


Proximity channeling during cyanobacterial phycoerythrobilin synthesis

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Substrate channeling is a widespread mechanism in metabolic pathways to avoid decomposition of unstable intermediates, competing reactions, and to accelerate catalytic turnover. During the biosynthesis of light-harvesting phycobilins in cyanobacteria, two members of the ferredoxin-dependent bilin reductases are involved in the reduction of the open-chain tetrapyrrole biliverdin IX α to the pink pigment phycoerythrobilin. The first reaction is catalyzed by 15,16-dihydrobiliverdin:ferredoxin oxidoreductase and produces the unstable intermediate 15,16-dihydrobiliverdin (DHBV). This intermediate is subsequently converted by phycoerythrobilin:ferredoxin oxidoreductase to the final product phycoerythrobilin. Although substrate channeling has been postulated already a decade ago, detailed experimental evidence was missing. Using a new on-column assay employing immobilized enzyme in combination with UV-Vis and fluorescence spectroscopy revealed that both enzymes transiently interact and that transfer of the intermediate is facilitated by a significantly higher binding affinity of DHBV toward phycoerythrobilin:ferredoxin oxidoreductase. Concluding from the presented data, the intermediate DHBV is transferred via proximity channeling.

Introduction

The biosynthesis of light-harvesting pigments is crucial for the efficient function of photosynthesis. While plants mainly employ chlorophyll for light harvesting, cyanobacteria, red algae, and cryptophytes use phycobilins, open-chain tetrapyrrole molecules, to capture light [1]. These pigments are covalently associated with proteins, which are then termed phycobiliproteins. In cyanobacteria, different types of phycobiliproteins are arranged to a phycobilisome [2]. These megadalton protein complexes sit on the thylakoid membrane and funnel the light via Förster resonance energy transfer to the photosystems in the membrane. Phycobilisomes are composed of a core of allophycocyanin (APC) and

radiating rods of phycocyanin (PC) and, depending on the species, phycoerythrin (PE) and phycoerythrocyanin (PEC), respectively [3]. The individual phycobiliproteins are constituted of α - and β -subunits building heterodimers and ultimately heterohexamers. Each subunit has between one and three covalently linked phycobilins bound. The two most abundant phycobilins in cyanobacteria are phycocyanobilin (PCB) and phycoerythrobilin (PEB) which are attached via conserved cysteine residues [2].

For many years, our laboratory has been interested in the biosynthesis of these open-chain tetrapyrrole pigments and we have mostly concentrated on

Abbreviations

BV, biliverdin IX α ; CV, column volume; DHBV, 15,16-dihydrobiliverdin; FDBR, ferredoxin-dependent bilin reductase; OCA, on-column assay; OSS, oxygen scavenging system; PebA, 15, 16-dihydrobiliverdin:ferredoxin oxidoreductase; PebB, PEB:ferredoxin oxidoreductase; PEB, phycoerythrobilin.

the biosynthesis of the pink pigment PEB [4–10]. The biosynthesis of PEB starts with the heme oxygenase reaction, in which heme is cleaved at the α -meso carbon to produce biliverdin IX α (BV) with the concomitantly liberation of carbon monoxide and ferric iron [11,12]. BV is then the substrate of nearly all members of the ferredoxin-dependent bilin reductases (FDBR). Biosynthesis pathways for PEB have been described in cyanobacteria, cryptophytes, and phages (viruses that infect bacteria) [1,5,7,13,14]. While the pathway in cyanobacteria and cryptophytes is identical and involves the subsequent action of two FDBRs, the one encoded by phages differs as it only requires a single enzyme to convert BV to PEB [6,7]. Cyanobacterial PEB biosynthesis starts with the reduction of BV by 15,16-dihydrobiliverdin:ferredoxin oxidoreductase (PebA) at the C15–C16 double bond to yield 15,16-dihydrobiliverdin (DHBV). DHBV is then the substrate of the subsequent enzyme PEB:ferredoxin oxidoreductase (PebB), which reduces the 2,3,3¹,3²-diene system of the A-ring of the tetrapyrrole and the final product PEB is formed (Fig. 1). The electrons required for this reduction step are provided by ferredoxin and each reduction requires two electrons [5]. Recent studies revealed two alternative PEB biosynthesis pathways encoded in phage genomes. The FDBR members PebS and PcyX are both able to convert BV in a single step to PEB, also via the intermediate DHBV [4,7] (Fig. 1). Although PebA and PebS share a high structural similarity, the reaction of PebA is terminated directly after DHBV formation, whereas PebS proceeds further to produce PEB via the intermediate DHBV [6]. PcyX on the other hand, although catalyzing formally the same reaction, does this by a different catalytic mechanism [8].

In this work, we focused on our previous postulate that PebA and PebB are involved in the metabolic channeling of DHBV. We provide evidence for proximity channeling of DHBV and, in addition, constructed a functional translational fusion of PebA and PebB. With this, we present some further understanding of the biosynthesis of PEB in cyanobacteria.

Results

Our previous work on phycoerythrobilin biosynthesis by PebA and PebB suggested an involvement of both enzymes in the metabolic channeling of DHBV [5]. The current study was performed to test whether both enzymes physically interact or whether the transfer proceeds via proximity channeling without a prolonged physical contact.

FDBRs are still active when immobilized onto affinity chromatography beads

The so-called ‘on-column’ assay (OCA) was established to probe for direct protein–protein interaction. First, we tested whether immobilization of GST-tagged PebA on affinity material retained enzymatic activity. Therefore, the complex of GST-tagged and BV loaded PebA was bound on GST-sepharose and an FDBR assay was performed as described. The initial green color of the bound complex turned into a violet color after a couple of minutes, indicating the formation of DHBV (Fig. 2A). Interestingly, conversion to DHBV was only observed when the OCA was performed under micro-oxic conditions, that is, when an oxygen scavenging system (OSS) was present. The OSS consists of glucose oxidase, glucose, and catalase and was initially introduced to stabilize substrate radicals during the reaction [14]. Although the OSS is also part of our regular FDBR assay in solution, it is dispensable for substrate conversion [14,15]. As substrate turnover of the OCA cannot be monitored using UV-Vis spectroscopy, an FDBR assay in solution was performed in parallel and monitored via UV-Vis spectroscopy. This assay was used to calculate the time for full substrate conversion. The product of this conventional assay was also used as the bilin standard for the subsequent HPLC analysis of the reaction products (Fig. 2B–D). Based on the control assays, the reaction time of all OCAs was accordingly set to 10 min. Performing an OCA with 10 μ M GST-tagged PebA:BV showed marginal amounts of DHBV in the wash fraction after washing with two column volumes (CV) of assay buffer. DHBV remained bound to PebA and eluted together with the enzyme upon washing with glutathione-containing elution buffer. HPLC analysis confirmed the identity of DHBV (Fig. 2B).

The intermediate DHBV is transferred from PebA to PebB on the column

Next, we tested whether addition of PebB to the immobilized PebA:DHBV complex will result in the interaction of PebA and PebB and, therefore, cause retention of PebB on the column. While we did not observe a retention of PebB on the immobilized PebA column, we did see a transfer of almost all PebA-bound DHBV to PebB, which was washed off the column with regular washing buffer. The transfer of DHBV was confirmed by HPLC analysis (Fig. 2C). Finally, GST-tagged PebA was eluted from the GST-sepharose with only a minor amount of DHBV remaining bound to it (Fig. 2C). These results indicate

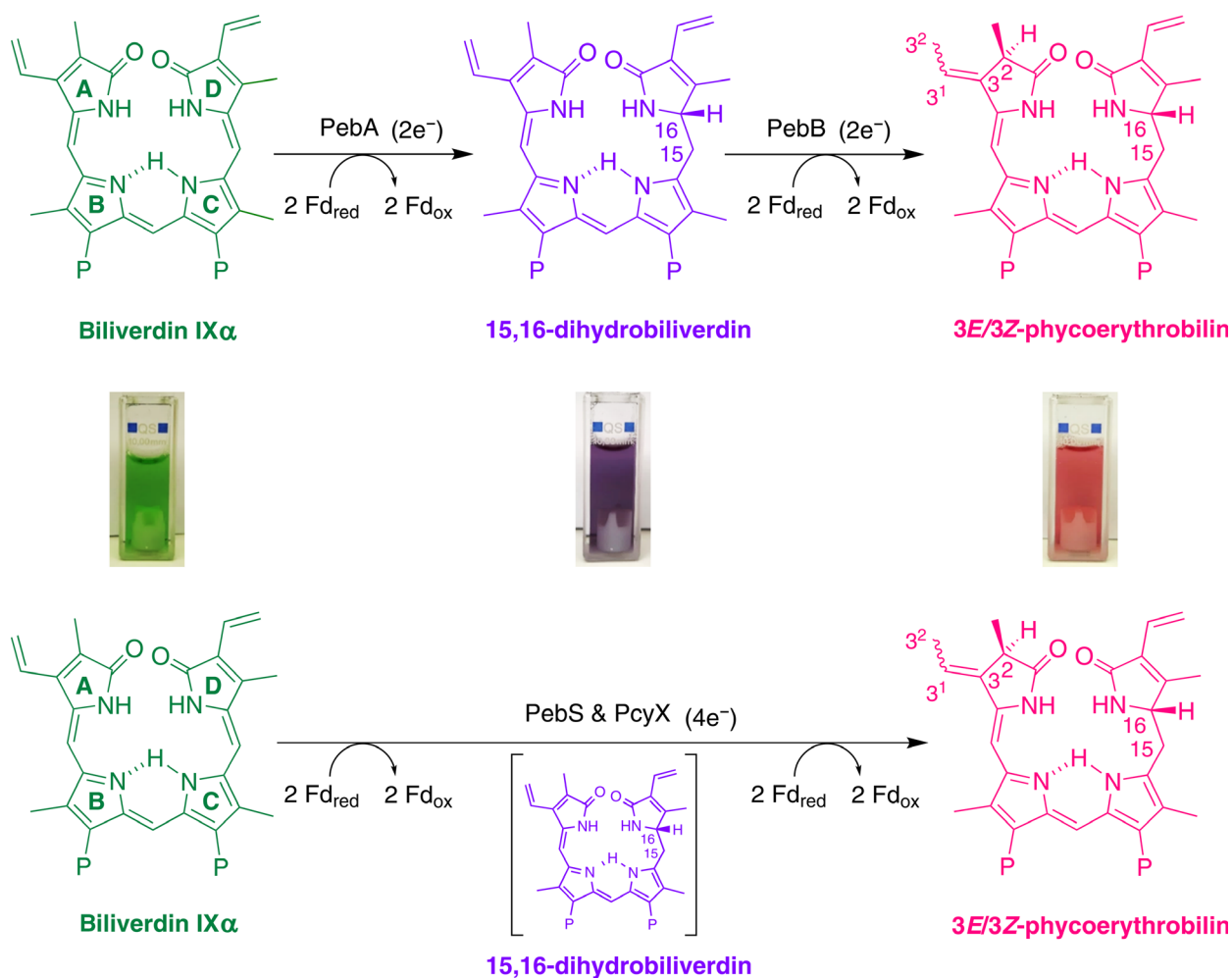


Fig. 1. The two known pathways for phycoerythrobilin synthesis. Overview of the two known pathways for phycoerythrobilin synthesis in cyanobacteria, red algae, cryptophytes, and phages. The green pigment biliverdin IX α is the product of the heme oxygenase reaction and serves as the natural substrate for all known FDBRs except PebB. The enzymatic conversion of biliverdin IX α to phycoerythrobilin is the result of a four-electron reduction step via the intermediate 15,16-dihydrobiliverdin. While cyanobacteria require the two FDBRs, PebA and PebB, to produce phycoerythrobilin, phages possess enzymes that are able to perform this two-step reaction in one. The FDBRs, PebS and PcyX, are able to convert biliverdin IX α to phycoerythrobilin as a single enzyme in a four-electron reduction step. The electrons needed for the reduction of biliverdin IX α and 15,16-dihydrobiliverdin are provided from ferredoxins (Fd) for both pathways. The change in color during pigment synthesis can be observed from green (BV) over violet (DHBV) to the final pink product (PEB). P, propionate side chain; PebA, 15,16-dihydrobiliverdin:ferredoxin oxidoreductase; PebB, phycoerythrobilin:ferredoxin oxidoreductase; PebS/PcyX; phycoerythrobilin synthase

a high affinity of DHBV to PebA, but an even higher affinity toward PebB since PebB is able to pull out DHBV from PebA.

PebA and PebB bound to a column are still functional and catalyze the reduction of BV to PEB

In addition to a single enzyme assay on the column, we next tested whether it is also possible to perform a coupled FDBR assay on the column, with both PebA and PebB fused to the GST-tag. For this set up, 10 μ M

of PebA:BV complex was added to the column and the reaction was performed as described above. After 5 min, 10 μ M of GST-tagged PebB was added to the column and incubated for another 5 min. The collected wash fraction contained DHBV and PEB indicating that DHBV was transferred to PebB and further reduced to the final product PEB. Interestingly, the affinity of PEB toward PebB appears low as PEB was eluted in the wash fraction and not bound to PebB (Fig. 2D). This is in agreement with earlier studies showing lower affinity of PebB to PEB than PebB to DHBV [5].

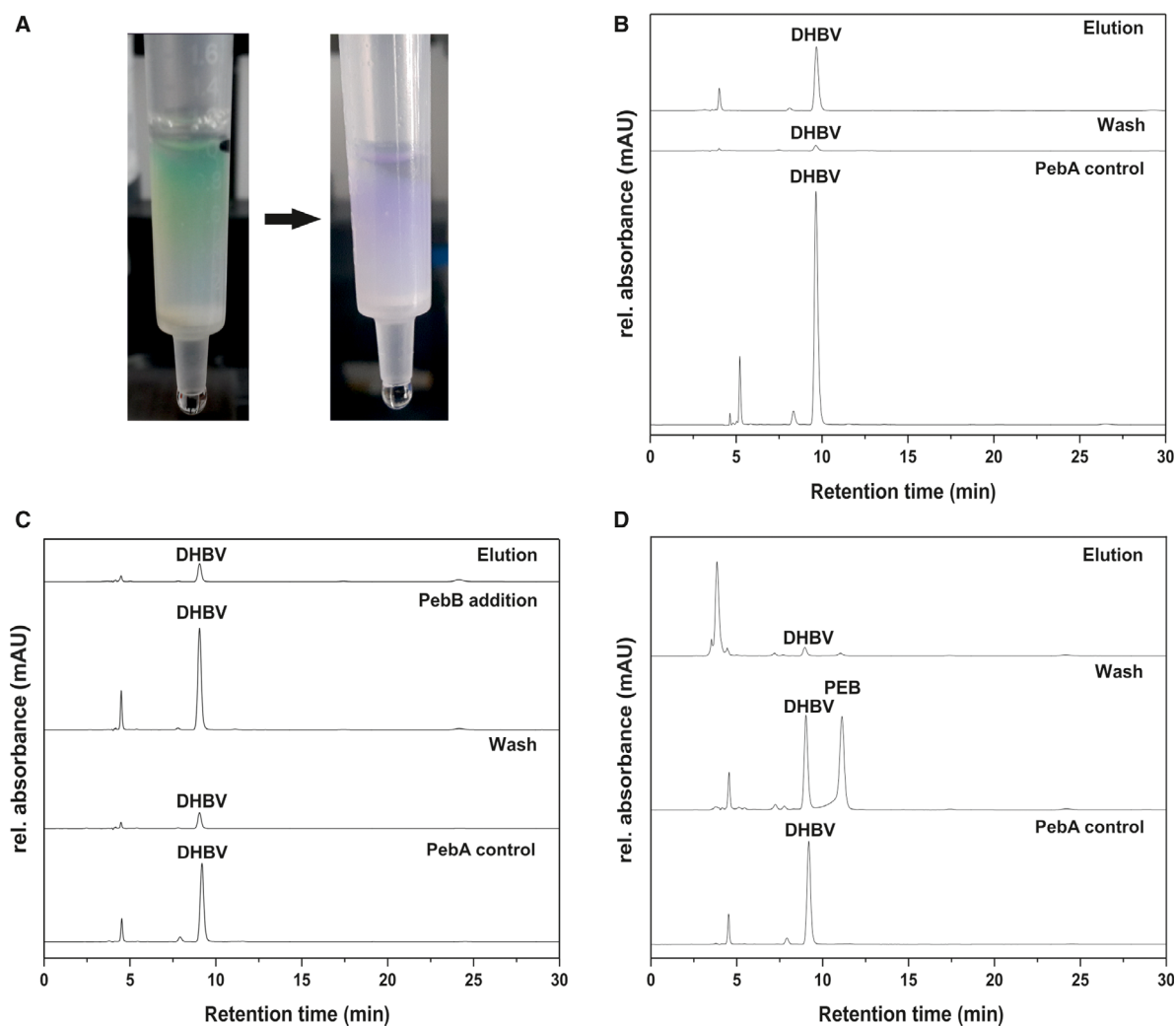


Fig. 2. Establishment of an 'on-column' bilin reductase assay to investigate the interaction between PebA and PebB. (A) 10 μM GST-tagged PebA preincubated with an equimolar concentration of BV was bound to 0.5 mL GST-sepharose and a ferredoxin-dependent bilin reductase assay was performed by adding all required components (including the oxygen scavenging system) to the column. After approximately 5 min, a change in color from green to violet was observed. Without the oxygen scavenging system no product formation was detectable (data not shown). (B) HPLC analysis of extracted bilins from 'on-column' assay (OCA) as described in A. After 10 min the column was washed with 2 CV washing buffer (wash) and the GST-tagged PebA was subsequently eluted with 3 CV elution buffer (elution). Fractions were collected and bilins were purified as described. (C) HPLC analysis of extracted bilins from OCA as described in A. After 10 min the column was washed with 2 CV washing buffer (wash) followed by an additional washing with 3 CV washing buffer containing 10 μM His-tagged PebB (PebB addition). Finally, the GST-tagged PebA was eluted with 3 CV elution buffer (elution). Fractions were collected and bilins were purified as described. (D) HPLC analysis of extracted bilins from OCA. A total of 10 μM GST-tagged PebA preincubated with an equimolar concentration of BV was bound to 0.5 mL GST-sepharose and a ferredoxin-dependent bilin reductase assay was performed by adding all required components (including the oxygen scavenging system) to the column. After 5 min, 10 μM of GST-tagged PebB was added to the column and incubated for another 5 min. Subsequently, the column was washed with 2 CV washing buffer (wash) and the GST-tagged proteins were finally eluted with 3 CV elution buffer (elution). Fractions were collected and bilins were purified as described. The bilins in all collected fractions were analyzed using HPLC on a Luna 5 μm RP C_{18} -column (Phenomenex, Aschaffenburg, Deutschland) with acetone and 20 mM formic acid (50 : 50/v/v) as the mobile phase at a flow rate of 0.6 mL \cdot min $^{-1}$. All reaction products were monitored at 560 nm.

UV-vis spectroscopy confirmed the elution of the FDBR: bilin complex from the column

The transfer of the intermediate DHBV was not only traced via HPLC analysis, it was also tested whether

the intermediate was still protein bound. Due to the alteration of the spectral properties of the bilins in their free and in their enzyme bound form, the collected OCA fractions were also monitored via

UV-Vis-spectroscopy. As a reference, 10 μM DHBV was dissolved in assay buffer and a whole spectrum was taken. Free DHBV showed an absorbance maximum at 560 nm, as described previously. Addition of 10 μM PebB to the free bilin led to a typical shift in the absorbance maximum from 560 nm to 606 nm, indicating the PebB:DHBV complex formation (Fig. 3A). UV-Vis spectroscopy of the collected fractions of the OCA (see Fig. 2C) revealed that DHBV was not present in the washing fraction. The majority of DHBV coeluted bound to PebB in the 'PebB addition' fraction. Here, the formation of the PebB:DHBV complex with its typical spectral properties resulting in an absorbance maximum of 606 nm was observed (Fig. 3B).

Substrate binding affinities of PebA or PebB suggest metabolic channeling

The obtained data from the OCA suggested that DHBV has a high affinity toward PebA and an even higher affinity toward PebB, which likely facilitates its transfer. In order to quantitate this observation, binding affinities of the recombinant produced enzymes to the intermediate DHBV were re-evaluated employing a more sensitive technique to the one previously described [5]. In these studies, the binding affinities of either PebA or PebB to DHBV were estimated via the fluorescent properties of the resulting enzyme: bilin complexes. Within this present study, the binding affinities of either PebA:DHBV or PebB:DHBV were also assessed using fluorescence titration experiments. However, a more rigorous analysis was performed using the MO.AFFINITY ANALYSIS Software (NanoTemper Technologies GmbH, Munich, Germany). To do so, unlabeled proteins were used and size exclusion chromatography was performed prior to the fluorescence measurements to avoid unspecific binding of the ligand DHBV due to impurities. Next, an increasing amount of DHBV was mixed with a constant concentration of each enzyme. The complex formation of the enzymes with the bilins affected the intrinsic fluorescent properties of this complex. Measuring this fluorescence of the FDBR:DHBV complex at an emission window of $\sim 700 \pm 40$ nm (excitation at 630 ± 20 nm) enabled us to calculate the dissociation constant of the bilin to the FDBRs via the initial fluorescence of this complex (Fig. 4). A dissociation constant (K_d) in the micromolar range was determined for both PebA and PebB (PebA to DHBV = $20.40 \mu\text{M} \pm 4.63 \mu\text{M}$; PebB to DHBV = $1.54 \mu\text{M} \pm 0.23 \mu\text{M}$). In contrast to earlier measurements [5], the affinity of DHBV is significantly

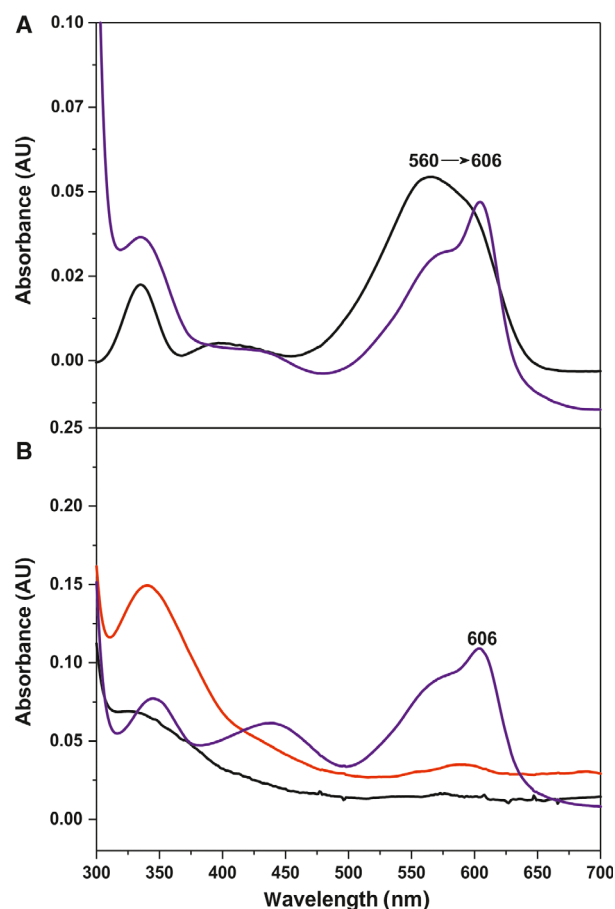


Fig. 3. Interaction between PebA and PebB results in the direct transfer of DHBV. (A) Binding spectra of PebB:DHBV complex in comparison to the protein free bilin via UV-Vis spectroscopy. A total of 10 μM PebB was incubated with an equimolar amount of DHBV in TES/KOH buffer, pH 7.5 (violet line). The black line shows protein-free DHBV with a concentration of 10 μM in the TES/KOH buffer, pH 7.5. (B) UV-Vis spectra of the transferred reaction product from an OCA with GST-PebA and His-tagged PebB. The OCA columns were prepared as described by adding 10 μM GST-PebA with an equimolar amount of BV to GST-sepharose and a ferredoxin-dependent bilin reductase assay was employed by adding all required components (including the oxygen scavenging system) to the column. After 10 min, the column was washed with 2 CV washing buffer (red line) and an additional washing with 3 CV washing buffer containing His-tagged PebB (violet line). The black line shows the absorbance of the flow through right after the column cap was removed. All spectra were smoothed with Origin® 2017.

(approx. 10-fold) lower toward PebA than toward PebB. However, in the light of the required transfer it seems reasonable, especially since we were able to show that the affinity is still high enough that a PebA:DHBV complex is maintained during the washing of the column. Therefore, these new results

strengthen our postulate for metabolic channeling of the intermediate DHBV.

A *PebA*/*PebB* translational fusion protein showed *PebS*-like activity

In cyanobacteria, the formation of PEB requires the activity of two subsequent FDBRs, *PebA* and *PebB*. Often, the encoding genes are located next to each other in either a bi- or a tricistronic operon together with a gene encoding for a heme oxygenase. In *Synechococcus* sp. WH8020, the genes encoding for *pebA* and *pebB* share an overlapping region. The *pebA* stop codon *TGA* is part of the *pebB* start codon *ATG* (Fig. 5). In order to generate a translational fusion

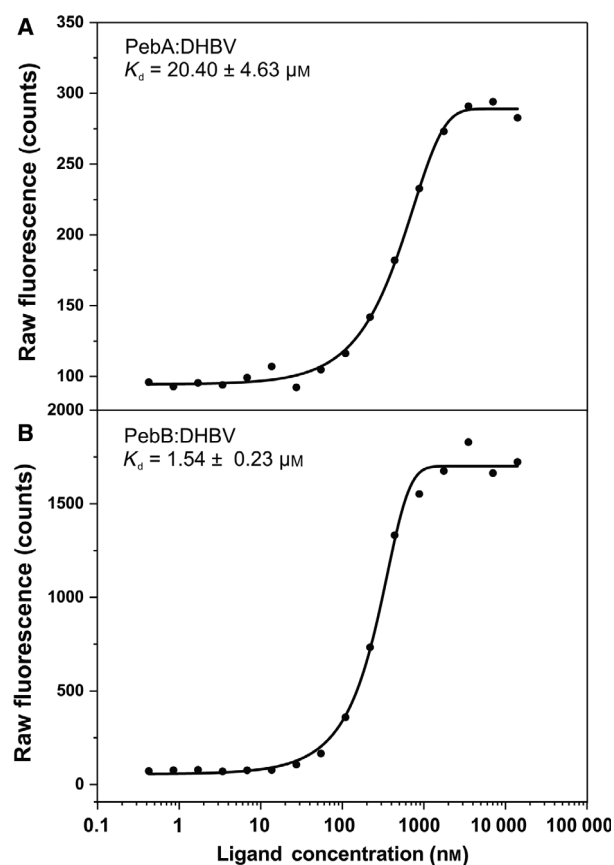


Fig. 4. Affinity of the biosynthetic intermediate 15,16-dihydrobiliverdin to *PebA* and *PebB* using initial fluorescence emission. Analysis of the substrate binding affinity of DHBV to the FDBRs *PebA* (A) and *PebB* (B) using fluorescent titration experiments with unlabeled proteins. The intrinsic fluorescence of DHBV was used to calculate the dissociation constant (K_d) with a fluorescence emission at ~ 700 nm. DHBV concentration was kept constant at $13 \mu\text{M}$, the concentration of either *PebA* or *PebB* was titrated in micromolar excess up to $75 \mu\text{M}$. Experiments were done in triplicate.

between *pebA* and *pebB*, a guanine base was inserted into the start–stop region of the *pebAB*-operon generating an artificial fusion of both enzymes, termed *PebAgB*. The newly generated codon *GTG* encodes for a valine residue, which now serves as a diminutive linker between *PebA* and *PebB* (Fig. 5). This fusion protein is significantly different to the phage encoded *PebS*, which is a homolog to *PebA* (and was originally annotated as such).

PebAgB was heterologously produced and purified using affinity chromatography (Fig. 5) as described. The fusion protein *PebAgB* was tested for activity and the established anaerobic bilin reductase assay was performed in comparison to the cyanophage-encoded bifunctional *PebS*. Enzyme activity was monitored for 10 min via UV-Vis spectroscopy (Fig. 6A). The initial absorbance (Fig. 6A, green line) of the FDBR-BV complex with absorbance at 680 nm decreased during the first few minutes of the reaction. Shortly thereafter, the formation of substrate radical intermediates was monitored indicated by an increase in absorbance at 450 nm and 750 nm [14]. Next, the formation of DHBV was observed, indicated by a simultaneous decrease in the radical absorbance and an uprising absorbance maximum at 580 nm (Fig. 6A, violet line).

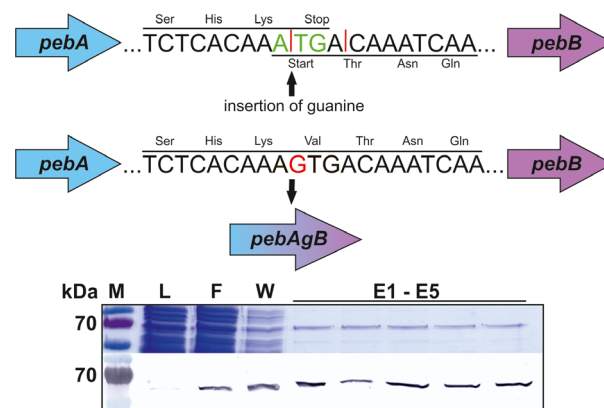
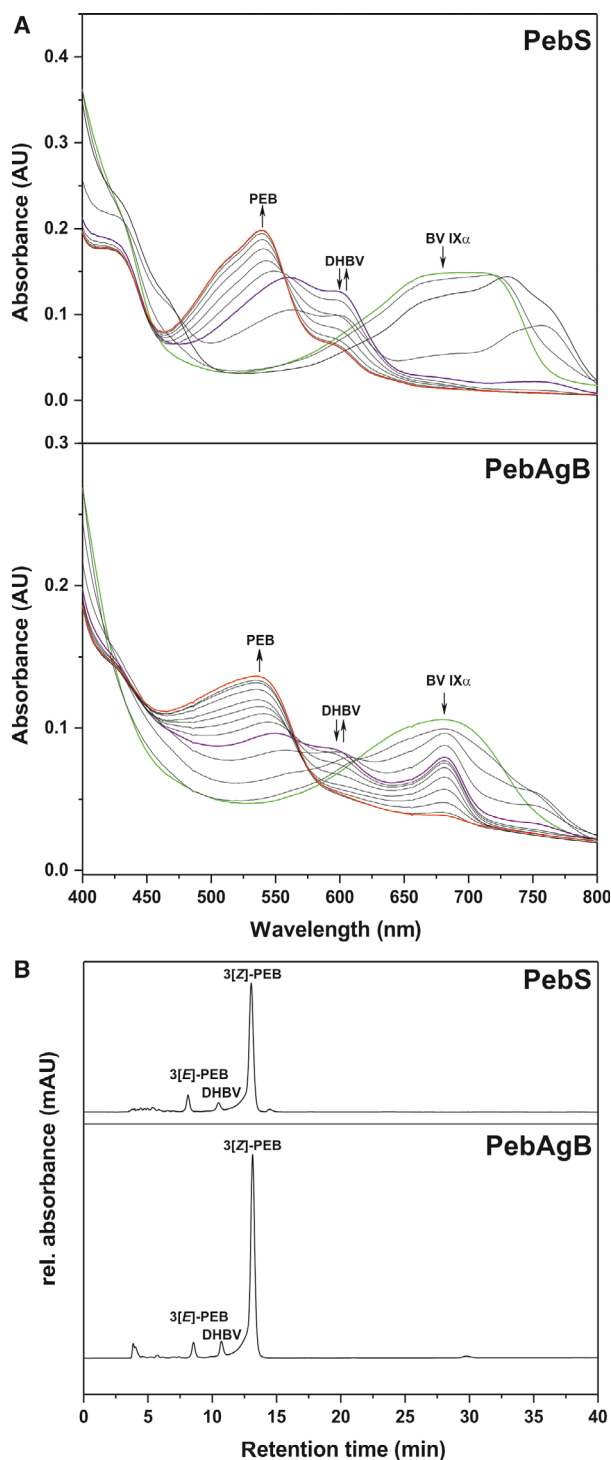


Fig. 5. Construction of the fusion protein *PebAgB*. The construction of the translational fusion protein *PebAgB* was mediated by an insertion of a guanine base into the start–stop (green color = start codon for *pebB*, red bars = stop codon for *pebA*) codon of the *pebAB*-operon via Quikchange[®]-mutagenesis. This insertion resulted in a frameshift of the *pebB* gene (violet arrow) into the translational frame of *pebA* (blue arrow) creating a codon for valine as a diminutive linker between both genes. The derived fusion gene *pebAgB* (blue-violet arrow) was created in the pASK-IBA-45⁺-vector (IBA Life Science GmbH) creating a N-terminal *strep*-tag fusion as well as a C-terminal *his*-tag fusion. Production of the fusion protein was followed by SDS/PAGE and western blot analysis (α -*strep* antibody). MW, molecular weight; L, lysate; F, flow; W, wash; E, elution.



In addition, a prominent absorbance was observed at ~ 680 nm, which decreased over time and with the subsequent formation of PEB. This likely represents the DHBV substrate radical observed during PebB-dependent conversion of DHBV [16]. The last steps of

Fig. 6. The fusion protein PebAgB shows PebS-like activity. (A) Time-resolved UV-Vis spectra of an anaerobic bilin reductase assay using $10 \mu\text{M}$ of PebS, respectively, PebAgB with equimolar amounts of BV. The PEB formation was tracked every 30 s for a maximum reaction time of 10 min. The starting absorbance of the FDBR:BV complex is indicated with a green line, the maximal absorbance of the derived intermediate DHBV is shown as the violet line. The red line displays the end of the PEB formation with the maximal product absorbance. Peak maxima are indicated with the corresponding bilin name (BV IX α , biliverdin IX α , DHBV, 15,16-dihydrobiliverdin, PEB, phycoerythrobilin). For further investigation the reaction products were purified for HPLC analysis. (B) The reaction products were analyzed with HPLC on a Luna $5 \mu\text{m}$ RP C $_{18}$ -column (Phenomenex, Aschaffenburg, Deutschland) with acetone and 20 mM formic acid (50 : 50/v/v) as the mobile phase at a flow rate of $0.6 \text{ mL}\cdot\text{min}^{-1}$. All reaction products were monitored at 560 nm. The PebS reaction products were used as a standard for bilin identification.

the reaction showed a shift of the absorbance maxima from 580 nm to 540 nm (Fig. 6A, red line) which indicated the further reduction of DHBV to PEB. The observed reaction steps of the PebAgB assay in parts resemble the spectral changes in the PebS reaction indicating that the fusion protein is able to convert BV via DHBV to PEB. The identity of the bilins was confirmed via HPLC analysis. The main product of the PebAgB reaction is not only 3(Z)-PEB but also 3(E)-PEB which is produced during this reaction (Fig. 6B). Comparison of the PebAgB-catalyzed conversion of BV with an assay containing both PebA and PebB revealed no significant changes in velocity (data not shown).

Discussion

Substrate or metabolic channeling is a very common phenomenon in metabolism to enhance enzymatic conversion, to protect unstable intermediates from degradation or from competing reactions. Several mechanisms for substrate channeling have been reported. A substrate can be moved from one active site to the other via a flexible arm of the protein, two active sites of two subsequent enzymes can form a tunnel through which the substrate is transferred, or the substrate can be moved via the surface of a protein through electrostatic interactions [17,18].

For over a decade, we have postulated with little experimental evidence that substrate channeling also plays a role during the biosynthesis of the pink tetrapyrrolic pigment PEB [5]. Within this present study we sought to provide better evidence of direct physical interaction of the two involved proteins, PebA and PebB. The channeling of the intermediate DHBV

makes sense in a physiological way, as DHBV is very unstable and prone to oxidative degradation [5,19]. Our data suggest that the interaction of PebA and PebB is not strong enough to trap or isolate a complex. Therefore, the formation of a tunnel in which DHBV is transferred from one active site to the other is unlikely. The recently solved crystal structure of PebB with bound DHBV furthermore argues against the formation of a tunnel. Here, we observed an unexpected horizontally flipped binding mode of DHBV [20]. This flipped binding mode suggests that DHBV has to briefly leave the active site of PebA in order to be bound by PebB. These data, together with our affinity measurements, would rather suggest a so-called proximity channeling, in which the two protein partners come close to each other and the transfer of the intermediate DHBV is facilitated by a much higher affinity to the second protein partner. Pulling out DHBV from the active site of PebA could then also involve the flipping of the molecule and promote the right positioning in the active site of PebB. Recent Brownian dynamics simulations suggest that indeed, the most effective reaction pathway is one in which the active sites are closely aligned. Furthermore, this study pointed out that when the active sites are too close to each other, the ability of the substrate to react with the first enzyme was hindered [21]. Overall, it appears that proximity channeling has only a minor role in enhancing reaction velocity. However, under *in vivo* conditions proximity channeling can have a more pronounced effect [22].

The formation of a functional translational fusion between PebA and PebB was not too surprising but did not help to prove or disprove any physical interaction between both proteins. Functional translational fusions have been described for other enzymes involved in tetrapyrrole biosynthesis. For instance, a functional fusion between cytochrome P450 reductase and heme oxygenase has been reported [23]. In addition, a synthetic fusion protein out of heme oxygenase, the FDBR PcyA, ferredoxin, and ferredoxin-NADP⁺-oxidoreductase has recently been reported [24]. This fusion protein is a valuable tool for the use of light-sensing phytochromes as a new optogenetic tool in mammalian cells. This photoreceptor employs an open-chain tetrapyrrole molecule as its chromophore. As mammalian cells lack the biosynthesis of phycobilins, researchers have now employed this quadruple fusion protein for the biosynthesis of phycocyanobilin in mammalian cells [24]. Judging from the time-resolved spectral analysis of BV turnover by the PebAgB construct compared to the bifunctional phage encoded PebS protein one can suggest that the

conversion within PebS might be different from that of PebAgB. While the conversion with the fusion protein clearly shows distinct absorbance for the intermediate and substrate radicals thereof, this is not seen for PebS (Fig. 6A). This might suggest that in PebS DHBV stays in the active site and is directly converted to PEB, while in PebAgB it is released and rebound by PebB. Future experiments on the exact mechanism and transfer of DHBV might help to unravel the proximity channeling during PEB biosynthesis in cyanobacteria.

Materials and methods

Chemicals and protein labels

All chemicals used in this study were ACS grade or better unless stated otherwise. All FDBR assay chemicals were purchased from Sigma-Aldrich except for BV, which was obtained from Frontier Scientific (Logan, UT, USA). Restriction enzymes, PhusionTM DNA polymerase and T4-DNA ligase were obtained from Thermo Fischer Scientific (Dreieich, Germany). The Monolith Protein Labeling Kit BLUE-NHS (amine reactive) was obtained from Nanotemper Technologies GmbH (Munich, Germany). Glutathion-SepharoseTM 4FF, PreScissionTM Protease and expression plasmid pGEX-6P-1 were obtained from GE Healthcare (Munich, Germany). The expression plasmid pASK-IBA45⁺ and Strep-Tactin[®] resin were obtained from IBA Lifesciences GmbH (Göttingen, Germany).

Cloning of expression constructs, the *pebA-pebB* operon, and site-directed mutagenesis to create a translational fusion protein

The overlapping genes *pebA* and *pebB* of *Synechococcus sp.* WH8020 were amplified via PCR from genomic DNA and ligated into the pASK-IBA45⁺ (IBA Lifesciences GmbH) fusing *pebA* with a 5'-*strep*-tag and *pebB* with a 3'-*His*-tag (pASK_{pebAB}). The primers used for amplification of the genes were *pebA*fwd: 5'- CGGAATTCC ATGTTTGATT CATTCTCAATG – 3' and *pebB*rev 5' - CTCTCGAGCG TAGATCAAAAAGCA CAGTG – 3'. The underlined sequences indicate the restriction enzyme sites *Eco*RI and *Xho*I. Both genes can be coexpressed and purified with appropriate affinity chromatography techniques (Strep-tactin-agarose for PebA, TALON[®]-metal affinity resin for PebB, data not shown). In addition, *pebA* and *pebB* were individually cloned into pASK-IBA45⁺ to generate the respective fusions to Strep-tag for PebA and His-tag for PebB. For *pebA* cloning the *pebA*fwd primer in combination with the *pebA*rev primer 5'-CTCTCGAGTCATTGTGAGAGGAGGA-3' was employed. Similarly *pebB* was amplified using the *pebB*fwd primer 5'-CTTGCTAGCCA TGACAAATCAAAGATTCAAAGC-3' together with the *pebB*rev primer listed above.

For the insertion of a guanine base into the cloned operon to generate a translational fusion between *pebA* and *pebB*, the QuikChange® Lightning Kit (Agilent Technologies, Waldbronn, Germany) was used by following the manufacturer's instructions. The above-mentioned construct pASK*pebAB* served as the template for the base exchange. The primer used for the site-directed mutagenesis (for clarity only the forward primer shown; the reverse primer is complementary to the forward primer) was 5'-CC GCCTCCTCCTCTCACAAGTGACAAATCAAAGATT CAAAAGC-3'. The site of mutation is shown underlined.

Production and purification of recombinant proteins

The production of recombinant PebA, PebB, and PebS as GST-fusions were performed as described previously and employed the plasmids pGEX*pebA*, pGEX*pebB*, and pGEX-*pebS* [5,6]. The Strep-tagged proteins encoded in the pASK-IBA vector were produced in a similar way in *E. coli* BL 21(DE3). LB medium containing the appropriate antibiotic was inoculated 1 : 100 with an overnight culture of *E. coli* BL21 (DE3) previously transformed with pASK-IBA45⁺-*pebAgB*, pASK*pebA*, or pASK*pebB*. The cells were cultivated at 37 °C with shaking (100 rpm, Innova® 44 New Brunswick Scientific, Eppendorf AG, Hamburg, Germany) until a final OD₅₇₈ of ~ 0.6–0.8 was reached. After a temperature shift to 17 °C, protein production was then mediated by an addition of 200 ng·mL⁻¹ anhydrotetracycline followed by an incubation for 18 h with shaking at 100 rpm. Cells were harvested by centrifugation for 10 min at 15 250 g (Sorvall™ LYNX™ 6000 centrifuge, rotor F9) and 4 °C. The cells were then stored at -20 °C or directly lysed for protein purification.

Harvested cells containing GST-tagged proteins were resuspended in 50–100 mL PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and 1 mg·mL⁻¹ of each lysozyme and DNase I (Applchem, Darmstadt, Germany) was added to the resuspended cells. Cell disruption was conducted by passage through a microfluidizer (Microfluidics Corporation, Newton, MA, USA) at 15 000 psi. This process was repeated twice under cold conditions. Then, the cell debris were sedimented by centrifugation for 1 h at 43 000 g and 4 °C (Sorvall™ LYNX™ 6000 centrifuge, rotor T29) and the supernatant was filtered with 0.45-µm RC filters (Carl Roth, Karlsruhe, Germany) for the following affinity chromatography. The cells containing the Strep-tagged proteins PebAgB or PebA and the His-tagged PebB were lysed the identical way as described above, but only the lysis buffer was exchanged to Buffer W (150 mM NaCl, 100 mM Tris/HCl, 1 mM EDTA, pH 8.0) according to the manufacturer's instruction. Filtered lysate was loaded on a 5-mL GST-trap FF column, 5-mL Strep-trap FF column, and affinity chromatography was performed according to the manufacturer's

instructions. Fractions with GST-tagged proteins were pooled and used for PreScission™ Protease cleavage (2 Units·mg⁻¹ protein) for tag removal overnight. Then, the protein solution was loaded onto another 5 mL GST-trap FF column to separate the tag from the protein. Proteins were then further purified using size exclusion chromatography (SEC). Each protein solution was loaded onto a HiLoad Superdex 75 pg 16/600 GL (GE Healthcare). The column was previously equilibrated with either assay buffer (25 mM TES/KOH, 100 mM KCl, pH 7.5) for FDBR assays or with Na-phosphate buffer (60 mM Na-Phosphate, 150 mM NaCl, pH 7.5) for affinity determination experiments. Fractions containing the FDBRs were pooled and concentrated using Vivaspin® concentrators (Sartorius, Göttingen, Germany) and the final protein concentration was determined using their absorbance at 280 nm [25]. The extinction coefficients used for concentration determination were calculated by using the protein calculator (<http://protcalc.sourceforge.net/>). For storage, a final concentration of 15% glycerol was added to the protein solutions, then frozen in liquid nitrogen and kept at -80 °C.

Anaerobic bilin reductase activity assay and bilin production

The bilin reductase assay was performed as described previously with a few modifications [14]. The employed assay contained final concentrations of 10 µM of enzyme and BV, 1 µM ferredoxin PetF from cyanophage P-SSM2 (Fd_p-SSM2), 0.1 µM PetH from *Synechococcus* sp. PCC7002 for the reduction of PetF [26]. The reaction was started by the addition of the NADPH-regenerating system containing 27 µM NADP⁺, 2.2 mM glucose-6-phosphate, and 0.37 U·mL⁻¹ glucose-6-phosphate dehydrogenase. The oxygen scavenging system (OSS) consisted of 50 U·mL⁻¹ glucose oxidase, 100 mM glucose, and 50 U·mL⁻¹ catalase. After full substrate conversion, the reaction was stopped by the addition of a 10-fold excess of 0.1% trifluoroacetic acid (TFA) to the assay. For the production of DHBV, the PebA assay was performed in an identical way, but after full substrate conversion, an additional 10 µM of BV was added to the assay solution [16]. This process was repeated at least five times to accumulate as much DHBV as possible (PebA-feeding assay). Enzymatically produced bilins were then isolated separating them on C₁₈-Sep-Pak columns (Waters, Milford, CT, USA) following lyophilization for 16 h in the Alpha 2-4 LSCplus lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

HPLC analysis and extraction of phycobilins

The reaction products of the bilin reductase activity assay were analyzed on a Luna 50 µm reverse phase C₁₈-column (Phenomenex, Aschaffenburg, Germany) performed on an

Agilent 1100 series HPLC. The column was equilibrated with the mobile phase containing 50% (v/v) acetone and 50% (v/v) 20 mM formic acid. The isocratic elution was performed with a constant flow rate of 0.6 mL·min⁻¹. To isolate the DHBV of the PebA-feeding assay, the reaction product was collected directly after the outlet of the DAD detector and immediately frozen at -80 °C for lyophilization as described above.

Development of an 'on-column' assay for FDBRs

Based on our previous results we suggested a transient interaction of both PebA and PebB during the conversion of BV to PEB. To get deeper insights into this interaction we developed an adapted FDBR assay with one interaction partner immobilized on a chromatography column. For this study, PebA was produced using the pGEX*pebA* construct providing a GST-fusion with this enzyme. In contrast to the manufacturer's protocol, no PreScissionTM-protease cleavage was performed and, therefore, the GST-PebA fusion protein was just further purified via gel filtration. The required PebB for this experiment was produced via the pASK*pebB* construct providing a C-terminally His-tag fusion. Due to the different tag, this protein will not bind to the column. The reaction time for the 'on-column' assay was calculated by a previously performed GST-PebA assay until a full substrate conversion was detected. This control assay was performed ahead of each 'on-column' assay. For all 'on-column' assays a reaction time of 10 min was used.

The so-called 'on-column' assay was performed by binding 10 µM of preincubated GST-PebA:BV on 500 µL GST-sepharose, equilibrated with an assay buffer (see above). Next, all required assay components were dissolved as described above and added to the column, which had been locked via the provided column cap (Bio-Rad Laboratories GmbH, Rüdigenheim, Germany). After an incubation time of 10 min, then the column cap was removed and the column was washed with 2 CV of assay buffer and followed by the elution of GST-PebA with elution buffer (see above). Each fraction was collected and diluted 1 : 10 with 0.1%-TFA. Produced bilins were then isolated according to the standard purification process and analyzed via HPLC.

The next 'on-column' experiment performed was identical to the previous one but after the first washing step, another washing step was added with three CV assay buffer containing 10 µM PebB (His-tagged). The subsequent washing, elution, and analysis were performed as described before. Here, the fractions were also analyzed via UV-Vis spectroscopy to verify the typical FDBR: bilin complex formation.

A coupled enzyme assay was performed by setting up the 'on-column' assay as in the previous experiments but after 5 min of reaction time an equimolar amount of GST-PebB (produced and purified identically to GST-PebA) was added to the column material. After an additional 5 min, the column was washed with 2 CV assay buffer followed

by the elution of the proteins with 3 CV elution buffer (see above). The subsequent washing, elution and analysis were performed as stated earlier. All obtained data were then normalized with Origin[®] 2017 by plotting the retention time of all produced bilins against the standards.

Fluorescence titration

To determine the affinity of PebA and PebB to DHBV, fluorescence titration experiments were performed. Therefore, a dilution series of either PebA or PebB in the micromolar (140 µM starting concentration) range in Na-phosphate buffer was prepared and a constant concentration of DHBV was added in the micromolar range (13 µM) to each sample. Protein-bilin affinity was then measured using MonolithTM NT.115 Standard Capillaries in a Monolith NT.115 instrument (Nanotemper Technologies GmbH, Munich, Germany). The measurements were performed at MST power 20 & 40% and LED power 20 & 40% using the 'red' filter of the instrument to detect the intrinsic fluorescence of the FDBR: bilin complex. The fluorescence titration experiments were done in triplicate and evaluated using the MO.AFFINITY ANALYSIS Software (NanoTemper Technologies GmbH).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MA and NFD designed the study and wrote the manuscript with help and input of all authors. MA, VH, and JH performed experiments. MA, VH, MN, and NFD analyzed data.

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