Differential Operation of Dual Protochlorophyllide Reductases for Chlorophyll Biosynthesis in Response to Environmental Oxygen Levels in the Cyanobacterium *Leptolyngbya boryana*[1]

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Most oxygenic phototrophs, including cyanobacteria, have two structurally unrelated protochlorophyllide (Pchlide) reductases in the penultimate step of chlorophyll biosynthesis. One is light-dependent Pchlide reductase (LPOR) and the other is dark-operative Pchlide reductase (DPOR), a nitrogenase-like enzyme assumed to be sensitive to oxygen. Very few studies have been conducted on how oxygen-sensitive DPOR operates in oxygenic phototrophic cells. Here, we report that anaerobic conditions are required for DPOR to compensate for the loss of LPOR in cyanobacterial cells. An LPOR-lacking mutant of the cyanobacterium *Leptolyngbya boryana* (formerly *Plectonema boryanum*) failed to grow in high light conditions and this phenotype was overcome by cultivating it under anaerobic conditions (2% CO_2/N_2). The critical oxygen level enabling the mutant to grow in high light was determined to be 3% (v/v). Oxygen-sensitive Pchlide reduction activity was successfully detected as DPOR activity in cell-free extracts of anaerobically grown mutants, whereas activity was undetectable in the wild type. The content of two DPOR subunits, ChlL and ChlN, was significantly increased in mutant cells compared with wild type. This suggests that the increase in subunits stimulates the DPOR activity that is protected efficiently from oxygen by anaerobic environments, resulting in complementation of the loss of LPOR. These results provide important concepts for understanding how dual Pchlide reductases operate differentially in oxygenic photosynthetic cells grown under natural environments where oxygen levels undergo dynamic changes. The evolutionary implications of the coexistence of two Pchlide reductases are discussed.

Chlorophyll *a* (Chl *a*), a tetrapyrrole pigment essential for photosynthesis, is synthesized from Glu via a complex pathway consisting of at least 15 reactions (e.g. von Wettstein et al., 1995; Fujita, 2002; Cahoon and Timko, 2003). The penultimate step of Chl *a* biosynthesis, protochlorophyllide (Pchlide) reduction, is catalyzed by two different enzymes (Fig. 1A); one is dark-operative Pchlide oxidoreductase (DPOR; Fujita and Bauer, 2003) and the other is light-dependent Pchlide oxidoreductase (LPOR; Rüdiger, 2003; Masuda and Takamiya, 2004). Although these two enzymes carry out the same stereo-specific reduction of the double bond of the D-ring to produce chlorophyllide *a* (Chlide), the direct precursor for Chl *a*, they are structurally very different and use completely different mechanisms. Thus, DPOR and LPOR are analogous enzymes (Galperin et al., 1998).

LPOR is a light- and NADPH-dependent enzyme belonging to a short-chain dehydrogenase/reductase (SDR) superfamily (Baker, 1994; Labesse et al., 1994; Jörnvall et al., 1999). Most angiosperms harbor multiple isoforms of LPOR, which seem to be differentiated to operate in different growth stages and in different tissues (Armstrong et al., 1995; Holtorf et al., 1995; Oosawa et al., 2000). However, only a single-copy gene is detected in pea (*Pisum sativum*) and cucumber (*Cucumis sativus*; Sundqvist and Dahlin, 1997; Fusada et al., 2000). The exclusive dependence on light for the LPOR reaction makes angiosperms etiolated when germinated in the dark. In etiolated seedlings, LPOR isoforms, such as POR-A and POR-B, accumulate in the prolamellar body of etioplasts in large amounts as ternary complexes (POR-A/B-NADPH-Pchlide). Upon illumination, a photochemical reaction occurs followed by nonphotochemical dark reactions to yield Chlide (Griffiths, 1991; Lebedev and Timko, 1999; Heyes et al., 2003), which triggers the greening process of the seedlings. After greening, a different set of LPOR isoforms, such as POR-B and POR-C, takes over reduction for a constant supply of Chl to assemble and reconstruct photosystems in green plants (Rüdiger, 2003; Masuda and Takamiya, 2004).

DPOR is a three-subunit enzyme consisting of ChlL, ChlN, and ChlB (BchL, BchN, and BchB in photosynthetic bacteria producing bacteriochlorophylls, respectively). The amino acid sequences of these subunits show significant similarity to the nitrogenase subunits

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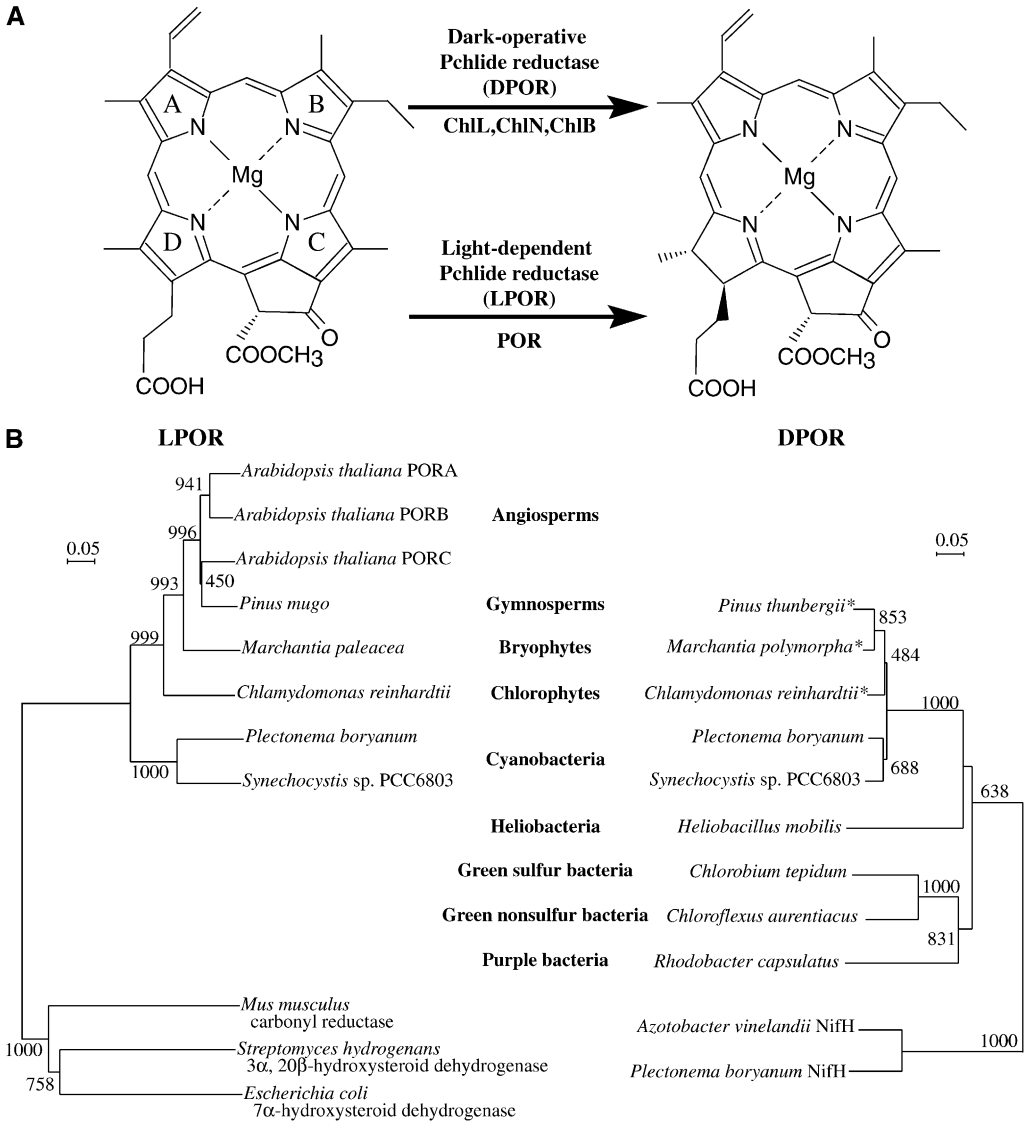

Figure 1. Two structurally unrelated Pchlide reductases. A, Pchlide reduction. The Pchlide D-ring is reduced by two different enzymes, DPOR and LPOR. B, Phylogenetic trees and distribution in extant phototrophs of two Pchlide reductases. The amino acid sequences of LPOR and DPOR (BchL/ChlL) were retrieved from GenBank as representative for each major taxon of extant phototrophs. Asterisks indicate that the genes are encoded in chloroplast genomes in these organisms. Each phylogenetic tree was constructed based on multiple sequence alignment by using ClustalX (version 1.81) and njplot. The three enzymes belonging to the SDR family and nitrogenase Fe-protein (NifH) were used as outgroups for the LPOR and DPOR trees, respectively. Bootstrap values produced by 1,000 replications are given in each node. The phylogenetic trees for LPOR and DPOR are essentially the same as those previously reported by Masuda and Takamiya (2004) and Xiong et al. (2000), respectively.

NifH, NifD, and NifK, respectively, which suggests that DPOR is a nitrogenase-like enzyme (Burke et al., 1993; Fujita, 1996; Armstrong, 1998). Nitrogenase is a complex enzyme comprising two essential component metalloproteins: Fe protein and MoFe protein (e.g. Rees and Howard, 2000; Christiansen et al., 2001; Igarashi and Seefeldt, 2003). Fe protein, the ATP-dependent reductase specific for the MoFe protein, carries one [4Fe-4S] cluster bridged between two identical subunits. The MoFe protein, which provides the catalytic centers, has two types of metalloclusters: the P-cluster (an [8Fe-7S] cluster) and the FeMo cofactor comprising [1Mo-7Fe-9S-X homocitrate]. The Fe protein is reduced by ferredoxin or flavodoxin and then the electrons are transferred from the [4Fe-4S] cluster of the Fe protein to the FeMo cofactor of the MoFe protein via the P-cluster. Eventually, dinitrogen is reduced to ammonia. The nitrogenase-like hypothesis of DPOR has been confirmed by the recent initial characterization of DPOR from the anoxygenic photosynthetic bacterium *Rhodobacter capsulatus* (Fujita and Bauer, 2000; Nomata et al., 2005, 2006a). Rhodobacter DPOR (RcDPOR)

requires ATP and dithionite (or reduced ferredoxin) for reaction, as well as nitrogenase, and consists of two separable components, L-protein and NB-protein, which are homologous to the Fe protein and MoFe protein, respectively. L-protein is a BchL homodimer, as is Fe protein (NifH homodimer), and NB-protein is a heterotetramer of BchN and BchB similar to the MoFe protein (NifD-NifK heterotetramer). These features strongly suggest that L-protein is an ATP-dependent reductase specific for the other catalytic component, as well as for the Fe protein, and that the other component, NB-protein, provides the catalytic centers for the double-bond reduction of the Pchlide D-ring similar to the MoFe protein. Recently, we have also confirmed that another nitrogenase-like enzyme, Chlide reductase, catalyzes Chlide B-ring reduction in the bacteriochlorophyll *a* biosynthesis of *R. capsulatus* (Nomata et al., 2006b).

As shown in Figure 1B, the two Pchlide reductases, DPOR and LPOR, have different evolutionary origins and distributions, implying that photosynthetic organisms have independently created two different molecular mechanisms to reduce the Pchlide D-ring. The distribution of the two enzymes among photosynthetic organisms probably reflects their distinct evolutionary histories. As shown in the phylogenic trees in Figure 1B, DPOR has evolved from ancestral genes common to nitrogenase and is distributed among anoxygenic photosynthetic bacteria, cyanobacteria, Chlorophytes, Pteridophytes, Bryophytes, and gymnosperms (Raymond et al., 2004). LPOR evolved from a large gene family of SDR distributed among all oxygenic phototrophs from cyanobacteria to angiosperms (Yang and Cheng, 2004). Thus, whereas anoxygenic photosynthetic bacteria use DPOR and angiosperms use LPOR as their only Pchlide reductases, most oxygenic phototrophs employ both DPOR and LPOR (Fig. 1B).

Nitrogenase is extremely sensitive to oxygen, which irreversibly destroys the metallocenters of both components. Thus, nitrogen-fixing organisms have evolved a number of strategies to protect nitrogenase from environmental oxygen (e.g. Hill, 1988; Oelze, 2000). For diazotrophic cyanobacteria, cellular integration of the two incompatible processes, nitrogen fixation and photosynthesis, is required because nitrogenase should be protected not only from atmospheric oxygen but also from photosynthetically produced oxygen (Gallon, 1992). In some filamentous cyanobacteria, this is achieved by development of heterocysts, which are diazotrophic cells providing sites for nitrogen fixation, where the cell environment is kept anaerobic by the absence of PSII and respiration activity (e.g. Wolk, 2000). The other mechanism occurs in some filamentous (nonheterocystous) and unicellular cyanobacteria and is a temporal separation of nitrogen fixation and photosynthesis (Bergman et al., 1997). Nitrogen fixation takes place predominantly in the dark phase of diurnal cycles in some nonheterocystous cyanobacteria growing under aerobic conditions. *Leptolyngbya boryana* (formerly *Plectonema boryanum*) is a nonheterocystous cyanobacterium showing diurnal cycles of nitrogen fixation and photosynthesis under anaerobic and continuous light conditions (Rai et al., 1992; Misra and Tuli, 2000). Such temporal separation mechanisms have been studied in some cyanobacteria (Gallon, 1992). By contrast, very few studies have been conducted on how the nitrogenase-like enzyme DPOR operates in oxygenic phototrophic cells.

We previously demonstrated that LPOR is essential for cyanobacterial cells to grow aerobically in high light conditions based on the high-light-sensitive phenotype of an LPOR-lacking mutant of the cyanobacterium *P. boryanum* (Fujita et al., 1998). This result suggested that DPOR no longer operates in conditions where oxygenic photosynthesis is very active and cellular oxygen levels are very high. Here, we report that anaerobic conditions are required for the maximal activity of DPOR to complement the loss of LPOR. We also determined the critical oxygen levels in environments for the mutant with DPOR as the sole Pchlide reductase to grow. Furthermore, we successfully detected oxygen-sensitive DPOR activity in cell-free extracts of mutant cells. These results provide important aspects to understand how DPOR operates in oxygenic cells grown under natural environments where oxygen levels undergo dynamic changes. In addition, evolutionary implications of the coexistence of two Pchlide reductases are discussed.

RESULTS

Growth Restoration of an LPOR-Lacking Mutant by Cultivation under Anaerobic Conditions

We previously found that the LPOR-lacking mutant YFP12 (Δ*por*) of *L. boryanum* could not grow under high light conditions (>130 μmol m^{-2} s^{-1}; Fujita et al., 1998). Given the nitrogenase-like features of DPOR in the anoxygenic photosynthetic bacterium RcDPOR (Fujita and Bauer, 2000; Nomata et al., 2005, 2006a), and its sequence similarity to *P. boryanum* DPOR (PbDPOR), we speculated that the high light-sensitive phenotype of YFP12 resulted from the oxygen-sensitive nature of PbDPOR. To test this, we cultivated YFP12 under aerobic and anaerobic conditions. YFP12 could not grow aerobically (Fig. 2, A and C; Fujita et al., 1998), but did grow under anaerobic conditions, even under high light, with a slightly longer doubling time (9 h) than the control strain YFD1 (6 h; Fig. 2, B and D). The Chl content of the cells was also determined (Fig. 2, E and F). Under anaerobic conditions, the Chl content of YFP12 is about 2- to 5-fold less than that of the control strain YFD1 throughout growth (Fig. 2F), suggesting that growth with a slighter longer doubling time is caused by Chl deficiency. The anaerobic growth capability of YFP12 is consistent with the hypothesis that PbDPOR is an oxygen-labile enzyme. In contrast, the DPOR-lacking mutant YFC2 (Δ*chlL*) grew as well

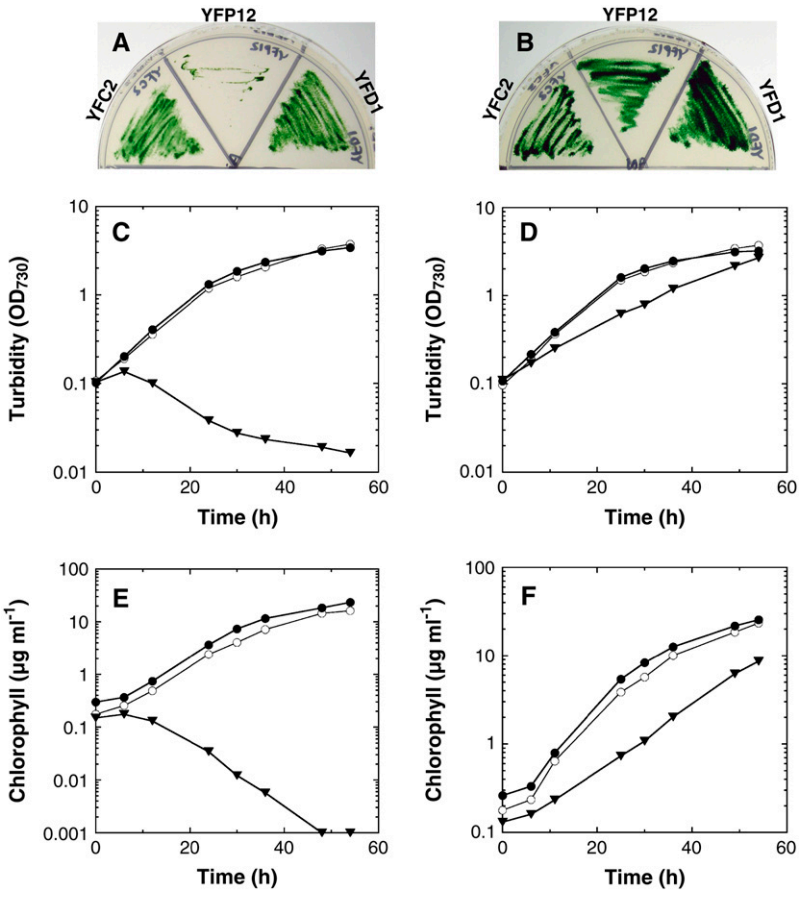

Figure 2. Restoration of the high light-sensitive phenotype of the LPOR-lacking mutant. Growth of the two mutants, YFP12 (LPOR lacking, Δ*por*) and YFC2 (DPOR lacking, Δ*chlL*), and the control strain YFD1 on BG-11 agar plates under high light (250–330 μmol m^{-2} s^{-1}) and aerobic (A) and anaerobic (B) conditions. Growth curves of YFP12 (black triangles), YFC2 (black circles), and YFD1 (white circles) under air bubbling (2% CO_2; C) and anaerobic (D) conditions are shown. Chl *a* content of the three strains was monitored with growth under air bubbling (E) and anaerobic (F) conditions.

as YFD1, with almost the same Chl content under both conditions (Fig. 2). These growth characteristics suggest that LPOR operates under both aerobic and anaerobic conditions and that the LPOR activity in YFC2 is sufficient to fully compensate for the loss of DPOR activity in this mutant.

Critical Oxygen Level for YFP12 under High Light Conditions

To determine how much oxygen is critical for growth inhibition of YFP12, this mutant was cultivated photoautotrophically under high light conditions in liquid media bubbling with mixed gas (2% CO_2 in N_2) containing various levels of oxygen from 0% to 21%. As shown in Figure 3A, YFP12 did not grow when bubbled with air (21% O_2, 5% O_2, or 4% O_2). When the oxygen level in the gas mixture was stepped down to 3%, YFP12 grew slowly with a long doubling time (20 h). YFP12 cells grown under 3% O_2 accumulated about 10 times as much Pchlide as did YFD1 cells (data not shown). Growth restoration with Pchlide accumulation suggested that a significant portion of DPOR survives to support Chl synthesis and that inactivation of DPOR still proceeds. As the oxygen level was further decreased, the growth rate of YFP12 gradually increased. Growth rates of YFP12 under various oxygen levels were compared with those of the control strain YFD1 (Fig. 3B). YFD1 grew at a constant rate throughout the conditions examined. The growth rate of YFP12 was slightly slower (67%) than YFD1, even under anaerobic conditions (0% O_2), suggesting that DPOR was partially inactivated by endogenously produced oxygen, or that maximal DPOR activity is still not enough to supply as much Chlide as is needed for maximal growth (see also Fig. 2, C and D). YFC2, the DPOR-lacking mutant (Δ*chlL*), grew as well as YFD1 under all conditions examined, demonstrating that LPOR is indeed an oxygen-insensitive enzyme. Figure 3C shows that Chl *a* content in cells is strongly correlated with the growth rate of YFP12, suggesting that Chl *a* content may be a major growth-limiting factor for YFP12. Under these conditions, approximately 0.6 μg mL^{-1}/optical density $(OD)_{730}$ was the lowest Chl content that still allowed YFP12 to grow photoautotrophically. These results suggest that the presence of 3% oxygen in the environment is the upper limit for DPOR to remain functionally useful in this cyanobacterium under high light.

DPOR Activity Is Detected in Cell-Free Extracts from Anaerobically Grown YFP12 Cells

Thus far, there have been no unambiguous reports of DPOR activity from oxygenic phototrophic organisms.

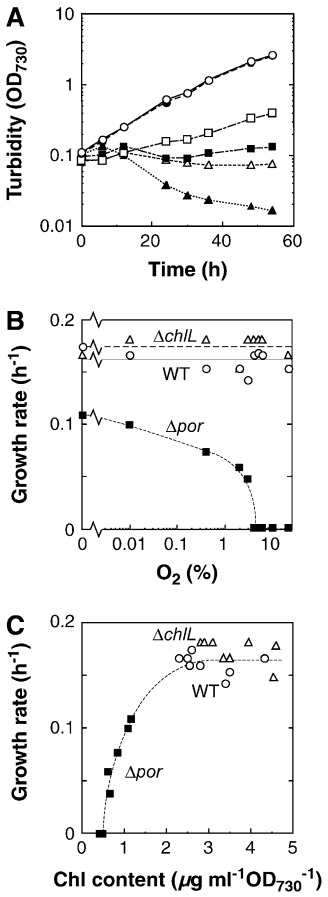

Figure 3. Oxygen level critical for growth of the LPOR-lacking mutant. A, Growth curves of YFP12 under various oxygen levels. YFP12 was cultivated under high light conditions with bubbling in 2% CO_2/air (21% O_2; black triangles), 5% O_2 (white triangles), 4% O_2 (black squares), 3% O_2 (white squares), 0.01% O_2 (black circles), and 0% O_2 (white circles). B, Change in growth rates of the three strains at various oxygen levels: YFD1 (white circles), YFC2 (white triangles), and YFP12 (black squares). Growth rate (h^{-1}) was estimated as the reciprocal of the initial doubling time. C, Relationship between growth rate and Chl content of the cells. Chl content of YFD1 (white circles), YFC2 (white triangles), and YFP12 (black squares) was determined in cells grown for 24 h at each oxygen level. Chl content ($\mu g\ mL^{-1}/OD_{730}$) was estimated by dividing Chl amounts per unit culture volume by turbidity (OD_{730}).

We devised an in vitro assay system in cell-free extracts from anaerobically grown YFP12 cells. All procedures, harvesting of cells, cell disruptions, preparation of cell-free extracts, and enzyme assays were carried out in an anaerobic chamber in a similar manner to the assay for RcDPOR (Fujita and Bauer, 2000; Nomata et al., 2005). To discriminate DPOR activity from LPOR activity, assays were carried out in the dark. As shown in Figure 4A, a clear increase in the absorption peak at 665 nm corresponding to Chlide was observed after incubation (traces a–f). The rate of Chlide formation was almost linear with time during the first 10 min (3.5 pmol min^{-1} mg^{-1} protein) and was followed by a slower increase until 60 min (Fig. 4B). By contrast, DPOR activity was at undetectable levels in cell-free extracts of YFD1 cells grown anaerobically (Fig. 4A, traces g–i), indicating that YFP12 cells grown anaerobically have much higher activity of DPOR than control cells. The ATP requirement for DPOR activity was examined (Fig. 4A, traces j–m). When ATP was not added, a slightly decreased amount of Chlide was formed after incubation (Fig. 4A, trace l). About one-half the amount of Chlide was formed with no addition of the ATP regeneration system (Fig. 4A, trace k). The cell-free extracts used in this assay seem to contain significant amounts of ATP or ADP because no desalting process was carried out. Thus, the exogenously added ATP regeneration system efficiently reproduced ATP, supporting DPOR activity. Similar retention of activity without either the ATP or the ATP regeneration system was observed in the initial characterization of nitrogenase with the crude extract that had not been desalted (Bulen et al., 1965). When both the ATP and ATP regeneration systems were omitted from the reaction mixture, no Chlide formation was detected (Fig. 4A, trace j). This result confirmed that PbDPOR is an ATP-dependent enzyme, as has been previously shown for RcDPOR (Fujita and Bauer, 2000).

The upper limit of oxygen level for the growth of YFP12 was 3% (Fig. 3, A and B). Then, DPOR activity in the cell-free extracts from YFP12 cells grown under 3% O_2 was compared with that grown under 0% O_2 (Fig. 4B). The initial rate of Chlide formation in the extract from the 3% O_2 condition (1.1 pmol min^{-1} mg^{-1}) was about one-third that of the anaerobically grown cells (3.5 pmol min^{-1} mg^{-1}). This result suggests that the increase in environmental oxygen level leads to a decrease in DPOR activity, resulting in growth retardation. DPOR activity detected in cells grown in 3% O_2 appears to be the minimal activity capable of supporting photoautotrophic growth of this cyanobacterium under these conditions.

To examine how PbDPOR activity is sensitive to oxygen, the extract was exposed to air for various times before the assay (Fig. 4C). Activity was very quickly lost upon exposure to the air, with an estimated half-life of approximately 10 min. This result clearly indicates that PbDPOR activity is very sensitive to oxygen and supports the results from the phenotypic analysis of the YPF12 mutant.

Two DPOR Subunits, ChlL and ChlN, Increase in Anaerobically Grown YFP12 Cells

To address why DPOR activity was stimulated in anaerobically grown YFP12 cells, the abundance of three DPOR subunits (ChlL, ChlN, and ChlB) and LPOR in YFP12 and YFD1 were examined in cells grown anaerobically for 24 h (Fig. 5A). Based on densitometric analysis of the purified subunits with known amounts in the same gel for western-blot analysis, we estimated the absolute amounts of DPOR subunits in YFD1 and YFP12 cells (Fig. 5B). Interestingly, ChlL and ChlN subunits were much more abundant in YFP12 cells than in YFD1 cells, whereas the level of ChlB was almost the

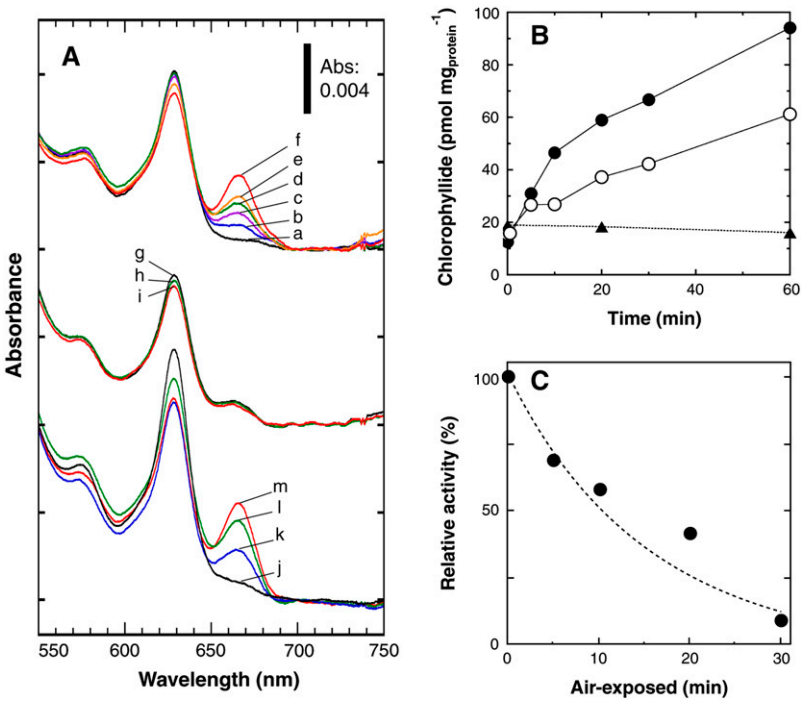

Figure 4. DPOR activity in cell-free extracts of anaerobically grown cells. Cell-free extracts were prepared from YFP12 and YFD1 cells grown anaerobically under high light for 24 h. DPOR assays were carried out with cell-free extracts from YFP12 (A, traces a–f) and YFD1 (A, traces g–i). Incubation periods were 0 min (a, black), 5 min (b, blue), 10 min (c, purple), 20 min (d, green), 30 min (e, yellow), and 60 min (f, red) for the assay with YFP12 extracts (0.34 mg protein), and 0 min (g, black), 20 min (h, green), and 60 min (i, red) for the assay with YFD1 extracts (0.54 mg protein). The reactions were stopped by the addition of acetone followed by phase partitioning with *n*-hexane. Absorption spectra of the lower phase were recorded. A (traces j–m), ATP requirement of the DPOR reaction. Trace m (red) represents the complete reaction containing 1 mM ATP and the ATP regeneration system, trace l (green) represents a reaction without ATP, trace k (blue) represents a reaction without the ATP regeneration system, and trace j (black) represents a reaction without ATP and the ATP-regeneration system. B, Time course of Chlide formation of the assay with cell-free extracts of YFP12 grown under 0% O_2 (black circles) and 3% O_2 (white circles), and YFD1 under 0% O_2 (black triangles). The amounts of Chlide in anaerobically grown (0% O_2) YFP12 and YFD1 extracts were calculated from the respective absorption spectrum (A, traces a–i). C, Oxygen sensitivity of DPOR activity. Aliquots of the cell-free extract of YFP12 were exposed to the air for various time periods at 4°C. After exposure, DPOR assays were carried out under anaerobic conditions as described. The DPOR activity after 30-min incubation under anaerobic conditions was taken as standard (100%) activity, which was 1.4 pmol min^{-1} mg^{-1}.

same as for YFD1. Increased levels of ChlL, ChlN, and ChlB in YFP12 compared with YFD1 were 3.6-, 3.8-, and 1.1-fold, respectively. Assuming that PbDPOR consists of two components, L-protein [ChlL homodimer: $(ChlL)_2$] and NB-protein [ChlN-ChlB heterotetramer: $(ChlN)_2(ChlB)_2$], as well as RcDPOR (Nomata et al., 2005; J. Nomata and Y. Fujita, unpublished data), the content of L-protein and NB-protein in the cells could be estimated (Table I). YFP12 cells contained 3.6 times as much L-protein as did YFD1 cells. Because ChlN content (10.3 pmol mg^{-1}) was much less than ChlB content (30.3 pmol mg^{-1}) in YFD1 cells, the NB-protein content was determined by the lower ChlN content to be 5.2 pmol mg^{-1}. In contrast, the ChlN content in YFP12 cells (39.3 pmol mg^{-1}) increased to be almost the same as for the ChlB content (32.8 pmol mg^{-1}), which was similar to that obtained for YFD1. NB-protein content in YFP12 cells was 16.4 pmol mg^{-1}, which was about 3.2 times higher than that in YFD1 cells. Consequently, the content of both L-protein and NB-protein in YFP12 was more than 3 times higher than in YFD1. This resulted in greater DPOR activity, perhaps to compensate for the loss of LPOR activity in the mutant.

In addition, the DPOR activity of YFP12 cells grown under 3% O_2 was decreased by about 70% compared with that of YFP12 cells grown under 0% O_2 (Fig. 4B). Western-blot analysis indicated that the amounts of ChlL and ChlN in 3% O_2-grown YFP12 cells were comparable to those in 0% O_2-grown YFP12 cells (data not shown). This result supports the idea that the increase of environmental oxygen level causes inactivation of DPOR enzyme activity leading to growth inhibition of YFP12.

Taken together, it is suggested that anaerobic restoration of YFP12 growth occurred for two reasons: (1) a marked increase in the abundance of ChlL and ChlN led to an increase in DPOR activity; and (2) the negative effect of the oxygen sensitivity of DPOR was effectively neutralized by the anaerobic conditions.

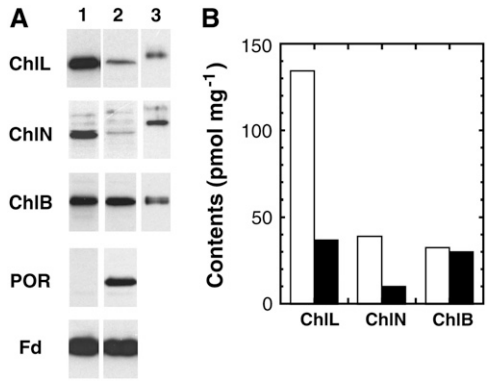

Figure 5. Western-blot analysis for DPOR subunits and LPOR in YFP12 and YFD1 cells (A) and the quantification of the DPOR subunits by densitometry (B). A, YFP12 (LPOR lacking, lane 1) and YFD1 (control strain, lane 2) were grown photoautotrophically under anaerobic conditions for 24 h. Cells were harvested, cell-free extracts were prepared and separated by SDS-PAGE (5.5 μg protein/lane), electrotransferred onto PVDF membranes, and immunostained with antisera against ChlL-6xHis, ChlN-6xHis, ChlB-6xHis, LPOR-6xHis (POR), and maize ferredoxin I (Fd). Purified ChlL-6xHis (11.5 ng), ChlN-6xHis (15.6 ng), and ChlB-6xHis (9.2 ng) proteins were loaded as quantification markers onto the same gel (lanes 3). Note that purified 6xHis tag proteins show a slight mobility difference due to the tags compared with the proteins in the crude extracts. B, Amounts of DPOR subunits in YFP12 (white bars) and YFD1 (black bars) were quantified densitometrically by NIH Image Version 1.63 based on the respective purified subunit with known amounts.

DISCUSSION

Cyanobacteria have two structurally unrelated Pchlide reductases, LPOR and DPOR, to produce Chlide, the direct precursor for Chl *a* (Fujita, 1996). In our previous work, we reported that the two Pchlide reductases are partially differentiated depending on light intensity; DPOR is the sole operative reductase in the dark, LPOR and DPOR compensate each other in low light conditions, and LPOR is essential in high light conditions (Fujita et al., 1998). Because cyanobacteria are widely distributed in nature in terrestrial, freshwater, and marine habitats (Whitton and Potts, 2000), there should be other environmental factors responsible for the differentiation of the two enzymes besides light intensity. In this article, we found that the oxygen level in the environments is another key factor. Even in high light conditions, where the LPOR-lacking mutant cannot grow aerobically (Fig. 2, A and C), the mutant grows well under anaerobic conditions (Fig. 2, B and D). This growth restoration suggests that DPOR, the sole Pchlide reductase in the mutant, is operative in anaerobic conditions, which is consistent with the nitrogenase-like features of DPOR.

P. boryanum exhibits diurnal reciprocal cycles of nitrogen fixation (N-phase) and photosynthesis (P-phase), which results in discontinuous growth as shown by stepwise increases in turbidity and protein content (Rai et al., 1992; Misra et al., 2003). By contrast, the LPOR-lacking mutant, which synthesizes Chl with only the nitrogenase-like enzyme DPOR, showed normal exponential growth (Fig. 2B) concomitant with a continuous increase in Chl content (Fig. 2D). In addition, the content of any DPOR subunits during anaerobic growth did not change with the diurnal cycle (data not shown). These results indicate that DPOR operates simultaneously in *P. boryanum* with photosynthetic oxygen evolution not being temporally separated from photosynthesis.

We determined the maximal oxygen level at which an LPOR-lacking mutant was capable of growing photoautotrophically. From an evolutionary point of view, it is highly possible that the environmental oxygen level during the Proterozoic era was a key factor in the evolutionary development of LPOR in ancient cyanobacteria. YFP12 could not grow in aerobic and high light conditions (Fig. 2A). Assuming that LPOR-lacking mutants of other cyanobacteria commonly show this phenotype, an implication is that the evolution of LPOR was critical for the survival of ancestral cyanobacteria that were growing at or near the surface of the ocean and exposed to strong solar irradiation in oxidative environments. The maximal oxygen level under which YFP12 could grow was 3%, coincident with a proposed atmospheric oxygen level of 0.03 atm (3% [v/v]) at 2.2 to 2.0 Ga (Rye and Holland, 1998), implying that LPOR had evolved by 2.2 to 2.0 Ga at the latest (Fig. 6). This assumes that the oxygen sensitivity of the current and ancestral forms of DPOR is the same; if ancestral DPOR was more sensitive than current DPOR, LPOR evolution may have

Table I. *Amounts of two components of DPOR in the control and YFP12 cells*

Amounts of the three DPOR subunits (ChlL, ChlN, and ChlB) in crude extracts of the control (YFD1) and YFP12 (Δ*por*) cells grown anaerobically for 24 h were quantified by the densitometric comparison shown in Figure 5.

Strain	ChlL		ChlN-ChlB			Component Ratio[c]
	ChlL	L-Protein[a] $[(ChlL)_2]$	ChlN	ChlB	NB-Protein[b] $[(ChlN)_2(ChlB)_2]$	
	pmol mg^{-1}		*pmol mg^{-1}*			
YFD1	37	18.5	10.3	30.3	5.2	3.6
YFP12	135	67.5	39.3	32.8	16.4	4.1

[a]L-protein $[(ChlL)_2]$ was estimated as one-half of ChlL. [b]NB-protein $[(ChlN)_2(ChlB)_2]$ was estimated as one-half of ChlN or ChlB with the lower content. [c]Component ratio is expressed as $(ChlL)_2/(ChlN)_2(ChlB)_2$.

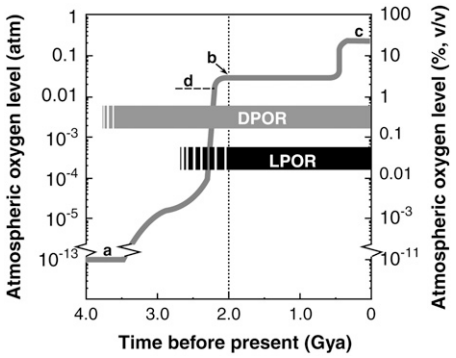

Figure 6. Proposed emergence time of two Pchlide reductases (DPOR and LPOR) in response to the rise of atmospheric oxygen level. When primary photosynthesis first arose on Earth, only trace levels of oxygen were in the atmosphere (10^{-13} atm; 10%–11% [v/v]; Kasting 1987; a). After oxygenic photosynthesis evolved, oxygen levels gradually increased, followed by a rapid rise between 2.4 and 2.2 Ga. The oxygen level then reached 0.03 atm (3% [v/v]) at about 2.2 to 2.0 Ga (Rye and Holland, 1998). The time when the oxygen level reached 3% is the Chl Pasteur point (b). By about 0.5 Ga, the oxygen level reached its present level (0.21 atm; 21% [v/v]; c). The oxygen level just above the oxygen oases (Kasting, 1992) is proposed to be 0.016 atm (1.6% [v/v]; d).

occurred much earlier than 2.2 Ga. DPOR evolved from nitrogenase-related genes and was the sole Pchlide reductase for Chl biosynthesis in ancestral cyanobacteria (earlier than approximately 2.7 Ga; Blankenship, 2001; Knoll, 2003; Grula, 2005). The trace level of atmospheric oxygen (approximately 10^{-13} atm; Fig. 6a) at that time (Kasting, 1987) was not sufficient to inhibit DPOR activity. However, evolution of oxygenic photosynthesis led to a gradual increase in the atmospheric oxygen level, which was followed by a rapid rise of about 2.4 to 2.2 Ga. The oxygen level reached 0.03 atm sometime between 2.2 and 2.0 Ga (Fig. 6b; Rye and Holland, 1998), causing oxygen inhibition of Chl biosynthesis in ancient cyanobacteria. The rapid rise in global oxygen level may have also driven the evolution of heterocysts to protect nitrogenase from oxygen (Tomitani et al., 2006). Extant cyanobacteria could be monophyletic descendants of the ancient cyanobacterial group that evolved LPOR (Yang and Cheng, 2004) because all cyanobacteria examined thus far, including *Gloeobacter violaceus* PCC 7421, which is thought to be the most deeply rooted cyanobacterium, contain both LPOR and DPOR (Nakamura et al., 2003). This implies that other ancestral cyanobacterial lineages that lacked LPOR became extinct at that time. This evolutionary scenario and the experimental evidence are consistent with the hypothesis previously proposed (Reinbothe et al., 1996).

The oxygen level of 0.3% (v/v) is called the Pasteur point, the level at which most extant aerobes and facultative anaerobes adapt from anaerobic fermentation to aerobic respiration (Berkner and Marshall, 1965; Runnegar, 1991). We have termed the critical atmospheric oxygen level (3% [v/v] in *P. boryanum* under high light) for Chl biosynthesis with only DPOR as the Chl Pasteur point. It is defined as the level above which the activity of the oxygen-sensitive Pchlide reductase DPOR is functionally insufficient and the oxygen-tolerant reductase LPOR becomes essential to survive (Fig. 3B). The Chl Pasteur point could provide a concept to help understand how dual Pchlide reductases operate in extant cyanobacterial cells. The oxygen level in natural environments undergoes dynamic changes throughout the day. For example, oxygen levels go up from 20% saturation in the morning to 150% saturation at noon in a vernal pool (Keeley, 1988). The oxygen level in cyanobacterial mats drops sharply to zero at a depth of 4 mm from the mat surface (Jørgensen et al., 1979). In environments where the oxygen level is below the Chl Pasteur point, DPOR significantly contributes to Chl biosynthesis.

We have detected DPOR activity in an oxygenic phototroph. Clearly established previous reports of DPOR activity in oxygenic phototrophs are lacking (Peschek et al., 1989a, 1989b; Forreiter and Apel, 1993). Three factors have made the detection of DPOR activity difficult in oxygenic phototrophs, including cyanobacteria: (1) Because these organisms have two Pchlide reductases, LPOR and DPOR, it has been difficult to discriminate between LPOR and DPOR activities; (2) as demonstrated here, DPOR activity is very sensitive to oxygen; and (3) the abundance of the DPOR subunits is very low and can be detected only with specific antisera (Fujita et al., 1989, 1996), suggesting that DPOR activity is too low to detect in wild-type cells (Fig. 4A). We overcame these difficulties as follows: (1) An LPOR-lacking mutant, YFP12, was used and the assay was performed in the dark, and ATP dependency, which is a DPOR-specific property, was also examined (Fujita and Bauer, 2000); (2) YFP12 was cultivated in anaerobic conditions and cell-free extracts were prepared in an anaerobic chamber, which was used for RcDPOR assay (Fujita and Bauer, 2000; Nomata et al., 2005); and (3) compared with the control strain YFD1, ChlL and ChlN levels were significantly increased in anaerobically grown YFP12 cells. This overexpression apparently resulted in stimulation of DPOR activity, allowing it to be detected by its activity in vitro (Figs. 4 and 5).

One may question why DPOR activity is stimulated by the increase in only two subunits. This could be explained by the coordinated increase of ChlL and ChlN proteins. In contrast to dynamic changes in ChlL and ChlN content, ChlB content was kept almost constant in the control and YFP12 cells (Fig. 5). Because ChlN content was much lower than ChlB content in control cells, ChlN content limited NB-protein content. ChlN content was markedly increased and reached a level equivalent to ChlB and formed active NB-protein with almost all existing ChlN and ChlB proteins (Table I). In accord with the rise of NB-protein level, the L-protein level also increased. Consequently, the content of both L-protein and NB-protein increased, resulting in an increase in DPOR activity. For maximal activity of a certain amount of NB-protein, more than

a 3-fold excess amount of L-protein is required in RcDPOR (J. Nomata and Y. Fujita, unpublished data). The coordinate increase of ChlL and ChlN content probably results from chromosomal localization of *chlL* and *chlN* genes in the same small operon in the *P. boryanum* genome. The *chlL* and *chlN* genes were previously shown to be cotranscribed (Fujita et al., 1991). Interestingly, the ratio of ChlL to ChlN was commonly about 3.5 in control and YFP12 cells (Fig. 5B). The L-protein to NB-protein ratio was kept constant at about 4 (Table I), which might be optimal for DPOR activity. The operon structure of the *chlL* and *chlN* genes is widely conserved from cyanobacterial genomes to chloroplast genomes (Fujita and Bauer, 2003). The *chlL-chlN* operon might provide a molecular mechanism to keep the L-protein to NB-protein ratio optimal for DPOR activity.

DPOR has been characterized previously in only the anoxygenic photosynthetic bacterium *R. capsulatus* (Fujita and Bauer, 2000; Nomata et al., 2005). As expected from its sequence similarity to nitrogenase, RcDPOR showed many features in common with nitrogenase, such as oxygen sensitivity, ATP dependency, and subunit composition. Because the photosynthetic apparatus (including bacteriochlorophyll) in *R. capsulatus* is synthesized only under anaerobic conditions (Sganga and Bauer, 1992), and the photosystem does not evolve oxygen, RcDPOR activity should be scarcely inhibited by oxygen in the natural environment where photosynthesis takes place. However, DPOR is widely distributed among oxygenic phototrophs, such as cyanobacteria and many photosynthetic eukaryotes (Fujita and Bauer, 2003), and not only environmental oxygen but also oxygen generated from PSII interferes with DPOR activity in their cells. Thus, it might be expected that the DPOR of oxygenic phototrophs acquired some degree of oxygen tolerance during evolution. However, PbDPOR activity was very sensitive to oxygen, with a half-life of about 10 min (Fig. 4C), which is a slightly shorter time than that of RcDPOR activity (about 40 min; Nomata et al., 2006a). This result suggests that at least cyanobacterial DPOR has not evolved to become more oxygen tolerant. The effect of oxygen level on nitrogenase activity was examined in *P. boryanum* strain 594 (Weare and Benemann, 1974). Nitrogenase activity was more than 90% inhibited by 6% oxygen, which is in good agreement with the case of photoautotrophic growth dependent on DPOR (3% is maximal oxygen level) in this study (Fig. 3, A and B). Indeed, only about 30% DPOR activity was detected in YFP12 cells grown under 3% O_2 compared with that of anaerobically grown YFP12 cells (Fig. 4B). It is suggested that PbDPOR conserves the vulnerability to oxygen just like nitrogenase. It may be that oxygenic phototrophs containing DPOR, including cyanobacteria, have developed some mechanisms to protect DPOR from oxygen rather than improving the oxygen tolerance of DPOR itself. It is noteworthy that the LPOR-lacking mutant grows as well as the wild type, with a normal level of Chl under low light ($<25\ \mu E\ m^{-2}\ s^{-1}$) even under aerobic conditions (Fujita et al., 1998). The light intensity of about $20\ \mu E\ m^{-2}\ s^{-1}$ gives one-sixth of the maximal rate of oxygen evolution achieved by saturating light intensity ($>100\ \mu E\ m^{-2}\ s^{-1}$) in *P. boryanum* (Kimata-Ariga et al., 2000). The oxygen-scavenging capacity of the protection system appears to be sufficient for low-level oxygen evolution by low light but to be limited less than the oxygen evolution rate near the maximum achieved by high light irradiation.

We assume that the higher oxygen concentration in cells grown aerobically under high light leads to inactivation of DPOR in vivo. This idea is supported by the marked decrease of DPOR activity in YFP12 cells grown under 3% O_2 in comparison with that of YFP12 cells grown under 0% O_2 (Fig. 4B). However, we do not exclude the possibility that DPOR is inactivated by reactive oxygen species (ROS) rather than oxygen itself in the cells. High light irradiation under aerobic conditions promotes ROS production in cyanobacterial cells. Many Fe-S proteins are inactivated not only by oxygen but also by ROS such as superoxide (Gardner, 1997). For example, aconitase B of *Escherichia coli*, which carries a [4Fe-4S] cluster in the active site, is inactivated by superoxide as well as by oxygen (Varghese et al., 2003). Thus, it is possible that the Fe-S clusters of DPOR are destroyed not only by oxygen but also by ROS in cyanobacterial cells. It might be a subject of future study to elucidate how DPOR is inactivated and protected in cyanobacterial cells.

It has been reported that the imposition of anaerobic conditions led to the accumulation of ChlL protein in the green alga *Chlamydomonas reinhardtii* (Cahoon and Timko, 2000). In the cyanobacterium *P. boryanum* YFD1, ChlL content is slightly increased under anaerobic conditions (data not shown). However, the accumulation of ChlL in anaerobically grown YFP12 was much more significant than the anaerobic induction measured for YFD1. This suggests that the marked accumulation of ChlL and ChlN results from a factor caused by the loss of LPOR rather than by anaerobic conditions. Cyanobacterium may have some mechanisms that sense Chl supply or accumulation of intermediates to induce the expression of the *chlL-chlN* operon. The molecular basis for the accumulation of ChlL and ChlN will be clarified in future studies.

Recently, Ouchane et al. (2004) showed that anaerobic and aerobic enzymes are involved in the biosynthesis of Pchlide at the level of fifth-ring (E-ring) formation by Mg-protoporphyrin monomethylester cyclase in many facultative phototrophs, such as photosynthetic bacteria and cyanobacteria. The anaerobic enzyme BchE appears to catalyze the cyclase reaction using water as the oxygen donor with some cofactors of the Fe-S center, *S*-adenosylmethionine and vitamin B_{12}, and to be inactive under high oxygen conditions because of the sensitivity of the Fe-S center to oxygen. BchE belongs to the radical SAM family of enzymes that catalyze a variety of radical reactions, such as coproporphyrinogen oxidase (HemN) and Lys

2,3-aminomutase (Layer et al., 2004). In contrast, the aerobic enzyme AcsF is an oxygen-tolerant di-iron-type monooxygenase that uses molecular oxygen. From an evolutionary perspective, it is possible that ancestral photosynthetic organisms may have only the anaerobic (BchE-type) cyclase, and the aerobic (AcsF-type) cyclase may have evolved later from the di-iron monooxygenase family to adapt to aerobic environments. Thus, the rise in the atmospheric oxygen level during the Proterozoic era is thought to have provided strong selective pressure for photosynthetic organisms to acquire not only LPOR, but also a host of other new enzymes in the Chl biosynthetic pathway, such as AcsF, to continue to effectively produce Chl under increasingly oxidative conditions. The concept of the Chl Pasteur point could be useful for understanding how such dual-enzyme systems operate in the extant photosynthetic organisms growing in environments with various oxygen levels.

MATERIALS AND METHODS

Cyanobacterial Strains, Mutants, and Cultivation Conditions

The mutants YFP12 (Δ*por*), YFC2 (Δ*chlL*), and YFD1 (used as a control strain resistant to kanamycin) were derived from *Plectonema boryanum* IAM-M101 strain *dg5* (Fujita et al., 1998; Kada et al., 2003). This strain was found to correspond to *Leptolyngbya boryana* UTEX 485 based on the nucleotide sequence of 16S rRNA (H. Arima, T. Sakamoto, and Y. Fujita, unpublished data; accession no. AB245143). The kanamycin resistance gene cartridge was introduced into the *por* gene, the *chlL* gene, and the intergenic neutral site between *chlL* and *chlN* in YFP12, YFC2, and YFD1, respectively. These strains were cultivated in BG-11 medium supplemented with 20 mM HEPES-KOH (pH 7.4) and 15 μg mL^{-1} of kanamycin sulfate. For growth monitoring, YFP12, YFC2, and YFD1 were cultivated in low light conditions (40 μmol m^{-2} s^{-1}) in BG-11 medium bubbled with 2% CO_2/air at 30°C for approximately 120 h. Aliquots of these cultures were then inoculated into fresh BG-11 medium in flat bottles to 0.1 of OD_{730}. Following this, they were cultivated for 24 h under high light conditions (250–330 μmol m^{-2} s^{-1}) with bubbling with 2% CO_2/air or 2% CO_2/N_2 containing various amounts of oxygen (0.5%–10%) that were prepared by mixing pure N_2, pure CO_2 (G1 grade; Japan Fine Products; oxygen impurity less than 5 ppm), and air (21% O_2) with an appropriate flow rate (total flow rate: 600 mL min^{-1}). To determine the growth curve, these precultures were inoculated into fresh BG-11 medium to 0.1 of OD_{730} and cultivated under the same conditions. For bubbling with 0.01% and 0% oxygen, premixed pure gas was used (Japan Fine Products). Culture bottles were illuminated by fluorescent lamps (FL20SS; National) at 30°C. Cell turbidity was determined by OD_{730} measured by a spectrophotometer (model U-3210; Hitachi Koki). For cultivation of cells on agar plates under anaerobic conditions, agar plates were incubated in an anaerobic jar (BBL GasPak anaerobic systems; Becton, Dickinson and Company). Pigments were extracted in 90% methanol and the Chl concentration was determined as described previously (Fujita et al., 1998).

Assay of Cyanobacterial DPOR Activity

YFP12 cells were grown in 600 mL of BG-11 medium under high light conditions with bubbling in N_2 or 3% O_2 in N_2 containing 2% CO_2 for 24 h. After the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea to the culture to stop oxygen evolution, the culture was transferred into an anaerobic chamber (model A; COY; Nomata et al., 2005) and approximately 400 mg of sodium dithionite was added to the culture. All subsequent procedures were carried out in the anaerobic chamber using solutions that had been degassed and stored in the chamber, and 1.7 mM sodium dithionite (final concentration) was added just before use to remove residual oxygen. Cells were harvested on a 0.22-μm membrane filter (Millipore) by sucking with a small vacuum pump in the chamber. The harvested cells on the filter were collected and suspended in 5 mL of lysis buffer (Fujita and Bauer, 2000). Cells were disrupted by three 30-s sonication bursts at 50% output (Sonifier 250 sonicator with a micro tip; Branson). The sonicate was then transferred to 10 PC tubes (Hitachi) and centrifuged at 110,000*g* for 1 h (RP65T rotor; Hitachi) at 4°C. The resultant supernatant fractions were collected as cell-free extracts and stored at 4°C in the chamber until used to measure DPOR activity.

DPOR assays were carried out in a 250-μL volume (Fujita and Bauer, 2000; Nomata et al., 2005) containing 2 μM Pchlide and 50 μL of the cell-free extract (approximately 8 mg mL^{-1} protein). All assays were performed in a small glass vial with an airtight butyl rubber cap at 30°C in the dark with gentle shaking. Pchlide was prepared from the culture medium of a *bchL*$^-$ mutant ZY5 (Nomata et al., 2005). The reaction was stopped by the addition of 1 mL of acetone and centrifuged at 17,000*g* for 5 min to collect the supernatant. Chl and carotenoids were removed by phase partitioning with 500 μL of *n*-hexane. The absorption spectra of the lower phase were recorded with a spectrophotometer (model V550; Jasco). The concentration of Chlide was estimated with the equation by Porra (1991) and corrected for the hexane concentration of the acetone phase by multiplication with the factor 0.606 (Nomata et al., 2005). To examine the oxygen sensitivity of DPOR activity, aliquots of the cell-free extracts were exposed to the air for various times on ice. After the samples were returned to the anaerobic chamber, dithionite was added at a final concentration of 1.7 mM to remove oxygen and the DPOR assay was carried out as described above.

Western-Blot Analysis

Harvested cells were suspended in extraction buffer {50 mM HEPES-KOH (pH 7.4) and 10 mM $MgCl_2$ [(culture volume in mL) × OD_{730}/20] mL}, and disrupted by sonication as described above. Phenylmethanesulfonyl fluoride was added (final 1 mM) just after sonication. Sonicates were centrifuged at 17,000*g* for 10 min at 4°C (TMP-11 rotor; Tomy Seiko). The resultant supernatants were collected as crude extracts for western-blot analysis. Protein concentrations of the crude extracts were determined using protein assay (Bio-Rad) with bovine serum albumin as the standard. Western-blot analysis was carried out essentially as described previously (Fujita et al., 1996). Proteins fractionated by SDS-PAGE were electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore). The PVDF membrane was incubated with affinity-purified antibodies (ChlL and ChlN) or antisera (ChlB, LPOR, and ferredoxin), and then with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad). Antisera against ChlB, LPOR, and maize (*Zea mays*) ferredoxin I were as described previously (Fujita et al., 1996, 1998; Kimata-Ariga et al., 2000). The specific protein bands were visualized by chemiluminescent substrate (ECL western blotting analysis system; Amersham Bioscience). The antibodies against ChlL-6xHis and ChlN-6xHis were purified by an epitope selection method after binding to PVDF membranes on which the purified ChlL-6xHis and ChlN-6xHis proteins were electrotransferred (Fujita et al., 1989).

Purification of ChlL-6xHis, ChlN-6xHis, and ChlB-6xHis Proteins

ChlL-6xHis, ChlN-6xHis, and ChlB-6xHis proteins were overexpressed with pQE-60 vector (Qiagen) in *Escherichia coli*. *E. coli* JM105 carrying the respective overexpression vector was cultivated at 37°C for 2 h in Luria-Bertani medium containing ampicillin (50 μg mL^{-1}) after inoculation with 1/500 volume of an overnight culture. Isopropyl 1-thio-β-D-galactoside was added to a final concentration of 1 mM and cultivation was continued for a further 5 h. The *E. coli* cells were harvested and disrupted by three 30-s bursts of sonication (Sonifier 250 sonicator with a micro tip) on ice. All 6xHis proteins were recovered in insoluble fractions (17,000*g*, 15 min) as inclusion bodies and solubilized in a urea buffer (8 M urea, 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 7.35 mM β-mercaptoethanol). The supernatant of the urea buffer (17,000*g*, 15 min) was loaded on a Sephacryl S-300 (1.5 × 72 cm) column that was equilibrated with the urea buffer. Thus, the fractionated ChlL-6xHis and ChlB-6xHis proteins gave almost single bands in Coomassie Brilliant Blue-stained profiles of SDS-PAGE. ChlN-6xHis was purified further by an affinity column (His Trap HP column; Amersham-Pharmacia Biotech) with a stepwise gradient of His (300 mM). Purified 6xHis fusion proteins were quantified spectrophotometrically based on the molar extinction coefficient values at 276 nm (ChlL-6xHis: 26,715; ChlN-6xHis: 51,500; and ChlB-6xHis: 51,070; Gill and von Hippel, 1989).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB245143.

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