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His74 conservation in the bilin reductase PcyA family reflects an important role in protein-substrate structure and dynamics

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ABSTRACT

Phycocyanobilin:ferredoxin oxidoreductase (PcyA) catalyzes the proton-coupled four-electron reduction of biliverdin IXα's two vinyl groups to produce phycocyanobilin, an essential chromophore for phytochromes, cyanobacteriochromes and phycobiliproteins. Previous site directed mutagenesis studies indicated that the fully conserved residue His74 plays a critical role in the H-bonding network that permits proton transfer. Here, we exploit X-ray crystallography, enzymology and molecular dynamics simulations to understand the functional role of this invariant histidine. The structures of the H74A, H74E and H74Q variants of PcyA reveal that a "conserved" buried water molecule that bridges His74 and catalytically essential His88 is not required for activity. Despite distinct conformations of Glu74 and Gln74 in the H74E and H74Q variants, both retain reasonable activity while the H74A variant is inactive, suggesting smaller residues may generate cavities that increase flexibility, thereby reducing enzymatic activity. Molecular dynamic simulations further reveal that the crucial active site residue Asp105 is more dynamic in H74A compared to wild-type PcyA and the two other His74 variants, supporting the conclusion that the Ala74 mutation has increased the flexibility of the active site.

Introduction

Phytobilins are heme-derived linear tetrapyrroles that perform important functions in oxygenic photosynthetic organisms including green plants, cyanobacteria and red algae [1,2]. When attached to proteins (biliproteins) via thioether linkages to cysteine residues, phytobilins function as light sensors and as light-harvesting antennae. Phytobilins attached to phytochromes [3,4] function to regulate photomorphogenesis in plants [5]. Cyanobacteria, red algae, and cryptomonad algae also utilize phytobilins in the light-harvesting phycobilisomes that absorb light and transfer the captured energy to the chlorophylls in the photosynthetic reaction centers [6,7]. Since biliproteins are necessary both for energy capture and for adaptation to the light environment, production of these linear tetrapyrroles is critical to photoautotrophic organisms in which they are present.

The linear tetrapyrrole phycocyanobilin (PCB),[2] comprising the chromophores of algal phytochromes and core phycobiliprotein antennae of cyanobacteria and red algae, is synthesized by the enzyme phycocyanobilin:ferredoxin oxidoreductase (PcyA; EC 1.3.7.5). A member of the ferredoxin-dependent biliverdin reductase (FDBR) family [8], PcyA catalyzes the multi-step reduction of biliverdin IXα(BV) to produce 3*Z*/3*E*-phycocyanobilin (3*Z*/3*E*-PCB). PcyA mediates four one-electron transfers to BV – a ferredoxin-dependent reaction that proceeds via two radical intermediates (first and third electron transfer) [9] and a stable two-electron reduced intermediate 18^1,18^2-dihydrobiliverdin (DHBV) to yield PCB (Scheme 1) [8,10]. DHBV is not detected under steady state conditions, suggesting that this intermediate remains bound to the enzyme until the endovinyl reduction is complete [8,10]. Although the three dimensional structure of PcyA has been extensively studied crystallographically [11–13], a mechanistic understanding of the structural basis for PcyA's reduction regiospecificity remains unclear.

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[2] *Abbreviations used:* PcyA, phycocyanobilin:ferredoxin oxidoreductase; FDBR, ferredoxin-dependent bilin reductase; PCB, phycocyanobilin; BV, biliverdin IXα; DHBV, 18^1,18^2-dihydrobiliverdin IXα; Fd, ferredoxin; FNR, ferredoxin:NADP oxidoreductase; RMSD, root mean squared deviation.

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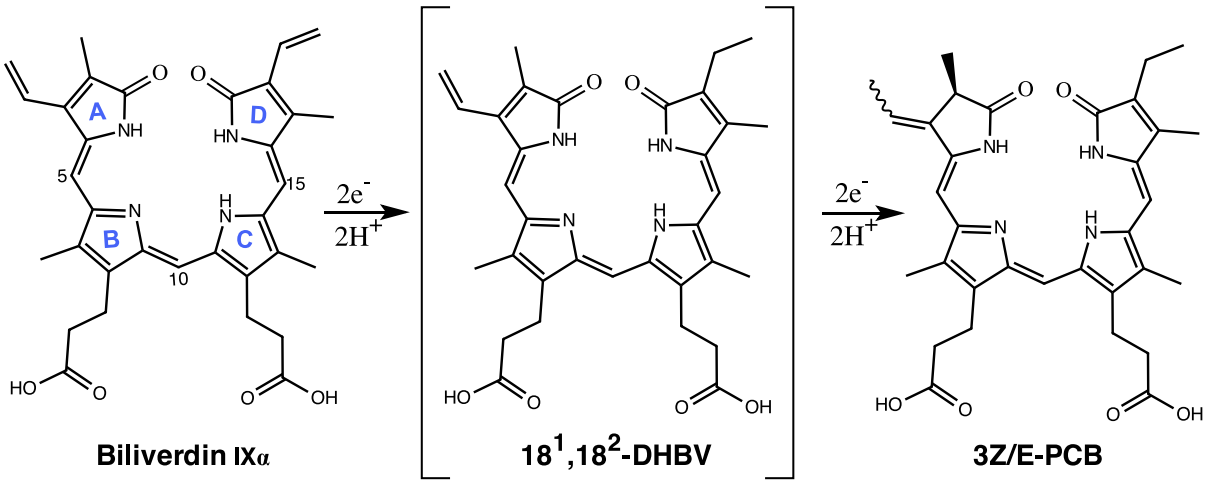

Scheme 1.

With each electron transfer, a proton must be transferred to the bilin substrate [9,12,14]. Site directed mutagenesis has been a powerful tool to help identify residues that mediate this proton donation to the tetrapyrrole substrate. Previously, His88 and Asp105 (*Synechocystis* sp. PCC 6803 PcyA numbering) were shown to be critical for the reduction of both vinyl groups [14]. Crystal structures reveal that these residues interact in the absence of substrate, but move apart upon BV binding to interact with the bilin substrate [11,12,15]. In the substrate-free PcyA structure, the protonated imidazolium group of His88 is ion paired with the carboxylate side chain of Asp105. NMR studies suggest that an imidazolium proton/His88 is transferred to Asp105 uponsubstrate binding, resulting in a mostly charge-neutral pocket with bound BV [13]. Substitution of either residue with a non-proton-donating amino acid prevents transfer of a second electron thereby stabilizing the one-electron reduced BVH**.** radical [16]. In addition to D-ring exovinyl group reduction, His88 also plays a critical role in A-ring endovinyl reduction since the H88Q variant is unable to reduce the A-ring vinyl group of exogenous DHBV [12]. Re-protonation of His88 therefore appears necessary for endovinyl reduction.

The crystal structures of PcyA show that conserved residue His74 is H-bonded via a bridging water molecule to His88 suggesting that His74 plays a role in His88 re-protonation. Moreover, the buried water between His88 and His74 is present whether or not substrate is bound. Interestingly, exchange of His74 with other residues capable of hydrogen bonding merely reduced the activity – i.e. H74Q, H74E and H74 N had 65%, 50% and 46% of wild-type PcyA activity, while the H74A variant was nearly inactive [14]. These data establish that His74 is not an essential proton donor, but implicate a role for this residue to position the bridging water molecule to mediate subsequent proton transfer to the reduced substrate.

The present work was undertaken to correlate the enzymatic activities of PcyA wild type and H74Q, H74E and H74A variants from the cyanobacterium *Synechocystis* sp. PCC 6803 with their corresponding X-ray crystal structures. Surprisingly, the presence of the bridging water is not correlated with the activity of the PcyA variant. While the presence of this bridging water in the H74A variant is apparently inconsistent with its role in proton transfer, a gap in the region near the Ala74 side-chain is observed in the H74A variant. We therefore performed molecular dynamics simulations of native PcyA as well as all three variants, H74Q, H74Q and H74A, in the presence of BV. These simulations reveal that the gap in the region near Ala74 in the H74A variant facilitates flipping of the conserved active site residue Asp105, leading to greater flexibility of BV in the active site. This increase of flexibility accounts for the reduced affinity of the substrate and the loss of activity for theH74A variant.

Materials and methods

Site-directed mutagenesis, expression and purification of Synechocystis PcyA

Synechocystis sp. PCC 6803 PcyA variants were generated from the wild type cloned into the expression vector pTYB12 (New England BioLabs, Ipswich, MA) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). PCR primers used were: ***H74A_F,***CAGTTTCGCAAAATG**GCG**TTGGAGTTGGCCAAG; ***H74A_R,***GTCAAAGCGTTTTAC**CGC**AACCTCAACCGGTTC; ***H74E_F,***CAGTTTCGCAAAATG**GAG**TTGGAGTTGGCCAAG; ***H74E_R,***GTCAAAGCGTTTTAC**CTC**AACCTCAACCGGTTC; ***H74Q_F,***CAGTTTCGCAAAATG**CAG**TTGGAGTTGGCCAAG; ***H74Q_R,***GTCAAAGCGTTTTAC**GTC**AACCTCAACCGGTTC. All new clones, characterized by DNA sequencing (Davis Sequencing, Davis, CA), were transformed into *Escherichia coli* strain BL21-DE3 (Stratagene, La Jolla, CA) for protein expression. After lactose induction at 15 °C for 19 h, variant proteins were purified according to the IMPACT-CN protein purification protocol (New England BioLabs, Beverly, MA) with the following modifications. Cell lysis buffer consisted of 50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 0.25 mM EDTA and 0.1% (v/v) Triton-X 100 and column equilibration buffer consisted of 50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 0.25 mM EDTA. On-column cleavage was performed by addition of 50 mM 2-mercaptoethanol to column equilibration buffer (50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 0.25 mM EDTA) followed by incubation at 4 °C for 48 h. The cleaved protein was eluted with 35 mL of the buffer (50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 0.25 mM EDTA). The purified protein was subsequently dialyzed in 20 mM Tris–HCl, pH 8.0 for 24 h, followed by concentration using Amicon Ultra 10,000 MWCO centrifugal tubes (Millipore Corp, Billerica, MA) to approximately 15 mg/mL. Concentrated PcyA protein was stored at −80 °C.

PcyA end-point assay

NADPH (Cat. No. N-1630), ferredoxin:NADP$^+$ oxidoreductase (FNR) (Cat. No. F-0628), glucose oxidase (Cat. No. G6125) and cat-