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HO1 and PcyA proteins involved in phycobilin biosynthesis form a 1:2 complex with ferredoxin-1 required for photosynthesis

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ABSTRACT

The *HO1* and *PcyA* genes, encoding heme oxygenase-1 (HO1) and phycocyanobilin (PCB):ferredoxin (Fd) oxidoreductase (PcyA), respectively, are required for chromophore synthesis in photosynthetic light-harvesting complexes, photoreceptors, and circadian clocks. In the PCB biosynthetic pathway, heme first undergoes cleavage to form biliverdin. I confirmed that Fd1 induced the formation of a stable and functional HO1 complex by the gel mobility shift assay. Furthermore, analysis by a chemical cross-linking technique designed to detect protein-protein interactions revealed that HO1 and PcyA directly interact with Fd in a 1:2 ratio. Thus, Fd1, a one-electron carrier protein in photosynthesis, drives the phycobilin biosynthetic pathway.

Structured summary:
MINT-7014657: *Fd1* (uniprotkb:P0A3C9) and *HO1* (uniprotkb:Q8DLW1) *bind* (MI:0407) by *comigration in non-denaturing gel electrophoresis* (MI:0404)
MINT-7014666: *HO1* (uniprotkb:Q8DLW1 and *Fd1* (uniprotkb:P0A3C9) *bind* (MI:0407) by *cross-linking studies* (MI:0030)
MINT-7014675: *PcyA* (uniprotkb:P59288) and *Fd1* (uniprotkb:P0A3C9) *bind* (MI:0407) by *cross-linking studies* (MI:0030)

1. Introduction

In cyanobacteria, phycocyanobilin (PCB), a chromophore of the phytochrome [1] family of photoreceptors [2], photosynthetic light-harvesting complexes, and circadian clocks [3], is synthesized from heme via biliverdin (BV) IXα (Fig. 1) [4]. PCB synthesis from heme comprises 2 enzymatic steps—the conversion of heme to BV IXα and reduction of BV IXα to PCB [5,6]. The thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 (Te) lacks genes required for biliverdin reductase (BVR) [7] and PebB [8] syntheses. Recently, cyanobacterial *HO1* and *PcyA* genes were cloned [5,6]. The amino acid sequence encoded by cyanobacterial *HO1* shows low but significant homology with those of heme oxygenases (HOs) of non-photosynthetic organisms. *PcyA* belongs to the bilin reductase family. The cyanobacterial *HO1* and *PcyA* genes encode HO and bilin reductase, respectively, which are required for phytochrome chromophore biosynthesis. *HO2*, an additional gene for HO, has recently been detected in the cyanobacterial genome; *HO2*, which is partly homologous to *HO1*, may be involved in phytochrome chromophore biosynthesis [6,9].

In this biosynthesis, HO1 and phycocyanobilin:ferredoxin oxidoreductase (PcyA) first form stable complexes with heme and BV IXα molecules, respectively. HO catalyzes stereospecific cleavage yielding BV IXα from heme, accompanied by the release of Fe^{2+} and carbon monoxide (CO) [10,11]; PcyA reduces BV IXα forming PCB. HO1 and PcyA depend on ferredoxin (Fd). The properties of these enzymes indicate that Fd, which functions as a one-electron carrier, is the preferred electron donor for HO1 and PcyA. Bacterial two-hybrid analyses are unable to detect protein–protein interactions between cyanobacterial HO1 or PcyA and Fd1 [12]. Thus, the redox partner of cyanobacterial HO and PcyA remains unidentified.

To understand the action mechanisms of TeHO1 and TePcyA and compare them with those of their counterparts occurring in plants, other cyanobacteria, and algae, I purified recombinant

Abbreviations: HO, heme oxygenase; PCB, phycocyanobilin; Fd, ferredoxin; PcyA, phycocyanobilin:ferredoxin oxidoreductase; BV, biliverdin; BVR, biliverdin reductase; CO, carbon monoxide; Te, *Thermosynechococcus elongatus* BP-1; LB, Luria–Bertani; PCR, polymerase chain reaction; β-ME, β-mercaptoethanol; FNR, ferredoxin-$NADP^+$ oxidoreductase; PAGE, polyacrylamide gel electrophoresis; NADPH, reduced nicotinamide adenine dinucleotide phosphate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; BS^3, bis-(sulfosuccinimidyl) suberate; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography

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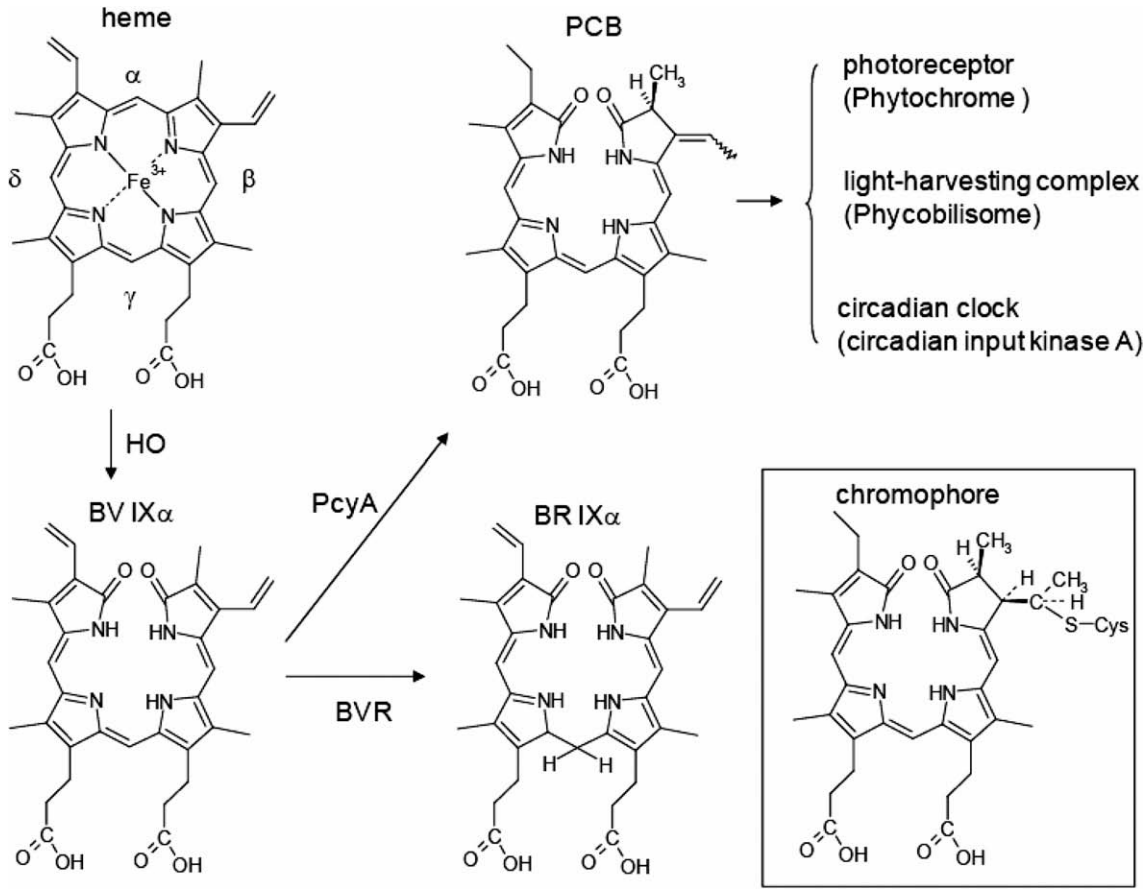

Fig. 1. Phycobilin biosynthetic pathway in cyanobacteria showing the formation of PCB from heme. The first step comprises HOI-catalyzed heme degradation.

TeHO1 and TePcyA expressed in *Escherichia coli* and characterized the reactions catalyzed by these enzymes. A chemical cross-linking analysis technique designed to detect protein–protein interactions demonstrated that HO1 and PcyA directly interacted with Fd1 (1:2); this indicates that TeHO1 and TePcyA are strongly expressed and depend on Fd. This report discusses these attributes in a functional context.

2. Materials and methods

2.1. Reagents

Enzymes for DNA manipulation (New England Biolabs); agar and organic nutrients for Luria–Bertani (LB) medium (Difco); hemin and BV IXα preparations for use as substrates and/or high-performance liquid chromatography (HPLC) standards (Frontier Scientific Inc. and Sigma); and HPLC-grade acetone, ethanol, water, and acetic acid (Nacalai Tesque) were purchased.

2.2. Cloning of relevant T. elongatus BP-1 genes

Standard procedures were adopted for most DNA manipulations. Gene sequences and their corresponding accession numbers were obtained from the CyanoBase database (Kazusa DNA Research Institute). All relevant genes were amplified from the genomic DNA of Te using polymerase chain reaction (PCR). All amplicons were sequenced to verify their fidelity.

2.3. Cloning of the HO1-encoding gene in T. elongatus BP-1

TeHO1NdeI (forward) and TeHO1XhoI (reverse) primers were used to amplify the *HO1* gene (tll0365). The resulting 0.72-kb PCR fragment was cloned into pET21b (+) with NdeI and XhoI (Novagen) to yield the pET21b–TeHO1 plasmid.

2.4. Cloning of the PcyA-encoding gene in T. elongatus BP-1

TePcyANdeI (forward) and TePcyAXhoI (reverse) primers were used to amplify the *PcyA* gene (tll2308). The resulting 0.71-kb PCR fragment was cloned into pET21b (+) digested with NdeI and XhoI (Novagen) to yield the pET21b–TePcyA plasmid.

2.5. Expression and purification of recombinant proteins

E. coli BL21 (DE3; Novagen) were transformed with either the pET21b–TeHO1 or pET21b–TePcyA plasmids and incubated overnight at 37 °C in a 100-mL LB medium containing 1% glucose. This culture (80 mL) was inoculated into LB medium (8 L) and grown at 37 °C up to the mid-log phase. Subsequently, 1 mM isopropyl β-D-thiogalactopyranoside was added to induce HO1 and PcyA; the medium was incubated overnight at 20 °C. The cells were harvested and lysed with a lysis buffer (50 mM HEPES-NaOH, pH 7.5) containing 10 mM β-mercaptoethanol (β-ME) and 50 mM NaCl; the cell lysate was sonicated on ice for 30 s and centrifuged at 50 000×*g* for 30 min. The resulting supernatant was loaded onto a DE52 column (Whatman). Next, 25% ammonium sulfate was added to the column. The fraction was separated by hydrophobic

column chromatography on a butyl-Toyopearl 650 M column (Tosoh) and further purified by hydroxyapatite column chromatography using a Cellulofine HAp column. The resulting protein fraction was concentrated (3.7 mL) on an Amicon Ultra-15 filter unit (10-kDa cutoff; Millipore) and ultimately purified by gel filtration on an XK 26/100 Sephacryl S-200 HR column (GE Healthcare UK Ltd.). The protein was concentrated (18 mg/mL) and rebuffered (50 mM HEPES-NaOH containing 100 mM NaCl and 10 mM β-ME; pH 7.5) using prepacked Sephadex G-25 gel filtration columns (NAP-10; GE Healthcare UK Ltd.).

2.6. Expression and purification of recombinant Fd1 and FNR

Expression and purification of recombinant Fd1 and ferredoxin-$NADP^+$-reductase (FNR) of *T. elongatus* were performed as described earlier [12].

2.7. Heme- and BV IXα-binding gel mobility shift assay

HO1 and PcyA were added to 10 μM hemin (final concentration) and a chemically synthesized BV IXα solution, respectively, in 50 mM HEPES-NaOH buffer (total volume, 500 μL; pH 7.5) containing 100 mM NaCl. The substrate complex containing HO1 or PcyA was detected by 15–25% native-gradient polyacrylamide gel electrophoresis (PAGE).

2.8. HO and PcyA assays

HO and PcyA activities were assayed at room temperature (25 °C) in 500 μL of 50 mM HEPES-NaOH buffer (pH 7.5) containing 50 μM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 mM glucose-6-phosphate (G6P), 0.5 units G6P dehydrogenase (G6PDH), 7.2 nM TeFNR, 2.4 μM and 0.24 μM TeFd1 (HO assay) and 0.5 μM TeFd1 (PcyA assay), 10 μM hemin substrate, and 10 μM TeHO1. The reactions were initiated with Fd. Heme cleavage and BV IXα reduction catalyzed by HO1 and PcyA, respectively, were monitored spectrophotometrically with a Shimadzu UV-2500PC UV–Visible spectrophotometer.

2.9. Gel mobility shift assay for HO1–Fd1 and PcyA–Fd1 complexes

HO1 and PcyA proteins were added to 50 mM HEPES-NaOH buffer (pH 7.5) containing 150 mM NaCl and 2 mM Fd1. HO1–Fd1 and PcyA–Fd1 complexes were detected by 15–25% native-gradient PAGE.

2.10. Chemical cross-linking analysis

The proteins were incubated at room temperature for 30 min in a conjugation buffer containing 20 mM HEPES-NaOH (pH 7.5) and 150 mM NaCl in the presence of the cross-linker bis-(sulfosuccinimidyl) suberate (BS^3). This reaction was terminated with a quenching buffer (final concentration of Tris–HCl, 50 mM; pH 7.5). The ionic strength, pH, protein concentration, and reaction time were optimized.

3. Results

3.1. Expression and purification of TeHO1 and TePcyA

E. coli BL21 (DE3) cells were transformed with pET21b–TeHO1 or pET21b–TePcyA. TeHO1 or TePcyA expressions were induced with isopropyl-β-D-thiogalactopyranoside. TeHO1 (27 kDa) and TePcyA (26.9 kDa), expressed as soluble proteins, were detected by sodium dodecyl sulfate (SDS)–PAGE (Fig. 2). Recombinant

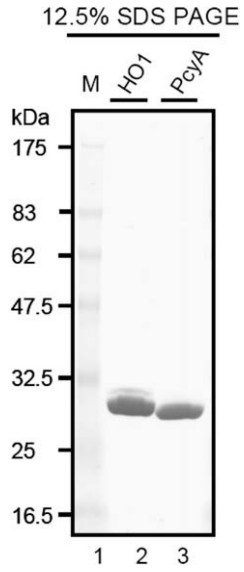

Fig. 2. Purification and SDS–PAGE analysis of recombinant TeHO1 and TePcyA. Lane 1 contains molecular mass markers; lane 2, purified TeHO1; and lane 3, purified TePcyA. Protein fractions in lanes 2 and 3 were used for further analysis.

TeHO1 and TePcyA were purified through anion-exchange, involving hydrophobic interactions and hydroxyapatite, and gel filtration chromatography; they were identified as single 27-kDa bands (Fig. 2, lanes 2 and 3). A 1-L culture yielded ~300 mg each of purified TeHO1 and TePcyA. Absolute activities of TeHO1 and TePcyA were maintained even after 6 months of storage (−30 °C) in 50 mM HEPES-NaOH buffer (pH 7.5) containing 100 mM NaCl.

3.2. Heme–HO1 and BV IXα–PcyA complex formation

It is unusual that HO and PcyA form stable complexes with heme and BV, respectively. To investigate whether recombinant TeHO1 and TePcyA also form such complexes, these proteins were incubated with heme and BV, respectively, and gel mobility shift assays involving native-gradient PAGE were performed to detect complex formation (Fig. 3). The absorption spectra of heme–TeHO1 and BV IXα–TePcyA complexes are provided (Supplementary data). The bands of the substrate complexes—heme–HO and BV IXα–PcyA—moved downward on the gel (Fig. 3). The results indicated that the heme–TeHO1 and BV IXα–TePcyA complexes were very stable.

3.3. Spectrophotometric evidence for HO and PcyA activities and phycobilin synthesis

To verify that TeHO1 and TePcyA are HO and bilin reductase, respectively, I monitored TeHO1- and TePcyA-catalyzed reactions spectrophotometrically (Fig. 4). When TeHO1 and heme were incubated with reduced TeFd1, the Soret peak present at 401 nm disappeared (TeHO1–heme complex) and two peaks with maxima at 534 and 575 nm appeared (Fig. 4A). These peaks indicate the presence of a ferrous dioxyheme complex [13]. When Fd was included in the assay as an electron carrier protein, the assay mixture produced a broad peak at 650–670 nm, indicating BV IXα formation (Fig. 4A inset). No BV IXα formation was detected when Fd was excluded from the assay (Fig. 4A). When TePcyA and BV IXα were incubated with reduced TeFd1, no peak was observed at 665 nm (TePcyA–BV IXα complex) but a peak with maxima at 724 nm appeared (Fig. 4B); this indicates the presence of an intermediate complex [14]. When Fd was included in the assay, the assay mixture peaked at ~600 nm, indicating PCB formation (Fig. 4B inset).

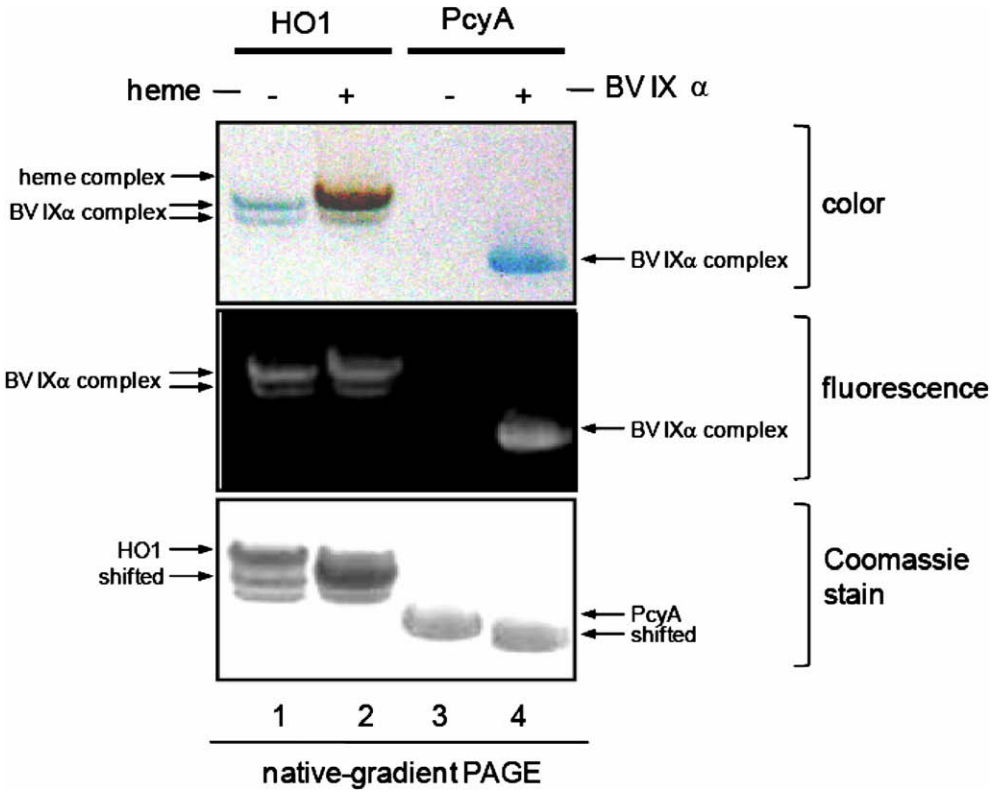

Fig. 3. Gel mobility shift assay involving native-gradient PAGE to detect the binding of HO1 and PcyA to their substrates. The proteins are identified by the colors emitted from BV IXα and heme (upper panel), UV-excited fluorescence of BV IXα (middle panel), and Coomassie blue stains (lower panel). HO1 (lanes 1 and 2) and PcyA (lanes 3 and 4) were separated by native-gradient PAGE (15–25%).

When Fd was excluded from the assay, no PCB formation was detected (Fig. 4B). Cyanobacterial HO and PcyA proteins use Fd as the principal reductant. In the control experiments, wherein Fd was excluded from the reactions (Fig. 4), BV IXα and PCB formation was not detected; this indicates that Fd is essential for these catalytic reactions.

3.4. Gel mobility shift assay and chemical cross-linking analysis

To investigate whether HO1 and PcyA form stable complexes with Fd1, the proteins were incubated with Fd1 functioning as a redox partner. Complex formation was detected by performing a gel mobility shift assay involving native-gradient PAGE (Fig. 5A). The band of the HO1–Fd1 complex moved downward on the gel (Fig. 5A, lane 4), indicating that the complex was stable in vitro. The PcyA–Fd1 complex was not detected by the gel mobility shift assay. The results indicate that HO1 interacts with Fd1 more aggressively than PcyA.

Chemical cross-linking was performed to analyze the oligomeric state of HO1 and PcyA in the presence of Fd1 in vitro. When HO1 or PcyA was incubated with Fd1 in the presence of BS^3, the complex bands stained with Coomassie brilliant blue were observed to be ~47.5 kDa (Fig. 5B, lanes 5 and 6). The calculated molecular masses of HO1, PcyA, and Fd1 are 27, 26.9, and 10.8 kDa, respectively; HO1 or PcyA appear to form ~38-kDa complexes with Fd1. No other 38-kDa cross-linked products were detected, suggesting a 1:2 ratio of HO1 or PcyA to Fd1 in the complex.

4. Discussion

The results described here indicate that the mechanisms underlying heme cleavage and BV IXα reduction, which together result in the production of PCB from heme, are conserved in *T. elongatus*, *Synechocystis* PCC6803 (HO1), and *Anabaena* PCC7120 (PcyA) (Supplementary data). Moreover, TeHO1 and TePcyA could to bind with heme and BV IXα, respectively; each formed stable complexes, which were directly observed by the gel mobility shift assay designed to detect substrate–enzyme interaction. These findings suggest that the molecular structure of the heme- or BV-binding site is conserved in thermophilic and mesophilic cyanobacteria.

Different aspects of HO1 and PcyA formation are conserved. Further, the source of reducing equivalents is highly conserved in organisms such as algae [15,16] and higher plants [17–19]. Cyanobacterial HO and PcyA use reduced Fd [5,9,13,14]. To investigate whether the biochemical properties of proteins are conserved in cyanobacteria and higher plants, the reductant requirements of thermophilic cyanobacterial enzymes were determined; the results demonstrated that reduced Fd1 sustains TeHO1 and TePcyA activities (Fig. 4). Moreover, Fd interacts with and directly promotes TeHO1 and TePcyA activities in the presence of NADPH and FNR; this finding supports the view that Fd functions as the only electron donor in vivo. This is probably because *T. elongatus* lacks genes of electron carrier proteins such as flavodoxin [20,21]. Fd seems to be used as an electron donor in all oxygenic photosynthetic organisms investigated thus far. Among ferredoxin-dependent enzymes, HO1 and PcyA have the unique ability of catalyzing 7- and 4-electron reduction of heme and BV IXα, respectively. However, it is unlikely that Fd transfers only one electron to its dependent enzymes because HO1 and PcyA interacted with Fd more than once. Thus, multiple interactions between HO1/PcyA and Fd1 may be weak. In fact, PcyA reacted with Fd1 at extremely high binding and dissociation rates (Supplementary data).

Some factors that affect electron transfer are electrostatic and hydrophobic protein interactions, overall dipole moment of

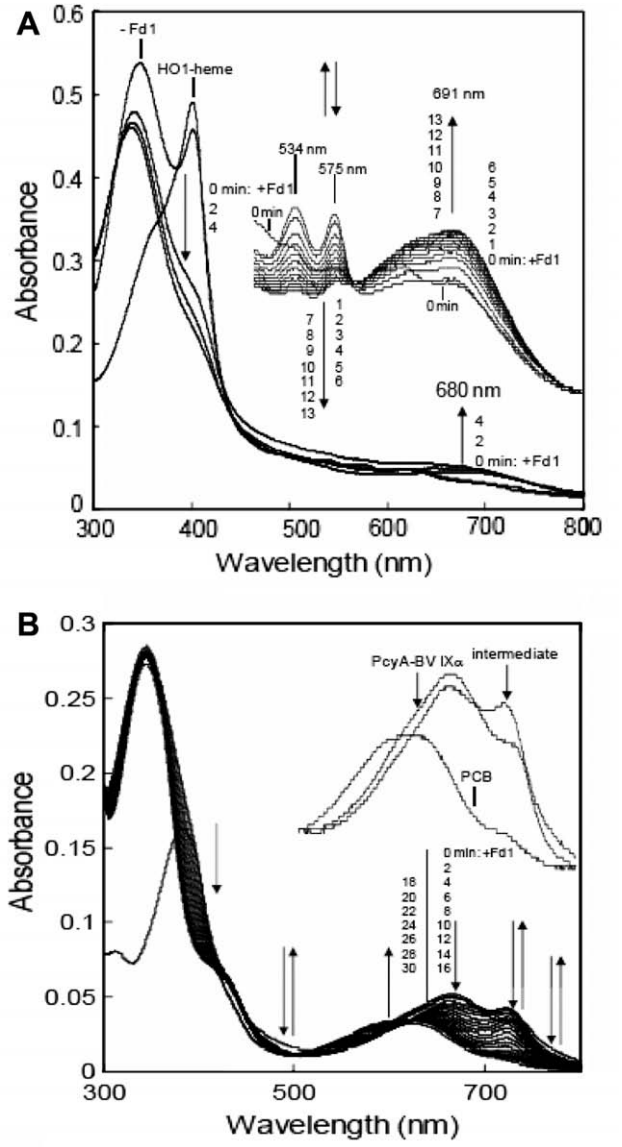

Fig. 4. Absorption spectra of HO1- and PcyA-catalyzed reactions. (A) The time course of the HO1-catalyzed heme conversion reaction was monitored spectrophotometrically at 2-min intervals after the sequential addition of purified Fd1. The spectrum recorded after 4 min indicates BV IXα formation. Inset: time-dependent changes in the spectra were monitored for 12 min at 1-min intervals. The spectra were recorded at the indicated times after adding Fd1 (final concentration, 0.24 μM). (B) Time-dependent changes in the spectra were monitored during the PcyA-catalyzed reaction; the spectra were recorded after adding NADPH, FNR, and Fd1. The absorbance shows an increase followed by decay at 480, 724, and 770 nm (double arrows), a decrease at 383 and 665 nm, and a subsequent increase at 600 nm (single arrows). Inset: absorption spectra of BV IXα-PcyA, intermediate complexes, and PCB in the PcyA-catalyzed reaction.

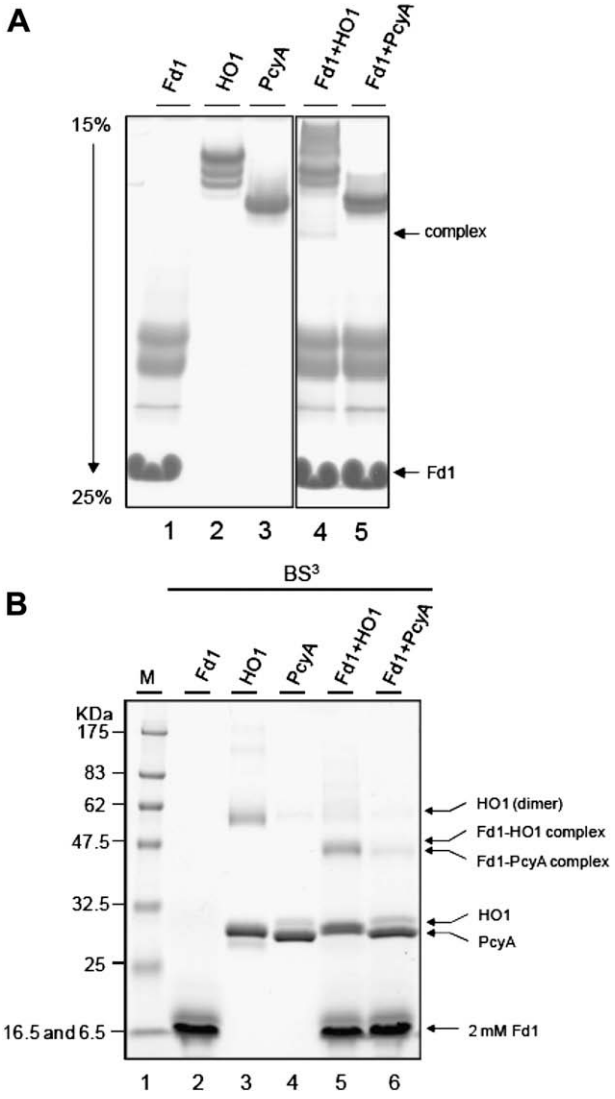

Fig. 5. Detection of Fd1-containing complexes. (A) Gel mobility shift assay involving native-gradient PAGE to detect an Fd1-containing complex. Lane 1 contains Fd1; lane 2, HO1; lane 3, PcyA; lane 4, HO1 and Fd1; lane 5, PcyA and Fd1. Arrows indicate the HO1–Fd1 complex and Fd1. (B) Chemical cross-linking of HO1 and PcyA with Fd1. SDS–PAGE (12.5%) showing patterns of cross-linked mixtures: molecular mass markers (1), Fd1 (2), HO1 (3), PcyA (4), Fd1 + HO1 (5), and Fd1 + PcyA (6). The arrows indicate Fd1, HO1, and PcyA. The chemical cross-linker BS^3 was used in all protein fractions (lanes 2–6).

partner proteins, certain aromatic groups in the electron transfer pathway, and the relative orientation of and distance between prosthetic groups in the complex. The interactions between Fd and its redox partners such as HO1 and PcyA mainly occur because of electrostatic and hydrophobic interactions. Here, the formation of cross-linked 1:2 complexes is demonstrated. The 1:2 complexes were formed later in the reaction, suggesting the existence of cooperation between the redox centers. This model predicts that a ternary complex involving 2 Fd molecules and 1 HO1 or PcyA molecule is formed. The Fe–S clusters of Fd should be close and nearly coplanar to the substrates of HO1 and PcyA.

To my knowledge, this is the first report on HO1 and PcyA characterization relating to interactions with Fd. The results of chemical cross-linking of HO1 and PcyA with Fd indicate that HO1 and PcyA interact with Fd1 and might produce a functional complex that would serve as an electron source for HO1 and PcyA in vivo. Further, an NADPH-dependent redox cascade involving purified recombinant TeFNR and TeFd1 was triggered, which demonstrated electron transfer from Fd to HO1 and PcyA (Fig. 4).

No recent study has demonstrated that HO1 and PcyA directly and/or indirectly interact with Fd. Here, it was demonstrated that TeHO1 and TePcyA feebly interacted with Fd. In cyanobacteria, Fd may function as a natural electron donor for HO1 and PcyA. Other electron carrier proteins are probably unable to serve as efficient redox partners for HO1 and PcyA in Te because the cyanobacterial genome lacks other electron carrier proteins such as flavodoxin, which can sustain reduction reactions catalyzed by HO and PcyA. In Te, reductants other than Fd involved in the phycobilin biosynthetic pathway remain unidentified. I conclude that

the catalytic conversion of heme to PCB may be dependent on Fd, which functions as a one-electron carrier protein, and that HO1 and PcyA form complexes with Fd1 in a 1:2 ratio.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.03.052.

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