Phycoerythrobilin Synthase (PebS) of a Marine Virus

CRYSTAL STRUCTURES OF THE BILIVIDERIN COMPLEX AND THE SUBSTRATE-FREE FORM

Received for publication, May 16, 2008, and in revised form, July 7, 2008. Published, JBC Papers in Press, July 28, 2008, DOI 10.1074/jbc.M803765200

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The reddish purple open chain tetrapyrole pigment phycoerythrobilin (PEB; Amax = 550 nm) is an essential chromophore of the light-harvesting phycobiliproteins of many cyanobacteria, red algae, and cryptomonads. The enzyme phycoerythrobilin synthase (PebS), recently discovered in a marine virus infecting oceanic cyanobacteria of the genus Prochlorococcus (cyanophage PSSM-2), is a new member of the ferredoxin-dependent bilin reductase (FDBR) family. In a formal four-electron reduction, the substrate biliverdin IXα is reduced to yield 3Z-PEB, a reaction that commonly requires the action of two individual FDBRs. The first reaction catalyzed by PebS is the reduction of the 15,16-methine bridge of the biliverdin IXα tetrapyrole system. This reaction is exclusive to PEB biosynthetic enzymes. The second reduction site is the A-ring 2,3,3′,3″-dieney system, the most common target of FDBRs. Here, we present the first crystal structures of a PEB biosynthetic enzyme. Structures of the substrate complex were solved at 1.8- and 2.1-Å resolution and of the substrate-free form at 1.55-Å resolution. The overall folding revealed an α/β/α-sandwich with similarity to the structure of phycocyanobilin:ferredoxin oxidoreductase (PcyA). The substrate-binding site is located between the central β-sheet and C-terminal α-helices. Eight refined molecules with bound substrate, from two different crystal forms, revealed a high flexibility of the substrate-binding pocket. The substrate was found to be either in a planar porphyrin-like conformation or in a helical conformation and is coordinated by a conserved aspartate/asparagine pair from the β-sheet side. From the α-helix side, a conserved highly flexible aspartate/proline pair is involved in substrate binding and presumably catalysis.

Phycoerythrobilin (PEB)4 is a reddish purple pigment belonging to the group of heme-derived open chain tetapyrrole molecules called bilins. It functions as a chromophore of light-harvesting phycobiliproteins of cyanobacteria, red algae, and cryptomonads. Covalently linked to phycoerythrins, and some phycocyanins, PEB is an essential component of many phycobilisomes (1–3). Besides this natural function, phycobiliproteins are also commonly used as fluorescent probes for biotechnological and biomedical applications (4, 5). The biosynthesis of all bilins starts with the common precursor molecule biliverdin IXα (BV), which is the product of oxidative cleavage of heme by the enzyme heme oxygenase (6). BV is then further reduced by highly specific ferredoxin-dependent bilin reductases (FDBRs) (7). This new family of radical enzymes, characterized by the lack of organic or metal cofactors (8, 9), is composed of several members, each targeting specific double bonds in the tetrapyrole system. Within the FDBR family, one can distinguish between two-electron and four-electron reductases. Phycocyanobilin:ferredoxin oxidoreductase (PcyA) is a four-electron FDBR and the best characterized member of this class of enzymes (8–13). It converts its substrate BV via the semirduced intermediate 181,182-dihydrobiliverdin (DHBV) to phycocyanobilin (PCB) (8). The required four electrons for the reduction are provided by the reduced form of the small acidic redox protein ferredoxin (Fd) (Fig. 1). In vivo, the electrons for Fd reduction most likely originate from photooxidized photosystem I and are then passed on to a variety of Fd-dependent enzymes (14). In contrast to the biosynthesis of PCB, two independent biosynthetic pathways leading to PEB have been described (Fig. 1). In phycoerythrin-containing cyanobacteria, BV is converted by two consecutive two-electron reductions to PEB (15, 16). The first reduction is catalyzed by 15,16-DHBV:ferredoxin oxidoreductase (PebA) and yields 15,16-DHBV. The product of this first reduction then serves as a substrate for PEB:ferredoxin oxidoreductase (PebB) to generate PEB. A second pathway leading to PEB has recently been described by discovery of a new member of the FDBR family (17). Phycoerythrobilin synthase (PebS; EC 1.3.7.6) catalyzes the Fd-dependent four-electron reduction of BV to PEB (Fig. 1). Biochemical analyses have established that the order of reduction is identical to that catalyzed by the consecutive action of PebA and PebB. The intermediate 15,16-DHBV is thought to
remain in the active-site pocket of PebS and not to be released during catalysis (17). This observation is in agreement with results on PebA and PebB from *Synechococcus* sp. WH8020 that suggest metabolic channeling of 15,16-DHBV directly from PebA to PebB (15). What is compelling about PebS is not only its novel activity but also its widespread distribution in wild cyanophage populations and absence in the respective host populations (17). Therefore, PebS is a biologically and biochemically exceptional enzyme.

Here, we present high resolution crystal structures of this novel enzyme, PEB synthase alone and in complex with its substrate BV. We provide further insights into the structural basis of substrate recognition and specific reduction of the tetrapyrrole system in this interesting family of radical enzymes.

**EXPERIMENTAL PROCEDURES**

**Recombinant Production and Purification of PebS**—Heterologous expression of cyanophage *pebS* was performed in *Escherichia coli* BL21(DE3) using a pGEX-6P-3 construct (GE Healthcare) for a translational fusion to glutathione S-transferase as described (17). The recombinant protein product resulted in nine additional N-terminal amino acids that remained after cleavage with PreScission™ protease (GE Healthcare). For production of selenomethionine-labeled protein, pre-cultures and expression cultures were grown in glucose (0.4%)-supplemented M9 (instead of Luria-Bertani) medium. 20 min prior to induction with 100 μM isopropyl β-D-thiogalactopyranoside, the cultures were cooled down, and an amino acid mixture containing l(+)-selenomethionine (50 mg/liter final concentration; Acros) was added to inhibit methionine biosynthetic pathways as described (18). Cells were harvested 15 h post-induction, lysed, centrifuged, and purified as described (17) with the following modifications: ultracentrifugation was carried out at 170,000 × g for 1 h, and all buffers were adjusted to pH 8.0 and contained 200 mM potassium chloride and up to the final dialysis step 5 mM dithiothreitol for selenomethionine-labeled proteins. The purified proteins were then concentrated using a nitrogen-pressurized stirred ultrafiltration cell with an *M*~r~ 10,000 filter cutoff (Millipore). The concentrated protein was incubated with an excess of BV (Frontier Scientific, Inc.) dissolved in Me2SO for 1 h on ice in the dark. To remove free BV and to reduce salt concentration, the protein solution was applied to a NAP™5 desalting column (GE Healthcare) and eluted with 25 mM TES-KOH (pH 8.0). The molar extinction coefficient (ε~280~) (19) and adjusted to the respective concentrations. The activity of the native and selenomethionine-labeled proteins was assayed as described (17).

**Crystallization of PebS and the PebS-BV Complex**—Crystallization conditions were screened by the sitting drop vapor diffusion method using the Classic, Cryo, and JSCG Suites (Qiagen), applying 200/100-nl and 100/100-nl mixtures of the protein solution (10.5 mg/ml)/reservoir solution incubated at 18 °C in the dark.

Initial native PebS-BV crystals (clusters of green thin plates) grew in a 1:1 mixture of protein solution (10.5 mg/ml) and reservoir solutions containing 1.6 M sodium citrate. These conditions were further optimized with the hanging drop vapor diffusion method, and final crystals grew within 14 days in a 10-μl drop. The drop was taken from a 1:1 mixture of protein solution (7.5 mg/ml) and 1.025 M sodium citrate containing 4% glycerol. The mixture was incubated for 15 h at 18 °C and centrifuged (2 × 30 min, 14,000 rpm) before placing the drop over a reservoir solution of 1.25 M sodium citrate at pH 8.0. Initial selenomethionine-labeled seleno-PebS (SePebS)-BV crystals (green)
PebS X-ray Structure

RESULTS

PebS Mediates the Transfer of Four Electrons from Ferredoxin to BV—PebS is the only second FDBR, after PcyA, known to perform a formal four-electron reduction (17). Biochemical analyses established that the recombinant protein is most active under slightly basic conditions (pH 8.0). The in vitro reduction is faster employing the recombinant phage-encoded Fd (PetF_PSSM-2) for electron delivery compared with the use of Synechococcus sp. PCC 7002 Fd, resulting in an estimated turnover of ~10 min⁻¹ for BV at room temperature (data not shown). This is likely due to more efficient electron transfer among the phage proteins. The A-ring reduction of BV results in two possible stereoisomeric ethylenediamine groups, 3Z and 3E. Discussions in the past have always led to the question whether the 3Z- or 3E-form is the primary product of certain FDBRs or whether they occur simultaneously. In this study, we confirmed 3Z-PEB as the primary product of PebS by absorbance spectroscopy (supplemental Fig. S1). However, ratios of 3Z-PEB and 3E-PEB obtained through high pressure liquid chromatography analyses are distorted because sample preparation tends to favor conversion to the 3E-isomer (7) (data not shown), the thermodynamically more stable isomer (30, 31). The formation of the 3Z-form as the primary product is in agreement with early studies on oat phytochromobilin synthase and other bilin reductases (7, 32, 33).

Structure Determination—X-ray data of PebS in three different crystal forms have been collected. Wild-type protein was crystallized with bound substrate BV (PebS-BV), and selenomethionine-labeled protein was crystallized with and without substrate (SePebS-BV and SePebS, respectively).

The structure of SePebS was determined by single wavelength anomalous dispersion phasing and refined at 1.55-Å resolution to an R-factor of 0.174 and a free R-factor of 0.194 (Table 1). This structure was substrate-free and subsequently used as a search model to determine the structures of the BV complexes. Both SePebS-BV and PebS-BV crystallized in space group P1 with similar cell dimensions. In both cases, four molecules were found in the asymmetric unit. The structures were refined to R-factors of 0.184/0.227 and free R-factors of 0.225/0.285 using data to 1.8- and 2.1-Å resolution, respectively (Table 1).

In all three structures, the N-terminal 20 residues were found to be disordered. Residues 51–56 form a flexible β-turn that was completely resolved only in chain D of PebS-BV. In the substrate-free form (SePebS), the active-site residues Asp²⁰⁶ and Pro²⁰⁷ could not be conclusively modeled. All Ramachandran plot outliers could be assigned to residues involved in either substrate coordination or crystal contact formation.

PebS Is a Globular Single Domain Protein—The PebS enzyme is a single domain globular protein with dimensions of ~45 × 36 × 33 Å, showing an α/β/α-sandwich fold (Fig. 2). A central seven-stranded antiparallel β-sheet is flanked by two α-helices on one side and three α-helices on the side of the substrate-binding pocket. The substrate BV is bound in a cavity formed by the β-sheet (proximal side) and by helices H3 and H4 and a long flexible loop between strands S6 and S7 (distal side), which closes in upon substrate binding. The flexibility of this region is illustrated in the C-α superposition of all nine copies of PebS present in the three crystal forms used in this study (Fig. 3).

The overall fold of PebS is similar to that of cyanobacterial PcyA from Synechocystis sp. PCC 6803 (Protein Data Bank...
codes 2D1E and 2DKE) (10, 11) and Nostoc sp. PCC 7120 (Protein Data Bank code 2G18) (12) (Fig. 4 and supplemental Table S1), which belong to the same family of FDBRs. Helix H3 is shorter than the corresponding helix of PcyA, and the flexible loop between strands S6 and S7 forms two short helices in PcyA that are not present in PebS (Fig. 4 and supplemental Fig. S2).

Both proteins bind the same substrate BV but specifically reduce it at distinct double bonds, yielding the isomers PCB (PcyA) and PEB (PebS).

**Substrate Recognition**—Clear electron density for the vinyl groups defines the orientation of the substrate with the propionate side chains facing toward the solvent (Figs. 2 and 5 and supplemental Figs. S3–S5). The A- and D-rings are buried inside the binding niche and are coordinated from the β-sheet by residues Asn88 (β-strand S4) and Asp105 (β-strand S5) (Fig. 5). Their side chains form a hydrogen bonding network with each other and the carbonyl oxygens of BV. These residues are comparably positioned in structure (supplemental Fig. S6A) and sequence alignment (supplemental Fig. S2) to the critical catalytic pair His88/Asp105 of PcyA (10, 12, 13). On the distal side, the substrate is flanked by two α-helices (H3 and H4) that are connected by a short linker containing an Asp/Pro pair (Asp206/Pro207) that is strictly conserved among FDBRs except in PcyA. Interestingly, different binding modes for the substrate BV were identified within different molecules in the crystallographic asymmetric unit of both crystal forms (Fig. 5 and supplemental Fig. S5). In SePebS-BV, three molecules show almost identical BV binding, with a water molecule being in hydrogen-bonding distance to all pyrrole nitrogens and to Asp206 (Fig. 5A). Here, BV is bound in a planar porphyrin-like conformation (supplemental Table S2), with the propionate side chains facing the solvent side. Within SePebS-BV, the fourth molecule in the asymmetric unit shows a different binding mode of BV. Asp206 is positioned closer to the pyrrole nitrogens, with one of the carboxylic oxygens replacing the pyrrole water (Fig. 5B). In the native structure PebS-BV, four different molecules are again found in the asymmetric unit. In three of these, the flexibility of the distal side is visible as Asp206 is rotated outside the active

### TABLE 1

Data collection and refinement statistics

| Dataset          | SePebS-1 (anomalous statistics) | SePebS-2 | SePebS-BV | PebS-BV |
|------------------|---------------------------------|----------|-----------|---------|
| **Data collection** |                                 |          |           |         |
| Beamline         | SLS PXII (30.6.07)              | SLS PXII (30.6.07) | SLS PXII (30.6.07) | SLS PXI (29.4.07) |
| Resolution (Å)   | 45-1.7 (1.8-1.7)                | 38-1.55 (1.6-1.55) | 49-1.8 (1.9-1.8) | 48-2.1 (2.3-2.1) |
| Cell parameters  |                                 |          |           |         |
| a, b, c (Å)      | 42.50, 72.47, 90.94             | 42.84, 72.45, 90.94 | 53.56, 67.57, 81.28 | 53.99, 70.04, 81.67 |
| α, β, γ          | 90°, 90°, 90°                   | 90°, 90°, 90°       | 92.26°, 109.07°   | 106.58° |
| Space group      | P212121                         | P212121     | P1        | P1      |
| Wavelength       | 0.97906                         | 0.9826     | 0.97887   | 0.98011 |
| Completeness (%) | 100 (100)                       | 98.8 (90.2) | 97.1 (95.9) | 97.5 (90.9) |
| Multiplicity     | 7.7 (7.5)                       | 26.3 (14.4) | 8.1 (8.2) | 4.3 (4.4) |
| Average I/σI     | 16.9 (4.8)                      | 34.1 (4.9) | 13.2 (5.0) | 12.8 (5.3) |
| Rsym (%)         | 8.9 (56.5)                      | 7.6 (64.1) | 10.5 (42.0) | 8.0 (29.7) |
| Rfree (%)        | 9.5 (60.7)                      | 7.7 (66.5) | 11.2 (44.9) | 9.1 (33.9) |
| Rmerge (%)       | 8.2 (34.6)                      | 4.8 (31.7) | 7.6 (26.7) | 9.0 (25.3) |
| **Phasing**      |                                 |           |           |         |
| SHELXD           |                                 |           |           |         |
| Resolution for phasing (Å) | 45–2.2                         |           |           |         |
| No. of scatterers found | 6                              |           |           |         |
| CC (all/weak)    | 55.69/36.66                    |           |           |         |
| PATOM             | 31.60                           |           |           |         |
| SHELXE resolution range (Å) | 45–1.7                 |           |           |         |
| Pseudo-free CC (correct/inverted) | 80.03/54.25 |           |           |         |
| **Refinement**   |                                 |           |           |         |
| PDB code         |                                 | 2VCL      | 2VCK      | 2VGR    |
| Resolution (Å)   | 38-1.55 (1.59-1.55)             | 49-1.80 (1.85-1.80) | 48-2.1 (2.15-2.1) |
| Rcryst (%)       | 17.4 (21.3)                     | 18.4 (21.9) | 22.65 (26.1) |
| Rfree (%)        | 19.4 (23.3)                     | 22.5 (NA) | 28.5 (NA) |
| No. of atoms     |                                 |           |           |         |
| Protein          | 1731                            | 6949      | 7010      |         |
| Biliverdin IXa   | 172                             | 172       | 172       |         |
| Water/glycerol   | 225/18                          | 575       | 339       |         |
| No. of side chains with alternate conformations | 5 | 9 | 0 |
| Average B-factor overall (biliverdin IXa) | 17.0 | 15.3 (21.9) | 29.0 (44.2) |
| **Ramachandran plot** |                                 |           |           |         |
| Favored          | 158 (88.3%)                     | 651 (88.7%) | 625 (83.9%) |
| Additional allowed | 21 (11.3%)                   | 82 (11.2%) | 111 (14.9%) |
| Generously allowed | 0                             | 0         | 3 (0.4%)  |
| Disallowed       | 0                               | 1 (0.1%)  | 6 (0.8%)  |
| **Root mean square deviation from ideality (protein atoms)** | |           |           |         |
| Bonds (Å)        | 0.018                           | 0.014     | 0.012     |         |
| Angles           | 1.73°                           | 1.47°     | 1.52°     |         |

*a* For definition of Rmerge and Rweighted, see Ref. 38.

*b* Rfree was calculated from 5% of data omitted either randomly (2VCL) or in thin shells (2VCK and 2VGR) from refinement.

*c* Values were calculated with Procheck (39).
site (Fig. 5C). The absence of Asp\textsuperscript{206} then allows the substrate to adopt a helical lock-washer conformation, with the A-ring shifted partially above the D-ring (supplemental Table S2). This position is visible in chains A (Fig. 5D and supplemental Fig. S4A) and C (supplemental Figs. S4C and S5C). This conformational change positions the second site of the reduction, the C-2 position of the 2,3,3\textsuperscript{1},3\textsuperscript{2}-diene system of BV, in proton-accepting distance to O-19.

**DISCUSSION**

FDBR enzymes catalyze the regio- and stereospecific reduction of BV to different bilin chromophores, which are essential for light harvesting in phycobilisomes and light sensing in phytochromes. Although the enzyme responsible for the four-electron reduction of BV to PCB has structurally and functionally been well characterized, no structural information has been available for an enzyme involved in the synthesis of PEB. The high resolution structures of PebS from the cyanophage PSSM-2 presented in this work shed light on the mechanisms of regiochemical control in related reductases that operate on the same substrate. At the same time, they raise questions as to whether there is a common conserved mechanism for the reduction of the A-ring vinyl group that takes place in all FDBRs except PebA.

PebS shows the same overall architecture as PcyA (Fig. 4), even though the conservation between the enzymes at the sequence level is rather low (14% identity, 25% similarity) (supplemental Fig. S2). However, critical residues for substrate binding are conserved, but distinct differences in the active-site environment seem to be the source of the chemical specificity.

**Initial Step: Binding and Protonation of BV**—The reaction starts with the binding of the substrate BV, which is accompanied by a structural change in the protein. The most obvious structural changes in PebS occur within a flexible loop that covers the active site (Fig. 3). Upon binding, BV and PebS form a spectroscopically distinct complex (supplemental Fig. S7). This appears to be a common phenomenon for biochemically characterized members of the FDBR family (8, 15, 40).\textsuperscript{5} Interestingly, the spectroscopic signatures for the complexes between FDBR and BV differ from each other, reflecting different binding modes or protein environments in the active sites. Although the long wavelength absorption of BV is blue-shifted upon binding to PcyA and HY2 (8, 40), it is red-shifted in the PebS-BV complex (supplemental Fig. S7). The PcyA-BV complex shows an additional shoulder in the near-infrared region (supplemental Fig. S7), which has been attributed to a protonated BV species as the initial step of the PcyA reaction (12). Protonation is thought to occur via the carbonyl oxygen-coordinating residue Asp\textsuperscript{105} (Synechocystis PcyA nomenclature). This residue (Asp\textsuperscript{105} in PebS) is highly conserved among almost all FDBRs (supplemental Fig. S2) and is involved in coordinating O-1 and O-19 of BV (Fig. 5). Interestingly, the BV complex of PebA from *Synechococcus* sp. WH8020 shows absorption properties more similar to those of PebS-BV than to those of PcyA-BV that may indicate the structural and mechanistic rela-

\textsuperscript{5} N. Frankenberg-Dinkel, unpublished data.
tionship of both 15,16-reduction enzymes (15, 17). With respect to the different binding modes of BV observed in the crystal structure, it is also likely that the absorption spectrum of the PB complex resembles a mixture of protonated and unprotonated species rather than only one particular BV species. Analysis of possible H-bonding patterns within the BV conformations found in our PebS structures suggests that protonation of the carbonyl oxygens of BV in the planar conformations is unlikely. In contrast, in the helical conformation, O-19 of BV is involved in a putative branched H-bond with Asp105. Consequently, a protonation at this position is likely, which would facilitate subsequent electron transfer. Helical conformations of BV are stable in solution but are destabilized if compensation for the intrachromophoric hydrogen bond is provided by other optimally oriented donor groups (34). Evidence for the presence of the helical conformation in PebS-BV complexes in solution was obtained by circular dichroism spectroscopy. The spectrum revealed a strong positive signal at ~380 nm that is weaker for PcyA-BV and a negative signal at ~680 nm (supplemental Fig. S8). These signs are characteristic for M-helical bilin conformations (35) like those in 2VGR chains A and C (Fig. 5D and supplemental Fig. S5C). In addition to the spectroscopic differences of the complexes, none of the observed binding modes of BV in PebS superposes perfectly with the situation in PcyA, reflecting the structural and functional divergence of the enzymes (supplemental Table S1).

On the basis of our spectroscopic data (supplemental Figs. S7 and S8) and the helical conformation of BV found in two molecules of the asymmetric unit (Protein Data Bank code 2VGR) (Fig. 5D and supplemental Fig. S5C), we propose a protonation of BV as the initial step in the PebS reaction. This is achieved via Asp105 to O-19 of the substrate BV in the helical conformation. In agreement with the postulated PcyA mechanism, this protonation is most likely not involved in the reduction of BV but rather generates a BV H⁺ cation that can readily accept one or two electrons from reduced Fd.

Coupled Electron-Proton Transfer—Common to all FDBRs is the direct coupling of electron and proton transfer steps. The electrons are provided by the single electron transfer protein Fd. Because of the lack of metal or organic cofactors within the FDBRs, the reactions are thought to occur via substrate radical intermediates, as has been shown previously for PcyA using
EPR spectroscopy (9). This has now also been proven for plant phytochromobilin synthase (40) and can be expected to be general to FDBRs.

Surface charge patterns of PcyA suggested an overlap of the Fd-docking site with the substrate-binding pocket (11). Hence, electron transfer could proceed, involving the propionate side chains of BV. It is generally accepted that electrons rapidly redistribute in the tetrapyrrole ring system. Although the existence of BV radicals has been shown experimentally for PcyA and HY2, the exact chemical nature of such a radical remains obscure. It can be expected that the unpaired electron is delocalized over the entire conjugated \( \pi \)-system. One might expect high and low spin densities at certain carbons, which are likely influenced by the conformation, binding environment, and protonation.

The First Reduction: D-ring—After the initial protonation of BV and a subsequent one (or two)-electron transfer to generate either a neutral or anionic radical, the first proton transfer(s) have to occur to finalize the first reduction. In contrast to PcyA, which performs a regiospecific reduction at the exo-vinyl group of BV, PebS has to transfer a proton stereospecific to C-16 to generate the \( R \)-configuration. Therefore, the proton must ultimately originate from the \( \beta \)-sheet side of the active site. Careful inspection of all structures failed to identify proton-donating residues in the vicinity of the C-15–C-16 double bond. This is in accordance with PebA homology models based on the PcyA structure (12) and also with a provisional PebA model based on PebS (2VCK chain C) (supplemental Fig. S6B). Therefore, both C-15–C-16 double bond-reducing enzymes (PebA and PebS) might work via a similar mechanism, different from PcyA, not involving direct protonation from active-site amino acid residues. The proposed reaction mechanism of PcyA is based on the concept of proton transfer involving the “critical pair” Asp\(^{105}/\text{His}^{88}\) and the carboxyl group of Glu\(^{76}\) alternating with direct electron transfer from Fd to the substrate. Central to this concept is a conserved proton transfer pathway leading from the solvent to the active-site residues, which allows initial formation of a BV H\(^{+}\) cation upon substrate binding and subsequent reprotonation and delivery of additional protons. In the PebS structure, no residues equivalent to Glu\(^{76}\) are found, and His\(^{88}\) is replaced by Asn\(^{88}\), thereby interrupting the proton relay and effectively inhibiting the D-ring vinyl reduction by the mechanism found in PcyA.

One possible explanation for the PebS (and possibly PebA) mechanisms lies in the observed structural heterogeneity of the PebS-substrate complexes in our crystals. They demonstrate that conformational changes of the complex can expose the C-15 and C-16 atoms of the C-D-methine bridge temporarily to the solvent. Therefore, the two protons for the full reduction could be donated from the bulk solvent, whereas the correct stereochemistry is enforced by the accessibility restraints imposed by the protein surface.

The Second Reduction—The second reduction, the reduction of the A-ring vinyl group, is the most common reaction in the FDBR family. Although PebA is the sole FDBR that does not catalyze this reduction, PebS is the only enzyme that combines A-ring vinyl group reduction with a preceding 15,16-reduction in one enzyme. The corresponding A-ring vinyl reduction of 18\(^1\),18\(^2\)-DHBV catalyzed by PcyA was proposed to proceed from the central Asp\(^{105}\) via His\(^{88}\) and O-1 to C-2, followed by a second proton from the bulk solvent to C-3\(^2\) upon product release (12). In Arabidopsis thaliana HY2, His\(^{88}\) is replaced by Asp\(^{116}\), which is postulated to be functionally different (40). Notably, in both PebB and PebS, His\(^{88}\) is replaced by Asn\(^{88}\), which cannot serve as the central proton donor. One possible solution for this discrepancy lies in the helical conformation observed in our PebS-BV structure. Here, C-2 is well positioned for protonation by Asp\(^{105}\) via O-19 to yield the \( R \)-stereochi-
event that should be controlled by the chemical state of the bound substrate and most likely will involve a subtle conformational response in PebS upon binding. These effects will be very difficult to assess until we are able to obtain structural information about the PebS-Fd complex.

Acknowledgments—We thank Drs. Carsten Köttling, Bernhard Kräutler, Wolf-Dieter Schubert, and Shih-Long Tu for helpful discussion. We also thank the beamline staff at the Swiss Light Source.

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