Functional Differentiation of Two Analogous Coproporphyrinogen III Oxidases for Heme and Chlorophyll Biosynthesis Pathways in the Cyanobacterium Synechocystis sp. PCC 6803

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Introduction

Chl a, the essential pigment for photosynthesis, is produced by a complex enzymatic system comprised of at least 15 different enzymes (Maqueo Chew and Bryant 2007, Tanaka and Tanaka 2007, Masuda and Fujita 2008). The first half of the system consisting of nine enzymes is shared in heme biosynthesis, and the latter half which consists of six enzymes is specific for Chl biosynthesis. A framework of the biosynthetic pathway of Chl has been developed by detailed molecular genetic analyses in photosynthetic bacteria and cyanobacteria (Bollivar et al. 1994, Suzuki et al. 1997). Taken together with an expanding collection of genome information of diverse photosynthetic organisms, it has been revealed that some reactions in the Chl biosynthetic pathway are catalyzed by more than two different enzymes with no structural similarity between them (Masuda and Fujita 2008), namely analogous enzymes (Galperin et al. 1998). The most extensively studied analogous enzymes in Chl biosynthesis are protochlorophyllide (Pchlide) reductases. The D-ring of Pchlide is trans-specifically reduced by two structurally unrelated enzymes, the light-dependent Pchlide oxidoreductase (LPOR) (Masuda and Takamiya 2004, Heyes and Hunter 2005) and the light-independent (dark-operative) Pchlide oxidoreductase (DPOR) (Fujita and Bauer 2008). These enzymes operate differentially on the light intensity and the environmental oxygen levels in the cyanobacterium Synechocystis sp. PCC 6803.

Coproporphyrinogen III oxidase (CPO) catalyzes the oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX in heme biosynthesis and is shared in chlorophyll biosynthesis in photosynthetic organisms. There are two analogous CPOs, oxygen-dependent (HemF) and oxygen-independent (HemN) CPOs, in various organisms. Little information on cyanobacterial CPOs has been available to date. In the genome of the cyanobacterium Synechocystis sp. PCC 6803 there is one hemF-like gene, sll1185, and two hemN-like genes, sll1876 and sll1917. The three genes were overexpressed in Escherichia coli and purified to homogeneity. Sll1185 showed CPO activity under both aerobic and anaerobic conditions. While Sll11876 and Sll1917 showed absorbance spectra indicative of Fe–S proteins, only Sll11876 showed CPO activity under anaerobic conditions. Three mutants lacking one of these genes were isolated. The Δsll1185 mutant failed to grow under aerobic conditions, with accumulation of coproporphyrinogen III. This growth defect was restored by cultivation under micro-oxic conditions. The growth of the Δsll11876 mutant was significantly slower than that of the wild type under micro-oxic conditions, while it grew normally under aerobic conditions. Coproporphyrin III was accumulated at a low but significant level in the Δsll11876 mutant grown under micro-oxic conditions. There was no detectable phenotype in Δsll11917 under the conditions we examined. These results suggested that sll1185 encodes HemF as the sole CPO under aerobic conditions and that sll11876 encodes HemN operating under micro-oxic conditions, together with HemF. Such a differential operation of CPOs would ensure the stable supply of tetrapyrrole pigments under environments where oxygen levels fluctuate greatly.

**Keywords:** Chlorophyll • Coproporphyrinogen III oxidase • Cyanobacteria • hemF • hemN • Synechocystis sp. PCC 6803.

**Abbreviations:** CPN, coproporphyrin III; CPgen, coproporphyrinogen III; CPO, coproporphyrinogen III oxidase; DPOR, light-independent protochlorophyllide oxidoreductase; Pchlide, protochlorophyllide; LPOR, light-dependent protochlorophyllide oxidoreductase; Pchlide, protochlorophyllide; PDA, photodiode array detector; PPgen, protoporphyrinogen IX; PPN, protoporphyrin IX; RT–PCR, reverse transcription–PCR; SAM, S-adenosylmethionine
Cyanobacteria are prokaryotes performing oxygenic photosynthesis like plants. Some cyanobacteria can grow in anaerobic environments such as microbial mats, lake sediments and soil (Stal and Moezelaar 1997). A genome comparison of cyanobacteria with other various prokaryotes suggests that cyanobacteria have both hemf and hemN genes in their genomes (Panek and O’Brien 2002). Thus, it is intriguing that the two analogous CPOs operate differentially in response to environmental oxygen levels in cyanobacteria. However, there has been little information on the physiological roles of different CPOs in cyanobacteria. Here we report the identification of genes encoding CPOs, HemF and HemN, by the in vitro activity of purified proteins, and the differential operation of the two different types of CPOs in response to environmental oxygen levels in the cyanobacterium Synechocystis sp. PCC 6803. This dual operation system of CPOs would ensure the stable supply of tetrapyrrole pigments under environments where oxygen levels fluctuate greatly.

Results

hemf-like and hemn-like genes in Synechocystis 6803

A hemf-like gene, sll1185, was found in the genome of Synechocystis sp. PCC 6803 (Synechocystis 6803) by a BLAST search with the amino acid sequence of HemF from E. coli (Troup et al. 1994). Sll1185 shows 55% identity to the HemF of E. coli. Similar levels of identity were detected in plants [45% to LIN2 from Arabidopsis thaliana (Ishikawa et al. 2001)], yeast [45% to Hem13p from Saccharomyces cerevisiae (Zagorec et al. 1988)] and humans (43% to CPX from Homo sapiens (Taketani et al. 1994)). Some residues proposed to be critical for the catalysis such as His113, His123, Arg127, His157, His187, Asp286, Arg287 and His312 (Breckau et al. 2003, Stephenson et al. 2007) are completely conserved in Sll1185.

Two hemn-like genes, sll1876 and sll1917, were found in the genome by a BLAST search with the amino acid sequence of HemN from E. coli (Troup et al. 1995). Sll1876 and Sll1917 show 49 and 27% identity, respectively, to the HemN of E. coli. A Gram-positive bacterium Bacillus subtilis has a HemN (Homuth et al. 1996) diverged from the E. coli HemN. Sll1917 shows higher similarity to the B. subtilis HemN (37% identity) than to that of the E. coli HemN. Both types of HemN are common members of the radical SAM family, which has a conserved Fe–S-binding motif (CxxxxC) to hold an oxygen-sensitive [4Fe–4S] cluster. This cysteine motif is conserved in both Sll1876 and Sll1917.

CPO activity of heterologously overexpressed Sll1185 and Sll1876

To examine the enzymatic activity of the three proteins Sll1185, Sll1876 and Sll1917, we subcloned their entire coding regions into an overexpression vector pASK-IBA5plus to construct pASK5Sll1185, pASK5Sll1876 and pASK5Sll1917,
respectively (Fig. 1A). All proteins have an affinity tag, a Strep-tag, in their N-termini. All three fusion proteins, Strep-Sll1185, Strep-Sll1876 and Strep-Sll1917, were successfully overexpressed in E. coli and purified to be almost single bands by Strep-Tactin affinity columns (Fig. 1B). The apparent molecular mass of the Strep-Sll1185, Strep-Sll1876 and Strep-Sll1917 proteins on the SDS–PAGE profile was in good agreement with the values of their estimated molecular mass, 40.3, 54.5 and 47.8 kDa, respectively. While there was no absorption in the visible region in purified Strep-Sll1185, purified Strep-Sll1876 and Strep-Sll1917 showed a broad absorption at around 410 nm (Fig. 1C, D, solid lines). Upon reduction of the proteins by dithionite, the broad absorption was significantly decreased (Fig. 1C, D, dotted lines). The spectra of Strep-Sll1876 are characteristic of Fe–S proteins such as ferredoxin and radical SAM family proteins that carry [4Fe–4S] clusters [HemN from E. coli (Layer et al. 2002); MiaB from Thermotoga maritima (Pierrel et al. 2003)]. The spectrum of As-isolated Strep-Sll1917 with a 330 nm peak and a subtle shoulder at 455 nm was slightly different from that of Strep-Sll1876, and these are typical features of the presence of a [2Fe–2S] cluster. Strep-Sll1917 thus appears to have a [2Fe–2S] cluster rather than a [4Fe–4S] cluster.

CPO assay was carried out under aerobic conditions with the purified Strep-Sll1185, Strep-Sll1876 and Strep-Sll1917 proteins (Fig. 2A, B). The conversion of CPgen to PPgen was monitored by the fluorescence spectral change (Fig. 2A). While neither CPgen nor PPgen show any fluorescence or absorption in the visible region, the respective oxidative products, CPN and PPN, show characteristic fluorescence emission peaks at 622 and 630 nm, respectively. As shown in Fig. 2A, only the pigment extract from the assay reaction with Strep-Sll1185 showed a fluorescence emission spectrum with a peak at 630 nm, while other reactions showed commonly fluorescence emission spectra of CPN with a peak at 622 nm. The formation of PPN was confirmed by HPLC analysis (Fig. 2B). PPN eluted at
10.2 min was detected only in the reaction with Strep-Sll1185. This result indicated that sll1185 encodes an active oxygen-dependent CPO HemF.

Given the oxygen-sensitive property of radical SAM enzymes and their unique requirement for SAM, an alternative CPO assay was carried out under anaerobic conditions with Strep-Sll1876 and Strep-Sll1917 purified under anaerobic conditions (Fig. 2C, D). The anaerobic CPO assay included SAM and NADH as a typical assay of radical SAM enzymes. In addition, some unidentified factors in the E. coli crude extract are required for in vitro assay of E. coli HemN (Layer et al. 2002). Thus, the crude extracts from E. coli and Synechocystis 6803, NADH and SAM were added in the assays under anaerobic conditions (C and D). Anaerobic assay mixtures contained purified Sll1185 (6.1 µg; red, trace b), Sll1876 (19.3 µg; cyan, trace c) and Sll1917 (16.9 µg; green, trace d). Chl a from the cyanobacterial crude extracts was detected as peak 3.
We also addressed the dependency of the HemN activity on *E. coli* and cyanobacterial extracts (Fig. 3). Surprisingly, a significant amount of PPN formation was detected in the assay without any extracts from *E. coli* and *Synechocystis* 6803 (Fig. 3, trace d). This result suggested that the cyanobacterial HemN catalyzes the CPO reaction independently of cellular extracts, in contrast to the *E. coli* HemN.

Transcript levels of the three genes *sll1185*, *sll1876* and *sll1917* in *Synechocystis* 6803

To examine whether these gene products differentially operate in response to environmental oxygen levels, we examined the transcription of the three genes, *sll1185*, *sll1876* and *sll1917*, in response to the environmental oxygen levels. The transcript levels of the three genes in wild-type cells grown photoautotrophically under aerobic and micro-oxic (Minamizaki et al. 2008) conditions were semi-quantified by reverse transcription–PCR (RT–PCR) (Fig. 4B). RT–PCR products derived from *sll1185* mRNA were detected almost equally in both cDNA preparations from aerobic and micro-oxic conditions. In contrast, those from *sll1876* and *sll1917* were much more abundant in the cDNA preparation from micro-oxic conditions than those from aerobic conditions, as previously shown for *sll1876* (Minamizaki et al. 2008). This result suggests that the expression of *sll1876* and *sll1917* is induced under micro-oxic conditions in contrast to the constitutive expression of *sll1185*.

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**Fig. 3** Crude extract dependence of anaerobic CPO monitored by HPLC analysis. Assay mixtures of Sll1876 (2.4 µg) contained both crude extracts from *E. coli* and *Synechocystis* 6803 (red, trace a), only cyanobacterial extract (4.0 µg; cyan, trace b), only *E. coli* extract (2.3 µg; green, trace c) and without any crude extracts (black, trace d). The HPLC profile of CPN and PPN (trace s) is identical to that in Fig. 2D.

**Fig. 4** (A) Gene arrangements of a hemF-like gene, *sll1185*, and two hemN-like genes, *sll1876* and *sll1917*, in the genome of *Synechocystis* 6803. The regions amplified by PCR to construct plasmids for targeted gene disruption are shown by horizontal solid thin bars. The regions amplified by RT–PCR are shown by horizontal solid thick bars. The regions replaced with a kanamycin resistance cartridge in the Δ*sll1185* and Δ*sll1876* mutants are shown by open bars. An open triangle indicates the BsiWI site where a spectinomycin resistance cartridge was introduced in the Δ*sll1917* mutant. (B) RT–PCR analysis for the transcript levels of *sll1185*, *sll1876* and *sll1917*. Total RNA was isolated from wild-type cells grown under aerobic (lane 1) and micro-oxic (lane 2) conditions. Cycle numbers of RT–PCR were 25, 30, 35 and 11 for *sll1185*, *sll1876*, *sll1917* and *rrn16Sa*, respectively. (C) PCR analysis to confirm the gene replacement in the isolated mutants. DNA fragments were amplified by PCR with forward and reverse primers used to construct plasmids for targeted gene disruption (Supplementary Table S1) from single colonies appearing on the selective agar plates. Shorter and longer fragments correspond to wild-type (WT) and mutant (KO) copies of the target genes, respectively. For Δ*sll1185*, transformants were segregated under aerobic (lane 3) and micro-oxic conditions (lane 6). As controls, the wild-type (lanes 2 and 5) and mutant (lanes 1 and 4) fragments were amplified from the plasmids carrying the wild-type and disrupted gene copies, respectively. A double mutant Δ*sll1876*/Δ*sll1917* was isolated from Δ*sll1917*. The gene replacement of *sll1876* in the double mutant was also confirmed (lane 7).
Isolation of mutants lacking one of the three genes

We isolated three mutants lacking one of these genes (Fig. 4A, C). Two mutants lacking sll1876 and sll1917 were successfully isolated by the normal segregation procedure under aerobic conditions (Fig. 4C). A double mutant lacking both sll1876 and sll1917 was also isolated from the sll1917-lacking mutant. For the sll1185 disruption, while kanamycin-resistant colonies appeared on the selective agar plates, the wild-type copy was eventually detected in the resultant kanamycin-resistant mutant (Fig. 4C, sll1185, lane 6). This segregation procedure suggested that sll1185 is essential for growth under aerobic conditions but is dispensable for growth under micro-oxic conditions. Mutants lacking sll1876, sll1876, sll1917 and sll1876-sll1917 are called sll1185, sll1876, sll1917 and sll1876/sll1917, respectively, hereafter.

Growth and Chl contents of the mutants

The physiological importance of these three genes in Synechocystis 6803 was examined by comparison of photoautotrophic growth under aerobic and micro-oxic conditions between the wild-type and the isolated mutants (Fig. 5). The ∆sll1185 mutant did not grow and turned yellow in the aerobic conditions (50 μmol photon m⁻² s⁻¹; Fig. 5A). The growth of ∆sll1185 was restored under micro-oxic conditions (Fig. 5B). This phenotype is consistent with the segregation procedure. In contrast, the sll11876 mutant showed the opposite growth behavior. ∆sll11876 grew well under aerobic conditions, but not under micro-oxic conditions (Fig. 5A, B). This phenotype was also observed in a double mutant ∆sll11876/∆sll1917. The ∆sll1917 mutant grew photoautotrophically like the wild-type on agar plates under both conditions. In liquid culture, the ∆sll1185 mutant persisted during several rounds of segregation under aerobic conditions. In liquid culture, the ∆sll11876/∆sll1917 mutant also grew well under micro-oxic conditions. At 621 nm, a characteristic of CPN. There was no detectable accumulation of CPN in the other two mutants ∆sll11876 and ∆sll11876 grown under aerobic conditions (Fig. 6A, B). We examined CPN accumulation in the mutants grown under micro-oxic conditions (Fig. 6C, D). While CPN was not detected in the HPLC profiles in any extracts (Fig. 6C), a significant accumulation of CPN was detected in the 2–4 min eluents from ∆sll1185 and ∆sll11876 (Fig. 6D, traces b and c). The CPN accumulation levels were similar in these mutants.

Significant amounts of CPN were excreted into the culture media of ∆sll1185 under aerobic and micro-oxic conditions and of ∆sll11876 grown under micro-oxic conditions (data not shown).

Taken together, it is suggested that Sll1185 is an exclusive CPO under aerobic conditions and that Sll11876 operates as an oxygen-independent CPO under micro-oxic conditions where Sll1185 also contributes almost equally as an oxygen-dependent CPO using oxygen endogenously evolved.

Discussion

We demonstrated that sll1185 and sll11876 encode oxygen-dependent and oxygen-independent CPOs, respectively, in the cyanobacterium Synechocystis 6803 by reconstitution of CPO activity with purified Sll1185 and Sll11876. Phenotypic analysis of the mutants lacking one of the three genes, sll1185, sll11876 and sll1917, suggested that Sll1185 is essential as the sole CPO under aerobic conditions and is dispensable but plays a significant role under micro-oxic conditions (Fig. 7), and that Sll11876 is dispensable under aerobic conditions but operates as an alternative CPO together with Sll1185 under micro-oxic conditions (Fig. 7). We propose to call sll1185 and sll11876 hemF and hemN, respectively.
CPO activity of the cyanobacterial HemF and HemN proteins

We have confirmed that slt1185 encodes HemF by the reconstitution of the CPO activity with purified Slt1185. An oxygen-dependent CPO assay system has been established with purified E. coli HemF protein (Warren and Shoolingin-Jordan 2002, Breckau et al. 2003). Interestingly, while the HemF activity depends on oxygen, Slt1185 showed CPO activity in the anaerobic assays as well as the aerobic assays (Fig. 2C, D). This apparent ‘oxygen-independent’ CPO activity has been reported previously (Breckau et al. 2003). It has been proposed that the affinity of HemF for oxygen is so high that oxygen is ‘loaded’
on the HemF protein once it is exposed to oxygen during cultivation and purification procedures. Such an ‘oxygen-loaded HemF’ catalyzes CPgen oxidation by a single turnover reaction. In our assay, Sll1185 was purified under aerobic conditions. The concentrations of Sll1185 and PPgen produced were 4.2 and 1.8 µM, respectively. This stoichiometry is consistent with this finding: about half of Strep-Sll1185 is in the ‘oxygen-loaded’ form and converts CPgen to PPgen by a single turnover.

HemN is a member of the radical SAM family that catalyzes a wide variety of reactions dependent on the 5′-deoxyadenosyl radical generated by the reductive cleavage of SAM (Wang and Frey 2007). Enzymes in this family carry a unique [4Fe–4S]
cluster ligated by three cysteine residues in the conserved cysteine motif (CxxxCxxC), and the bound SAM molecule serves as the fourth ligand. Since the [4Fe–4S] cluster is sensitive to oxygen, anaerobic conditions are required for the HemN assay. Here we have confirmed that Sll1876 showed CPO activity, indicating that sll1876 is the hemN ortholog in *Synechocystis* 6803. A high molecular mass fraction from *E. coli* cellular extract was required for the HemN activity of *E. coli* (Layer et al. 2002). Interestingly, the cyanobacterial HemN showed a significant CPO activity even in the absence of crude extracts from *E. coli* and the cyanobacterium (Fig. 3). The cyanobacterial HemN might function without any extra factors.

**Induction of the contiguous genes sll1874, sll1875 and sll1876 under micro-oxic conditions**

Previously we reported that three contiguous genes, sll1874, sll1875 and sll1876 (Fig. 4A), are induced commonly under micro-oxic conditions (Minamizaki et al. 2008), which was confirmed in this study (Fig. 4B). Thus, the three consecutive genes appear to form a common transcriptional unit regulated in response to the environmental oxygen levels. The sll1874 (*chlA*) gene encodes the ChlA protein involved in the formation of the E-ring of Chl a (Minamizaki et al. 2008), and the sll1875 (*ho2*) gene encodes a heme oxygenase (SynHO-2; Sugishima et al. 2005, Zhang et al. 2005). Since all three genes encode enzymes involved in tetrapyrrole biosynthesis, this gene arrangement might provide a regulatory basis to supply a variety of tetrapyrrole pigments such as Chl, heme and bilins under low oxygen levels. This contiguous arrangement is conserved in *Synechococcus* sp. PCC 7002, *Thermosynechococcus elongatus* and some *Cyanothecae* strains.

**Essential role of hemF under aerobic growth in other photosynthetic organisms**

A null mutant of sll1185 (Δsll1185) was successfully isolated when the segregation procedure was carried out under anaerobic conditions (Fig. 4C). As expected from this segregation process, sll1185 was found to be essential for aerobic growth since the Δsll1185 mutant failed to grow under aerobic conditions (Fig. 5). Such indispensability of hemF was also shown in some photosynthetic organisms such as a photosynthetic bacterium *R. sphaeroides* (Zeilstra-Ryalls and Schornberg 2006) and plants (Kruse et al. 1995, Ishikawa et al. 2001, Williams et al. 2006). Transformants of tobacco (*Nicotiana tabacum*) with antisense RNA of the cDNA encoding CPO showed a broad range of growth retardation and necrosis indicating oxidative damage (Kruse et al. 1995). A deficiency of CPO activity (the lin2 mutation) causes lesion formation in *A. thaliana* (Ishikawa et al. 2001). Maize (*Zea mays*) has two closely related hemF homologs, cpx1 and cpx2. While cpx2 encodes a mitochondrial CPO, the cpx1 gene encodes a plastid CPO to contribute to Chl biosynthesis. A null mutant lacking cpx1 develops extensive necrotic lesions in the light, suggesting that Cpx1 is the sole CPO in maize plastids (Williams et al. 2006).

Among 15 reactions in the Chl a biosynthesis pathway, there are three oxygen-requiring reactions, CPgen oxidation, PPgen oxidation and E-ring formation. It is reasonable for photosynthetic organisms adapting aerobic environments where oxygen is readily available to use preferentially the oxygen-dependent enzymes. For example, under aerobic conditions the photosynthetic bacterium *R. gelatinosus* uses HemF dominantly for the common biosynthetic pathway of heme and bacteriochlorophyll, together with an oxygen-dependent Mg-PPN monomethylster cyclase AcsF in the bacteriochlorophyll-specific pathway, which are mutually regulated by a transcriptional regulator FnrL (Ouchane et al. 2007). Furthermore, oxygenic photosynthetic organisms such as cyanobacteria and plants evolve oxygen from their own PSII, which would meet the needs of oxygen for the HemF reaction. At the same time, the endogenously evolved oxygen would eventually inactivate the oxygen-independent CPO, HemN, with the oxygen-labile Fe–S cluster. Thus, the primary use of...
the oxygen-dependent CPO is a very rational strategy for oxygenic phototrophs.

**Two homologous hemN-like genes, sll1876 and sll1917**

In the genome of *Synechocystis* 6803 there are two *hemN*-like genes, *sll1876* and *sll1917*. The successful reconstitution of the CPO assay with purified Sll1876 supports that *sll1876* encodes an active HemN. Thus, we concluded that *sll1876* is the *hemN* ortholog in *Synechocystis* 6803. The CPN accumulation in the Δsll1876 mutant under micro-oxic conditions was less evident than that in the Δsll1185 mutant under aerobic conditions (Fig. 6). However, when 5-aminolevulinate was fed for 24 h under micro-oxic conditions, CPN accumulation in the Δsll1876 mutant was at a much higher level than that of the Δsll1185 mutant (Supplementary Fig. S2). This observation indicated that Sll1876 may contribute as a dominant CPO to the tetrapyrrole biosynthesis under some oxygen-limited conditions.

Under micro-oxic conditions, even though the Chl content of Δsll1185 was significantly lower than that of Δsll1876 (Fig. S5), the CPN accumulation in these two mutants was comparable (Fig. 6D). One may have questions about this discrepancy. The Chl contents of the two mutants grown on agar plates rather than liquid cultures were commonly about 80% of that of the wild type (data not shown), which is consistent with the comparable CPN accumulation (Fig. 6D). There would be some conditional difference between liquid and agar cultures as mentioned in the growth characteristics.

In contrast to Sll1876, Sll1917 showed no CPO activity. In addition, there was no detectable phenotype of the Δsll1917 mutant. What is the function of the other *hemN*-like gene *sll1917*? While Sll1917 has an amino acid sequence characteristic of the radical SAM family, Sll1917 showed an absorption spectrum somewhat different from that of Δsll1185 and seems to have a [2Fe–2S] cluster rather than a [4Fe–4S] cluster. *sll1917* may have become a pseudogene or have evolved to encode a protein with some function different from CPO.

A phylogenetic tree of HemN from many organisms was constructed (Supplementary Fig. S3). HemN is largely divided into two sister groups; ‘HemN I’ and ‘HemN II’, which include HemNs from *E. coli* and *B. subtilis* as the representative strains, respectively. Sll1876 and Sll1917 are classified as HemN I and HemN II, respectively. Two cyanobacteria, *Thermosynechococcus elongatus* and *Anabaena* sp. PCC 7120, have both HemN I and HemN II-type HemNs. In contrast, most cyanobacteria have only the HemN I-type HemN, suggesting that the HemN I-type HemN is the authentic HemN in these cyanobacteria. HemN I-type Sll1876 is the authentic HemN rather than HemN II-type Sll1917 in *Synechocystis* 6803. It will be of interest to examine if HemN I-type HemN is active in other cyanobacteria.

As shown in Supplementary Fig. S3, some prokaryotes other than cyanobacteria have multiple *hemN* genes. *Bacillus subtilis* has two *hemN* genes, *hemn* and *hemz* (Homuth et al. 1999), and both are categorized into ‘HemN I’. Since a double mutant lacking *hemN* and *hemZ* grows under both aerobic and anaerobic conditions, neither *hemN* nor *hemZ* is indispensable for *B. subtilis*. *Bradyrhizobium japonicum* has two *hemN* genes (Fischer et al. 2001) that are closely related and categorized into ‘HemN I’. Only one *hemN* (*hemN1*) encodes a functional HemN while the function of the other (*hemN2*) is still unknown. The photosynthetic bacterium *R. gelatinosus* also has dual HemN I-type HemNs, HemN1 and HemN2. Only one (*hemN2*) encodes the functional HemN to support photosynthetic growth, rather than the other (*hemN1*) (Ouchane et al. 2007). Another photosynthetic bacterium *R. sphaeroides* also has *hemZ* and *hemN* categorized into HemN I and HemN II, respectively. Both hemN homologs are regulated to be induced under oxygen-limited conditions by the combined action of FnrL and PrrA (Yeliseev and Kaplan 1999, Oh et al. 2000).

As mentioned above the *hemN* genes seem to have been highly diversified to operate under some specific conditions, and some *hemN* genes might have become pseudogenes. Co-existence with the analogous gene *hemF* and/or a homologous gene *hemN* might have accelerated the diversification of *hemN* during evolution.

**Strategies to supply Chl under environments in various oxygen tensions**

In the biosynthetic pathways of Chl and heme, there are at least three reactions catalyzed by analogous enzymes. One is the Pchlide D-ring reduction catalyzed by DPOR and LPOR. The second is E-ring formation catalyzed by BchE and ChlA (*AcsF/Crd1/CHL27*). The third is CPgen oxidation catalyzed by HemN and HemF. Interestingly, one enzyme of each of these analogous pairs, HemN, BchE and DPOR, carries commonly oxygen-labile Fe–S clusters and is inactivated by oxygen. The other enzymes, LPOR, ChlA and HemF, are oxygen tolerant and two of them even require oxygen for the catalysis. Such common features of the analogous pairs may be evolutionary traces of photosynthetic organisms having adapted to the fluctuating oxygen levels in the environments.

The effect of an aerobic environment on the analogous enzymes seems to differ somewhat. In Pchlide reduction an aerobic environment is merely a negative factor for the operation of DPOR since oxygen irreversibly inactivates the Fe–S clusters in the DPOR components (Yamamoto et al. 2009). LPOR is just an oxygen-tolerant enzyme. In contrast, in CPgen oxidation while HemN is readily inactivated by oxygen as well as DPOR, HemF is not only oxygen tolerant but also oxygen dependent. Thus, an aerobic environment brings about two-sided effects on CPgen oxidation; one is a negative effect on the oxygen-labile HemN and the other is a positive effect on HemF.

Extant cyanobacteria thrive in a variety of environments where the oxygen levels undergo dynamic changes. From an evolutionary aspect, the environmental oxygen level during the Proterozoic era could be a key factor in the evolution of oxygen-tolerant enzymes in ancient cyanobacteria (Yamazaki et al. 2006). When the first photosynthetic organisms emerged
on the earth, the oxygen concentration was only a trace level. Under such conditions, many metalloenzymes with oxygen-sensitive Fe–S clusters were evolved to build up a primitive biosynthetic pathway of Chl (or bacteriochlorophylls). Upon the evolution of oxygen-evolving photosynthesis, the oxygen level of the earth has gradually increased followed by a quick increase called the ‘Great Oxidation Event’ 2.2 gigayears ago (Holland 2006). During the transition from anaerobic to aerobic environments, the pre-existing metabolic pathway with oxygen-sensitive metalloenzymes could undergo a drastic reconstruction to evolve a set of alternative oxygen-tolerant enzymes to substitute the respective oxygen-sensitive enzyme. The Chl biosynthesis pathway provides an interesting model to explore the details of the great metabolic reconstitution event during the Great Oxidation Event. Two typical examples have been reported so far. One is LPOR as the alternative for DPOR (Yamazaki et al. 2006) and the other is the oxygen-dependent Mg-PPN monomethylester (MPE) cyclase (ChlA/AcsF/Crd1/CHL27) as the alternative for the oxygen-sensitive MPE cyclase (BchE) (Ouchane et al. 2004). We consider oxygen-dependent CPO as a third example. HemF could have evolved to substitute the oxygen-tolerant CPO HemN during the Great Oxidation Event. Ancient cyanobacteria have developed many oxygen-tolerant enzymes; subsequently a mechanism to regulate the transcription such as Fnrl has been developed to optimize the expression of the new oxygen-tolerant enzymes in response to the environmental oxygen tensions. The elaborate mechanism comprised of the enzymes and the regulatory system provides a molecular basis to ensure the constant supply of tetrapyrrole pigments such as Chl and heme to allow cyanobacteria to thrive in any habitats with various oxygen tensions from anaerobic to hyperoxic environments on the Earth.

Materials and Methods

Cyanobacterial strains and growth conditions

Synechocystis sp. PCC 6803 (Synechocystis 6803) and its derivative strains used in this study were cultivated in BG-11 medium as described (Minamizaki et al. 2008). For cultures of the gene-disrupted mutant with a kanamycin and a spectinomycin resistant cartridge, the above medium was supplemented with 1.5 µg ml\(^{-1}\) kanamycin sulfate and 15 µg ml\(^{-1}\) spectinomycin, respectively. Liquid cultures were bubbled with 2% CO\(_2\) in air (for aerobic conditions) under continuous illumination provided from fluorescent lamps (−50 µmol m\(^{-2}\) s\(^{-1}\); FLR40SW, Hitachi). For agar plate culture, BG-11 liquid media were solidified with 1.5% agar (BactoAgar, Becton, Dickinson and Company, Sparks, MD, USA). For growth under micro-oxic conditions, liquid cultures were bubbled with 2% CO\(_2\)/N\(_2\) that were prepared by mixing pure N\(_2\) (99.999%, Nagoya Nissan, Nagoya, Japan) and CO\(_2\) with an appropriate flow rate. For cultivation on agar plates, agar plates were incubated in an anaerobic jar (BBL GasPak anaerobic systems; Becton, Dickinson and Company).

Overexpression of sll1185, sll1876 and sll1917 in E. coli and purification of the proteins

Three plasmids for overexpression of Sll1185, Sll1876 and Sll1917 were constructed as follows. The entire coding regions of sll1185, sll1876 and sll1917 were amplified by PCR from genomic DNA of Synechocystis 6803 with primer pairs described in Supplementary Table S1 by a standard thermal cycle (KOD-plus DNA polymerase, Toyobo, Osaka, Japan). After digestion with Bsal, the amplified DNA fragments were cloned into the Bsal site of pASK-IBA5plus (IBA, Göttingen, Germany) to form pASK5Sll1185, pASK5Sll1876 and pASK5Sll1917, respectively. The N-terminus of the Strept-Tacin was carried out as described (Yamamoto et al. 2008). For anaerobic culture all procedures after harvest were carried out in the anaerobic chamber (model A, COY Laboratory Products, Grass Lake, MI, USA) as described (Yamazaki et al. 2006).

Preparation of CPgen, CPO assay and HPLC analysis

CPgen was prepared by reduction of CPN with sodium amalgam (Grandchamp and Nordmann 1982). Aliquots of CPN were resolved in 0.05 N KOH (300 µl) and reduced by the addition of 0.5 g of sodium amalgam (5% Na; Wako) followed by shaking for 10 min. After the pink color of CPN disappeared, the mixture was neutralized by the addition of 300 µl of Tris-buffer (0.25 M Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA) and disrupted by sonication (Sonifier model 250; Branson, Danbury, CT, USA). The soluble fractions were obtained by centrifugation of the sonicates (15,000 rpm, 4°C, 30 min). Affinity purification with Strept-Tacin was carried out as described (Yamamoto et al. 2008). For anaerobic operation all procedures after harvest were carried out in the anaerobic chamber (model A, COY Laboratory Products, Grass Lake, MI, USA) as described (Yamazaki et al. 2006).
The proteins were precipitated by the addition of methanol (final 90%). Fluorescence emission ($\lambda_{em}$ at 400 nm) and absorption spectra of the supernatants prepared by centrifugation (15,000 rpm, 10 min at 4°C) were recorded with a spectrophotometer (model FP777w, Jasco, Hachioji) and spectrophotometer (model V-550, Jasco), respectively.

The CPO assay under anaerobic conditions was carried out in a 100 µl volume containing 15 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 2 mM SAM, 0.5 mM NADH, 6 µM CPgen, 4 µg of the crude extract of *Synechocystis* 6803, 2–300 µg of the crude extract of *E. coli* with pASK-IBA5plus, and 2–20 µg of purified Strep-Sll1876 or Strep-Sll1917 (Layer et al. 2002). The crude extract of *E. coli* was prepared by the same procedure as the supernatants of the protein purification described above. The crude extract of *Synechocystis* 6803 (wild type) grown under micro-oxic conditions was prepared in the same manner as *E. coli* crude extracts. The assay mixtures were incubated in the dark for 120 min at 37°C under anaerobic conditions. The reaction was stopped by the addition of 10 µl of hydrogen peroxide (31%). Fluorescence emission ($\lambda_{em}$ at 400 nm) and absorption spectra of the supernatants (15,000 rpm, 10 min at 4°C) were recorded. For HPLC analysis of the CPO assay, aliquots (20 µl) of the supernatant were loaded onto a 4.6×150 mm Symmetry C8 3.5 µm column (Waters). Pigments were separated as described (Zapata et al. 2000). Pigments were detected by absorption at 405 nm and in parallel by photodiode array detector (PDA). The HPLC system was a Shimadzu LC series (Shimadzu, Kyoto, Japan). CPN and PPN were used as standards.

### Preparation of RNA and RT–PCR

Total RNA and cDNA were prepared essentially as described (Minamizaki et al. 2008). RT–PCR was carried out as described with specific primers (Supplementary Table S1).

### Construction of plasmids for gene disruption and transformation of *Synechocystis* 6803

Plasmids for gene disruption were constructed as follows (Fig. 4, Supplementary Table S1). DNA fragments containing the target genes were amplified by PCR from genomic DNA of *Synechocystis* 6803 with primer pairs (Supplementary Table S1) by a standard thermal cycle (KOD-plus DNA polymerase). The amplified DNA fragments were cloned into the BamH I site of pUC118. The recombinant plasmids carrying sll1185 and sll1876 were digested by Aval and HindIII, respectively, to remove most parts of their coding regions. A kanamycin-resistant cartridge amplified from pMC19 (Fujita et al. 1992) was inserted into the blunted Aval and HindIII sites to construct the sll1185- and sll1876-disrupted plasmids, respectively. For disruption of sll1917 the recombinant plasmid was digested by *BsiWI*. A spectinomycin-resistant cartridge was amplified from pJN3 (Nomata et al. 2006) and inserted into the blunted *BsiWI* site to construct the sll1917-disrupted plasmid.

*Synechocystis* 6803 was transformed with the plasmid constructed as above (3 µg; GeneElute Plasmid Mini-prep Kit, Sigma) and the kanamycin- and spectinomycin-resistant colonies were segregated to isolate homozygous mutants (Minamizaki et al. 2008). For the isolation of the sll1185-disrupted mutant (Δsll1185) the kanamycin-resistant colonies that appeared on the first selective agar plates were picked up and cultivated under micro-oxic conditions. Complete replacement of the wild-type copy with the disrupted copy was examined by ‘colony PCR’ as described (Minamizaki et al. 2008).

### Heme stain

Cells of the wild type and mutants were grown photoautotrophically under aerobic and micro-oxic conditions on agar plates for 7 d. For the sample of Δsll1185 under aerobic conditions, cells were grown under micro-oxic conditions for 3 d followed by incubation under aerobic conditions for 4 d. Membrane fractions were prepared as precipitates of crude extracts by centrifugation (15,000 rpm, 15 min at 4°C), and proteins in the membrane fractions were separated by SDS–PAGE without boiling with β-mercaptoethanol (Feisnner et al. 2003). For heme stain the proteins separated by SDS–PAGE were transferred onto a PVDF membrane (Immobilon P, Millipore). The signals from c-type cytochromes were visualized by a sensitive chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Scientific, Yokohama, Japan) (Feisnner et al. 2003) with a lumino-image analyzer (LAS-3000mini, Fujifilm, Tokyo, Japan).

### Pigment extraction and spectroscopic analysis

Pigments were extracted from cells grown under aerobic or micro-oxic conditions on agar plates for 7 d. Because Δsll1185 does not grow under aerobic conditions, it was grown under micro-oxic conditions for 3 d and incubated under aerobic conditions for 4 d to prepare cells grown aerobically. Pigments were extracted in 90% methanol as described (Minamizaki et al. 2008). For cells grown micro-oxic conditions the pigment extraction procedure was carried out in an anaerobic chamber (Yamazaki et al. 2006). For 5-aminolevulinate feeding, cells of the wild type and mutants were grown photoautotrophically under micro-oxic conditions on agar plates for 3 d followed by incubation with 5-aminolevulinate (3 mM) for 24 h. Aliquots (20 µl) of the methanol extracts were loaded onto a 4.6×150 mm Symmetry C8 3.5 µm column (Waters) and separated as described (Zapata et al. 2000). Pigments were detected by absorption at 405 nm and in parallel by PDA. Eluates from 2 to 4 min were collected by a fraction collector (model FRC-10A, Shimadzu) and the fluorescence emission spectra of the fractions were recorded with excitation at 400 nm (model FP777w). CPN and PPN were used as standards. The standard pigments CPN and PPN were purchased (coproporphyrin III dihydrochloride, Frontier Scientific, Logan, UT, USA; protoporphyrin-‘IX’, BioChemika, Fluka, Buchs, Switzerland). To examine CPN excretion into the culture media, cells grown on agar plates were...
suspended in water and aliquots of the supernatants were mixed with methanol (final 90%) followed by recording fluorescence emission spectra ($\lambda_{em}$ 400 nm, model FP777w). Determination of Chl contents was carried out as previously described (Minamizaki et al. 2008).

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


