-DHBV, PebB was added and the reaction monitored for additional 8 min (spectra shown in grey, initial time point in bold). Spectra were taken every 30 sec. Possible radical absorption is indicated by an asterisk, development of the spectra over time is indicated by arrows. The final spectrum of each reaction is shown in black. **B**. EPR measurements for the WT have been performed as described previously [10] and under experimental procedures with samples taken from the reaction at indicated time points. All spectra are on the same scale and were recorded at T=40 K, a microwave power of 20 μ W and 1 mT field modulation (see text and [10] for further experimental details).

Figure 4. Overall structure of PebA. Shown is the protein backbone in cartoon representation, rainbow colored from blue (N-terminus, labeled N) to red (C-terminus, labeled C). The bound substrate BV and the two catalytic residues D84 and D205 are shown as stick models. Secondary structure elements are labeled.

Figure 5. Stereoview of the active site of PebA. Shown is a cartoon representation of both PebA (green) and PebS (yellow, PDB code 2VCK, chain C), superposed based on the C α -atoms of the central β -sheet. The two catalytic aspartates and the substrate are shown as sticks and are labeled. Water molecules present in the PebA structure are shown as red spheres.

Figure 6. Substrate binding pockets of PebA, PebS and PcyA. A. PebA (2X9O), PebS (2VCK, chain C), and PcyA (2D1E) active sites are shown with bound substrate BV (green) which is oriented with the D-ring left and the A-ring right. BV and active site residues (salmon) in the 3.6 Å sphere of BV are represented as stick models. Ordered water molecules in the active site are shown as red spheres. B. Proteins are shown in cartoon representation superposed with the molecular surface surrounding the substrate. The two residues of PebA discussed in the text are labelled (V65 and F211, replacing I86 and M212 of PebS, respectively).

PebS_D105		PebA_D84		PebB_D107	
D105N	-	D84N	-	D107N	-
D105E	-	D84E	15,16-DHBV	D107E	(PEB)
PebS_D206		PebA_D205		PebB_D231	
D206N	15,16-DHBV	D205N	15,16-DHBV	D231N	-
	15,16-				
D206E	DHBV/PEB			D231E	PEB

Table 1. Catalytic activities of protein variants of the two conserved aspartate residues. Homolog residues in each FDBR are shown in the same row.

-, no activity observed; DHBV, dihydrobiliverdin; PEB, phycoerythrobilin (as products of the reaction), brackets indicate that only trace amount are detectable.

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B

Data set	peba-10	neba-19
Beamline	SLS X10SA	SLS X10SA
Resolution (Å)	34-2.0 (2.07-2.0)	40 2-1 55 (1 59-1 55)
Cell parameters		
(a.b.c: Å)	42.25, 39.28, 71.39	42.42, 39.35, 71.27
$(\alpha, \beta, \gamma; degrees)$	90, 105.77, 90	90 105.6 90
Spacegroup	P21	P2 ₁
Wavelength	0.978	0.978946
Completeness (%)	99.3 (92.9)	97.7 (93.8)
Multiplicity	7.6 (6.1)	4.3 (3.4)
Average I/σI	24.5	11.3
	(6.7)	(4.8)
R _{sym} (%)	6.3	7.4
Sym ()	(24.7)	(22.0)
R_{meas} (%) ^b	6.8	8.3
incus ()	(26.9)	(25.8)
R_{mrgd-F} (%) ^b	5.6	8.6
	(21.5)	(28.2)
Phasing		
Phasing Power	1.13 overall	
C	3.45 at 5.5 Å	
	>1 above 2.4Å	
Refinement		
Resolution (Å)		40-1.55
R _{crvst} (%)		17.9
$R_{\text{free}}(\%)^{c}$		22.5
Number of atoms		
Protein		1929
Biliverdin		43
Water		322
Average B-factor Protein		19.7 (21.1)
(Biliverdin)		
Root mean square deviation		
from ideality		
Bonds (Å)		0.007
Angles (degrees)		1.164

Table 2. Data Collection and Refinement Statistics ^a

^a Data in parentheses represent values in the highest resolution bin. ^b For definition of R_{meas} and R_{mrgd-F} see [23]. ^c R_{free} calculated from 5% of randomly selected reflections.

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Figure 2.



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Figure 3.



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