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Overexpression and characterization of dark-operative protochlorophyllide reductase from *Rhodobacter capsulatus*☆

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Abstract

Dark-operative protochlorophyllide oxidoreductase (DPOR) plays a crucial role in light-independent (bacterio)chlorophyll biosynthesis in most photosynthetic organisms. However, the biochemical properties of DPOR are still largely undefined. Here, we constructed an overexpression system of two separable components of DPOR, L-protein (BchL) and NB-protein (BchN-BchB), in the broad-host-range vector pJRD215 in *Rhodobacter capsulatus*. We established a stable DPOR assay system by mixing crude extracts from the two transconjugants under anaerobic conditions. Using this assay system, we demonstrated some basic properties of DPOR. The Km value for protochlorophyllide was 10.6 μM. Ferredoxin functioned as an electron donor to DPOR. Elution profiles in gel filtration chromatography indicated that L-protein and NB-protein are a homodimer [$(BchL)_2$] and a heterotetramer [$(BchN)_2(BchB)_2$], respectively. These results provide a framework for the characterization of these components in detail, and further support a nitrogenase model of DPOR.
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1. Introduction

Protochlorophyllide (Pchlide) is an important intermediate in the biosynthesis of chlorophylls (Chls) and bacteriochlorophylls (BChls). Two different enzymes in photosynthetic organisms are capable of reducing a double bond in the D-ring of Pchlide in a stereo-specific manner to form chlorophyllide (Chlide) [1–3]. One enzyme is the light-dependent Pchlide oxidoreductase (LPOR; NADPH-Pchlide oxidoreductase, EC 1.3.1.33), which catalyzes D-ring reduction using NADPH as the reductant. LPOR is a rather novel enzyme in that it requires light absorption by the substrate Pchlide to catalyze the bond reduction. Because LPOR is the sole Pchlide reduction system in angiosperms (flowering land plants), dark-grown angiosperm seedlings fail to synthesize Chl and instead accumulate large amounts of Pchlide bound to LPOR. Upon exposure to light, the Pchlide bound to LPOR is rapidly converted to Chlide. LPOR has been studied extensively because it is a key enzyme in the greening process of angiosperms and can be readily isolated and assayed for enzymatic activity [4–6].

In contrast to the dependence on light for the "greening" (Chl synthesis) of angiosperms, some photosynthetic organisms such as nonflowering land plants, algae, cyanobacteria, and anoxygenic photosynthetic bacteria are capable of synthesizing Chls and BChls in the dark. These organisms have an unrelated Pchlide reductase enzyme called dark-operative Pchlide oxidoreductase (DPOR; light-independent Pchlide oxidoreductase), which catalyzes double-bond reduction in a light-independent manner [1,7,8]. For more than a century, DPOR had been considered a mysterious enzyme, in large part because of the absence of a reliable assay system, which has hampered efforts at purification [7,8]. A significant advance in understanding

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DPOR was facilitated by a series of genetic studies that revealed that three genes, *bchL*, *bchN*, and *bchB* (the orthologs in Chl-synthesizing organisms are *chlL*, *chlN*, and *chlB*, respectively), encode subunits of DPOR in *Rhodobacter capsulatus*, *Plectonema boryanum*, and *Chlamydomonas reinhardtii* [1,7–9]. Interestingly, the DPOR subunits, BchL, BchN, and BchB, exhibit significant sequence similarity to the nitrogenase subunits, NifH, NifD, and NifK, respectively, suggesting that the mechanism of reducing the D-ring of Pchlide may be similar to the reduction of dinitrogen by nitrogenase [7]. Nitrogenase is a complex, well-characterized metalloenzyme consisting of two separable component proteins, referred to as Fe protein and MoFe protein. Fe protein is a homodimer of NifH, containing one [4Fe–4S] cluster, which is coordinated by two Cys that are donated by each NifH subunit. Fe protein functions as an ATP-dependent electron donor to the MoFe protein complex where dinitrogen is reduced to ammonia. Oxidized Fe protein is subsequently reduced by ferredoxin or flavodoxin [10]. The amino acid sequences of BchL/ChlL exhibit ~30% identity to that of NifH protein, including a well-conserved ATP-binding motif and four Cys residues, two of which are thought to be involved in chelating a [4Fe–4S] cluster [7]. Therefore, it has been hypothesized that BchL/ChlL functions as an ATP-dependent reductase for BchN/ChlN–BchB/ChlB, which reduces Pchlide with electrons from BchL/ChlL [1,7,8].

The other two DPOR subunits, BchN/ChlN and BchB/ChlB, exhibit sequence similarity to both of NifD and NifK proteins, albeit at a lower level of 25%–30% similarity (including three Cys residues in NifD and one Cys residue in NifK) [1,7]. A heterotetramer of NifD and NifK forms the MoFe protein complex, which contains two novel metalloclusters that occur in pairs. One is the P cluster, which is an [8Fe–7S] cluster, and the other is the FeMo-cofactor comprising [1Mo–7Fe–9S–X homocitrate] [11]. The P cluster is thought to shuttle electrons from the Fe protein to the FeMo-cofactor where the reduction of nitrogenase occurs.

In a previous study, we described the isolation of DPOR from *R. capsulatus*, together with an initial description of an assay system for its activity [12]. That study demonstrated that DPOR consists of two separable components, L-protein (BchL) and NB-protein (BchN-BchB), and that DPOR activity is dependent on ATP and a reducing agent such as dithionite. Based on its functional and sequence similarities to nitrogenase, L-protein was suggested to function as an ATP-dependent electron donor to NB-protein, which provides the catalytic site for Pchlide reduction. However, further biochemical characterization of each component was not undertaken because both components were too labile to purify in sufficient amounts. In this study, we established a stable DPOR assay system by mixing crude extracts from two transconjugants overexpressing L-protein and NB-protein in *R. capsulatus*. Using this stable assay system, we have extended our biochemical analysis of the DPOR components, determining the Km value for Pchlide, its dependence on ferredoxin, and the molecular mass of each component. These results provide further support for a nitrogenase model of DPOR [12].

2. Materials and methods

2.1. Strains and culture conditions

A photosynthetically competent *R. capsulatus* mutant DB176 (ORF176::Km^R; [13,14]) was used as the host strain to overexpress S-tag-modified *bchL* and *bchN* genes. Preculture of the transconjugants were grown heterotrophically in PY medium [15] overnight with vigorous shaking at 200 rpm in the dark at 34 °C. To prepare crude extracts, the transconjugants were grown photosynthetically in an RCV medium [16] illuminated with incandescent lamps (100 W, National, Osaka, Japan) for 3 days at 34 °C. PY and RCV media contained 5 μg ml^{-1} kanamycin and 0.5 μg ml^{-1} streptomycin for the maintenance of plasmids.

2.2. Construction of overexpression plasmids

To overexpress BchL and BchN as S-tag fusion proteins, pJRD-SFX-bchL10 and pJRD-SFX-bchNB111 were constructed, respectively (Fig. 1). A chimeric DNA fragment consisting of the *puc* promoter, S-tag, and Factor Xa cleavage site was excised from pYCSFX by *Bam*HI and *Kpn*I digestion [12]. The 324-bp fragment was then ligated into the *Bam*HI and *Kpn*I sites of the broad-host-range vector pJRD215 [17], yielding pJRD-SFX. A pair of primers, bchLf2 (5′-GTGGTACCAAGCCCGCGCGACGATATTCC-3′) and bchLr2 (5′-ACGGTACCTCAGTCGAATCCCAGAAGTTC-3′), was used to amplify the entire coding region of *bchL* by the polymerase chain reaction (PCR). Another pair of primers, bchNf2 (5′-GCGGTACCGAGCCTCGATTCGCCGACTTT-3′) and bchBr2 (5′-CTGGTACCTCATCGTGCATAATGGGCCTT-3′), was used to amplify a 2.8-kb chromosomal region covering the entire contiguous coding regions of *bchN* and *bchB*. PCR was performed with KOD DNA polymerase (KOD-Plus, Toyobo, Osaka, Japan) using the genomic DNA of *R. capsulatus* as the template. Amplified *bchL* and *bchN-bchB* fragments (935 bp and 2869 bp, respectively) were digested with *Kpn*I (underlined above), and ligated into the *Kpn*I site of pJRD-SFX, yielding the final overexpression plasmids pJRD-SFX-bchL10 and pJRD-SFX-bchNB111, respectively. The amino acid sequences of the S-tag fusion proteins were identical to those previously reported [12].

2.3. Isolation of transconjugants

The plasmids pJRD-SFX-bchL10 and pJRD-SFX-bchNB111 were transferred into *R. capsulatus* DB176 cells

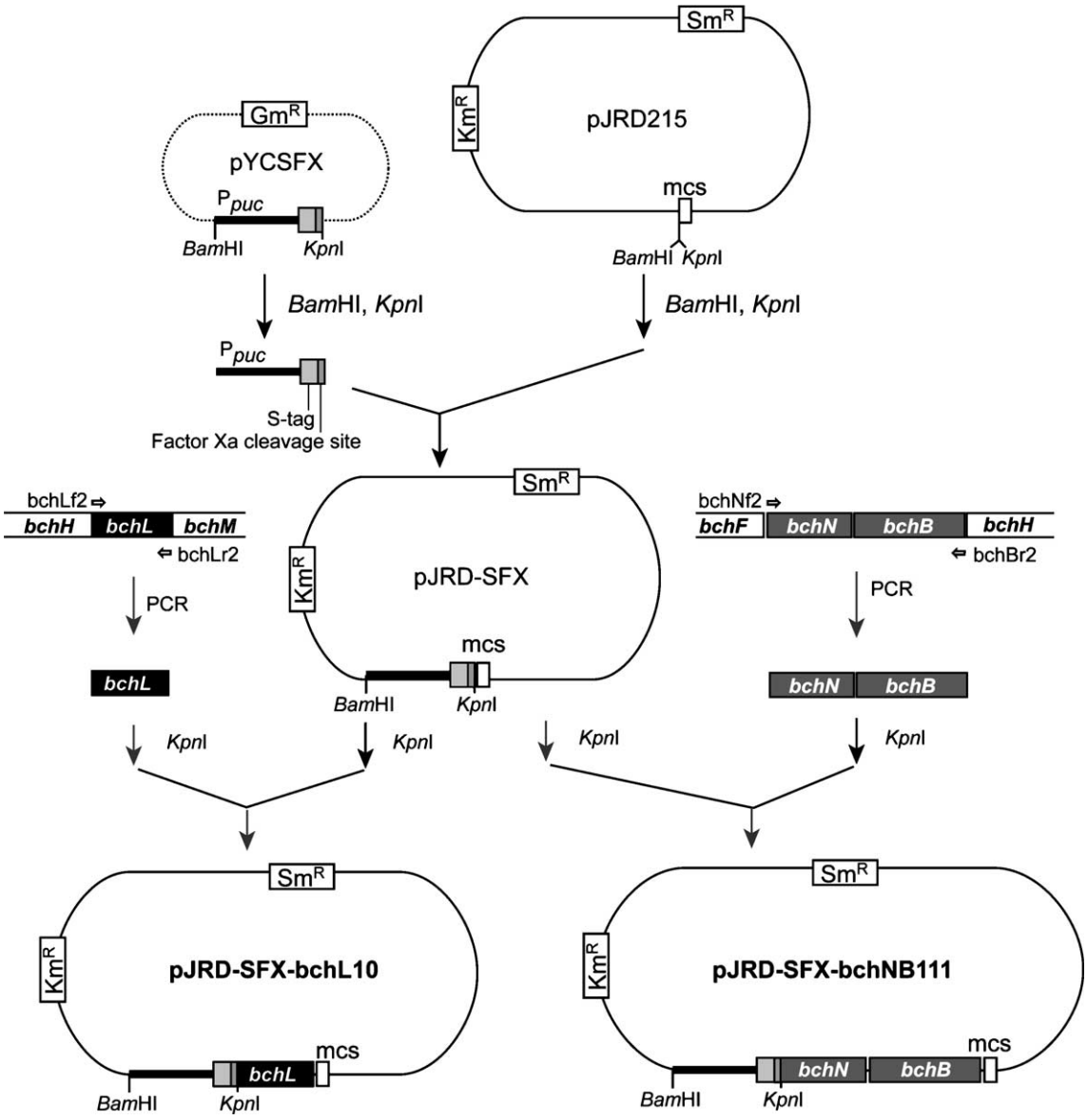

Fig. 1. Construction of plasmids used to overexpress DPOR L-protein and NB-protein as S-tag fusion proteins. A chimeric DNA fragment consisting of the *puc* promoter, S-tag, and Factor Xa cleavage site excised from pYCSFX was ligated into the *Bam*HI and *Kpn*I sites of pJRD215 [17] to form pJRD-SFX. Two plasmids, pJRD-SFX-bchL10 and pJRD-SFX-bchNB111, were constructed by ligating PCR amplified *bchL* and *bchN-bchB* fragments, respectively, from the chromosome into the *Kpn*I site of pJRD-SFX. The plasmids thus constructed were introduced into DB176 cells by triparental mating. mcs, multicloning site; Gm^R, gentamycin-resistance marker; Km^R, kanamycin-resistance marker; and Sm^R, streptomycin-resistance marker.

by triparental mating with *E. coli* strain JM105 containing the relevant plasmids [18]. Transconjugants were selected on PY plates containing rifampicin (100 μg ml^{-1}), kanamycin (10 μg ml^{-1}), and streptomycin (0.5 μg ml^{-1}). Transconjugate strains expressing high levels of S-tag L-proteins and S-tag NB-protein were designated YCL10 and YCNB111, respectively.

2.4. Preparation of crude extracts

Crude extracts of YCL10 and YCNB111 (protein content about 4 mg ml^{-1}) were prepared essentially as described previously [12]. Cultures were grown photosynthetically in a screw capped 500 ml bottle for 3 days, and then transferred into an anaerobic chamber (model A, COY, Grass Lake, MI) containing 1% H_2, 5% CO_2, and 94% N_2. About 25 mg of sodium dithionite was added to the collected cultures, and the cells were then harvested by ultracentrifugation at 7500×*g* for 15 min at 4 °C (RP42 rotor; Hitachi, Japan) in 80PC (Hitachi) tubes to keep the cells relatively anaerobic during centrifugation. All subsequent procedures were carried out in an anaerobic chamber using solutions that had been degassed and stored in an anaerobic chamber, with 1.7 mM sodium

dithionite (final concentration) added just before use to remove residual oxygen. Cell pellets were suspended in lysis buffer [12] and the cells disrupted by four 30 s sonication bursts at 50% output (Sonifier 250 sonicator with a micro tip; Branson, Danbury, CT). The sonicate was then transferred to 30PC tubes (Hitachi) and centrifuged at 37,000×*g* for 30 min (RP50-2; Hitachi) at 4 °C. The resultant supernatant fractions were collected as crude extracts and stored at 4 °C until use in activity assays.

2.5. Assay of Pchlide reduction

Assays of DPOR activity were carried out in a 250 μl volume containing 100 mM HEPES–KOH (pH 7.4), 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2 mM ATP, 20 mM creatine phosphate, 21 units μl^{-1} creatine phosphokinase, 0.68 mM sodium dithionite, 1–20 μM Pchlide, and 12.5 μl of enzyme extract(s). All assays were performed at 34 °C for 30–90 min in a small glass vial with an airtight butyl rubber cap. Pchlide was prepared from the culture medium of a *bchL*$^-$ mutant ZY5, as described previously [12]. When ferredoxin was used as an electron donor, ferredoxin (ferredoxin III, [19]), ferredoxin-$NADP^+$ oxidoreductase (FNR, [20]), and NADPH were added to the reaction mixtures instead of dithionite, at final concentrations of 10 μM, 10 nM, and 0.2 mM, respectively. The reactions were stopped by the addition of 1 ml of acetone. BChls and carotenoids in the extracts were removed by phase partitioning with 500 μl of hexane. The absorption spectra of Pchlide and Chlide in the lower phase were then recorded with a Jasco V550 spectrophotometer (Jasco, Hachioji, Japan). The concentration of Chlide in the lower acetone phase was estimated by the equation described by Porra [21], and corrected for the hexane concentration of the acetone phase by multiplication with a factor 0.606.

2.6. Gel filtration

Extracts from YCL10 and YCNB111 were fractionated by size exclusion on a pre-packed HiLoad 16/60 Superdex 200 pg column (Amersham Biosciences, Piscataway, NJ), equilibrated with 50 mM HEPES–KOH (pH 8.0), 100 mM NaCl, 10 mM β-mercaptoethanol, 1 mM DTT, and 1.7 mM sodium dithionite on an ÄKTAprime chromatography system (Amersham Biosciences) housed in an anaerobic chamber. Fractions containing L-protein and NB-protein components were identified by fluorometry using a fluorescent substrate for the S-tag–S-protein complex that shows RNase activity (FRETWorks S-Tag Assay Kit; Novagen) using a Jasco fluorometer (model FP-777W). Fractions of YCL10 or YCNB111 were also assayed for DPOR activity by mixing with the complementary crude extracts of YCNB111 (for L-protein) or YCL10 (for NB-protein), respectively, as described above.

3. Results

3.1. Overexpression of S-tag BchL, and S-tag BchN and BchB

In our previous study, we affinity-purified S-tag L-protein and S-tag NB-protein from recombinant *R. capsulatus* strains that were unable to grow photosynthetically because of a genetic disruption of BChl biosynthesis [12]. These strains produced low yields of DPOR because the DPOR subunits were expressed from a chromosomally encoded promoter that has maximal activity under photosynthetic growth conditions. To overcome these limitations, we constructed new photosynthetically competent DPOR expression strains in which the strong *puc* promoter from the light-harvesting II operon drives the expression of either S-tag L-protein or S-tag NB-protein. To increase gene dosage, we placed the *puc*-expressed DPOR subunits on the multi-copy plasmid pJRD215 (pJRD-SFX-bchL10 and pJRD-SFX-bchNB111, Fig. 1). To further drive maximal expression, we also used the *R. capsulatus* strain DB176, in which the chromosomally encoded *idi* gene (ORF176) is disrupted by insertion of a kanamycin resistance cartridge [13]. Expression from the *puc* promoter is significantly elevated in DB176 over that observed in wild-type cells [14]. Finally, DB176 is able to grow photosynthetically, which is the growth condition under which the *puc* promoter is maximally active.

Fig. 2 shows the overexpression of S-tag L-protein in strain YCL10 (DB176 containing pJRD-SFX-bchL10) and S-tag NB-protein in strain YCNB111 (DB176 containing pJRD-SFX-bchNB111) when grown under photosynthetic

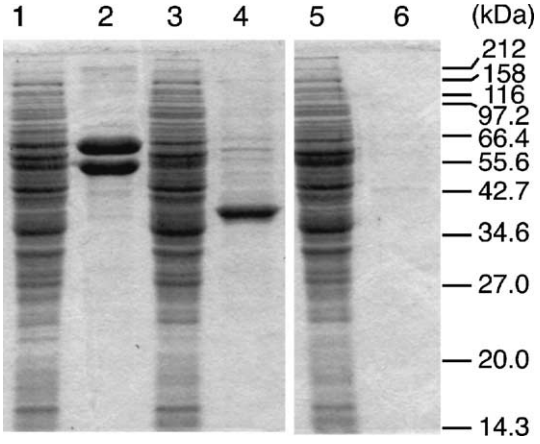

Fig. 2. SDS-PAGE to confirm the overexpression of L-protein and NB-protein in DB176. Crude extracts of YCNB111 (lane 1) and YCL10 (lane 3) cells grown photosynthetically were prepared as described in Materials and methods. Aliquots (10 μl) of S-protein agarose slurry were added to 250 μl of crude extracts to pull down S-tag BchL and S-tag BchN specifically with the agarose. The agarose pellets were washed twice and suspended in 10 μl of loading buffer. The slurries (5 μl) from YCNB111 (lane 2) and YCL10 (lane 4) were loaded into each lane. As a negative control, crude extract was prepared from DB176 cells (lane 5), and the S-protein agarose slurry prepared as above with crude extracts of DB176 was loaded (lane 6).

conditions. Compared with the crude extract of DB176 without plasmids (Fig. 2, lane 5), strain YCNB111 had significant additional bands with apparent molecular masses of 52 kDa and 60 kDa (Fig. 2, lane 1). To confirm that these bands represent the overexpression of the S-tag NB-protein complex, S-tag BchN protein was "pulled down" with S-protein agarose that specifically binds to S-tag proteins. The peptide profile in Fig. 2, lane 2 shows two bands indicative of the complex formation between BchN and BchB proteins that has been shown previously [12]. The expression of L-protein occurred at a lower level in strain YCL10 although a faint but significant band with an apparent molecular mass of 36 kDa was detected (Fig. 2, lane 3). This band also comigrated with the "pulled down" L-protein with S-protein agarose affinity (Fig. 2, lane 4). There was no protein bound to S-protein agarose in the control crude extract from DB176 cells that do not carry an expression plasmid (Fig. 2, lane 6). The apparent molecular masses of the three proteins, 36 kDa (S-tag BchL), 52 kDa (S-tag BchN), and 60 kDa (BchB), are in good agreement with the calculated molecular masses of these proteins; 36,046 Da, 48,671 Da, and 57,191 Da, respectively.

3.2. DPOR activity in crude extracts

DPOR activity was examined in the crude extracts (Fig. 3). There was a very small Chlide peak at 665 nm generated in the crude extract of YCL10 (in which only the L-protein was overexpressed) or in crude extracts of YCNB111 (in which only the NB-protein was overexpressed) (Fig. 3, dotted and dashed lines, respectively). However, when both crude extracts were added to the assay, a marked Chlide peak appeared; this peak was about five times higher than the sum of each peak generated from crude extracts with only one overexpressed component. The synergetic effect on Chlide formation of mixing the extracts indicates that overexpressed S-tag L-protein from YCL10 must interact with S-tag NB-protein from YCNB111 to form an active complex.

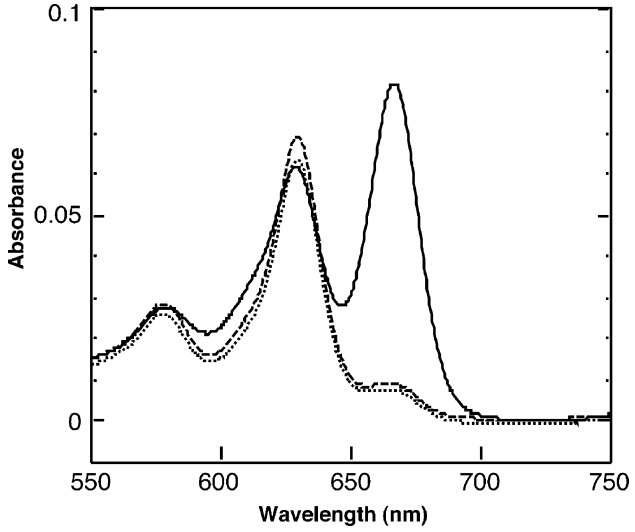

Fig. 3. Synergetic enhancement of Chlide formation by crude extracts of YCL10 and YCNB111. The DPOR assay was performed with crude extract from either YCL10 (dotted line) or YCNB111 (dashed line) or both crude extracts (solid line) for 60 min at 34 °C under anaerobic conditions. Pchlide was added at a concentration of 20 μM into these reactions. The amount of protein in the assay was about 50 μg for each crude extract. After the reaction, pigments were extracted in 80% acetone followed by phase partitioning with hexane. Absorption spectra of the lower phases were recorded.

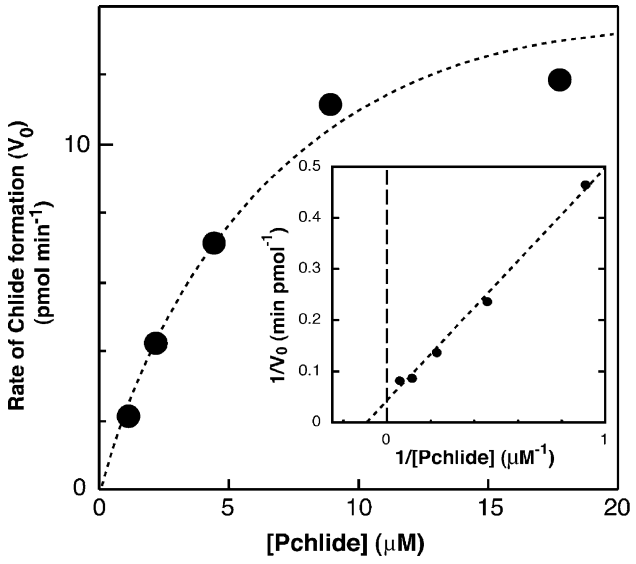

Fig. 4. Determination of Km value of DPOR for Pchlide using the assay with crude extracts. The rate of Chlide formation was measured at varying Pchlide concentrations (1.1, 2.2, 4.4, 8.9, and 17.7 μM). The Lineweaver–Burk plot used to determine the Km and Vmax of DPOR for Pchlide is shown in the inset.

Both activities of the components of the crude extracts were stable for more than 6 months when maintained anaerobically at 4 °C, with no significant loss of activity. In contrast, both activities of the components were rapidly lost during purification procedures, such as affinity chromatography with S-protein agarose. Quick loss of pale brownish color of the components upon purification suggests that some Fe–S clusters that have been proposed previously [7,12] are present in the components and that they are rapidly lost even in the anaerobic conditions we used. Attempts to purify the active components have so far been unsuccessful. Some basic properties of DPOR were determined with the stable assay system using crude extracts.

3.3. Determination of the kinetic parameters

To determine the Km value of DPOR for Pchlide, we measured the initial rate of Chlide formation over a range of Pchlide concentrations using the assay system with crude extracts. As shown in Fig. 4, the relation between the rate of Chlide formation and Pchlide concentration follows Michaelis–Menten kinetics. The apparent Km for Pchlide

was calculated to be 10.6 μM, and Vmax was determined to be 232 pmol min^{-1} $mg_{(total\ protein)}^{-1}$.

3.4. Ferredoxin-dependent activity

We examined whether ferredoxin acts as an electron donor for DPOR. For this analysis, we set up a series of assays using a ferredoxin from maize as the reductant. We also included a root type ferredoxin:$NADP^+$ oxidoreductase (FNR) from maize and NADPH as the system with which reduced ferredoxin enzymatically generates (Fig. 5). The spectrum of this reaction, shown in Fig. 5, indicated that Chlide was clearly formed when the maize ferredoxin was used as reductant instead of dithionite (trace b). No Chlide was detected above the background level when either ferredoxin, FNR, or NADPH was omitted from the reaction (Fig. 5, traces c, d, and e). This is the first experimental evidence that ferredoxin functions as the electron donor for DPOR.

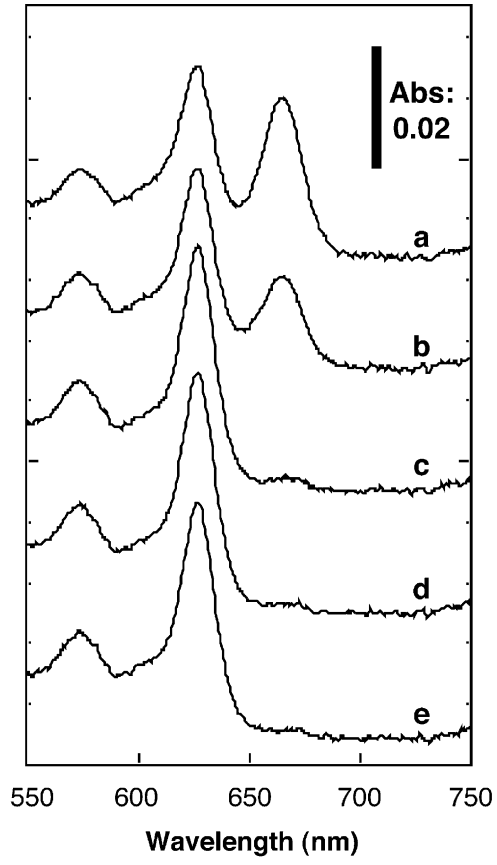

Fig. 5. Ferredoxin-dependent Pchlide reduction by crude extracts from YCL10 and YCNB111. Pchlide was added at a concentration of 2 μM to these reactions. As the electron donors, 0.68 mM dithionite (trace a) or 10 μM ferredoxin from maize with a reduced ferredoxin regeneration system (1 nM FNR from maize and 0.2 mM NADPH) (trace b) was used. Control reactions were carried out by omitting one component of the reduced ferredoxin regenerating system; ferredoxin (trace c), FNR (trace d), or NADPH (trace e). The reactions were performed at 34 °C for 1.5 h. After the reaction, pigments were extracted as described in Fig. 3, and the absorption spectra of the lower phases were recorded.

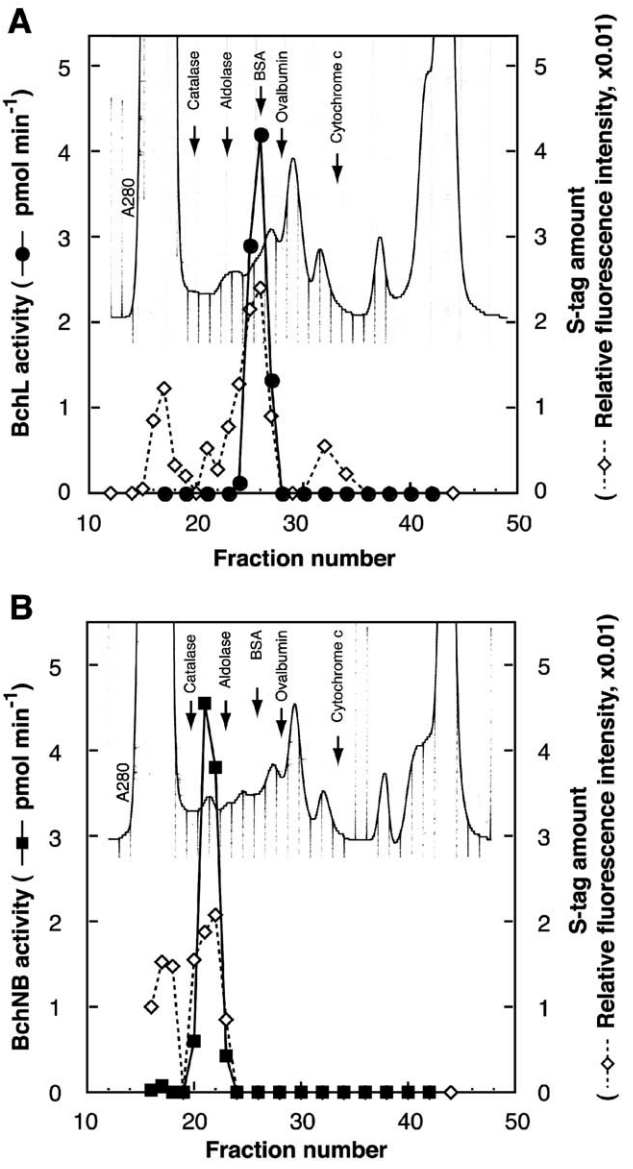

Fig. 6. Gel filtration chromatography of crude extracts of YCL10 (A) and YCNB111 (B). Crude extracts (500 μl) were fractionated on a prepacked column Superdex 200 pg column under anaerobic conditions. The activity of the respective component (closed symbols) was assayed with the complementary crude extract. The elution of S-tag protein was monitored with a FRETWorks S-Tag Assay Kit and a fluorometer (open diamonds). The elution of proteins was also monitored by absorption at 280 nm (solid line). The molecular mass markers used were catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (BSA; 67 kDa), ovalbumin (43 kDa) and cytochrome *c* (12 kDa).

3.5. Molecular mass of the two components

The molecular mass of the L-protein and NB-protein DPOR components was determined by gel filtration chromatography. For this analysis, crude extracts of YCL10 and YCNB111 were size fractionated by gel filtration chromatography. Fractions containing L-protein or NB-protein were then monitored by mixing the chromatography fractions with the appropriate crude extracts of the

other component and assaying for DPOR activity. The presence of DPOR subunits with S-tags was also confirmed by fluorometric measurement of RNase activity reconstituted by the binding of S-protein with the S-tag fusion protein. Whereas the activities of L-protein and NB-protein were dramatically decreased during gel filtration chromatography, the assay system with complementary crude extracts was sensitive enough to detect both fractionated activities (Fig. 6). The L-protein eluted in fractions that co-eluted with bovine serum albumin (BSA), which has a molecular mass of 67 kDa (Fig. 6A). Because S-tag BchL has a calculated molecular mass of 36,046 Da, this indicates that L-protein is most likely a homodimer in solution (72 kDa calculated molecular mass). Similar analysis of the NB-protein complex indicated that it fractionates with a molecular mass of 200 kDa (Fig. 6B). Because the monomeric molecular masses of S-tag BchN and BchB are 48,671 Da and 57,191 Da, respectively, and these proteins are in equimolar amounts ([12]; Fig. 2, lane 2), the molecular mass of 200 kDa indicates that NB-protein exists in solution as an (S-tag $BchN)_2(BchB)_2$ heterotetramer that has a calculated molecular mass of 212 kDa.

4. Discussion

4.1. Nitrogenase-like features of DPOR

The results of this study, coupled with those of our previous analysis [12], indicate that DPOR has considerable similarity to nitrogenase. Specifically, we now know that DPOR consists of two separable components: the L-protein (BchL) and the NB-protein (BchN-BchB) ([12]; Fig. 3). We also know that DPOR requires ATP hydrolysis for activity, using either dithionite [12] or ferredoxin as electron donors (Fig. 5), and that L-protein and NB-protein are a homodimer and a heterotetramer, respectively (Fig. 6). Therefore, DPOR and nitrogenase enzymes not only share primary structural similarities, but also share several biochemical features.

4.2. Overexpression of DPOR components and development of a crude extract assay system

In this study, we have developed a stable assay system with crude extracts prepared from two transconjugants that overexpress L-protein and NB-protein. Crude extracts of YCL10 and YCNB111 cells contained significant amounts of plasmid-mediated overexpressed L-protein and NB-protein, respectively (Fig. 2), as well as small amounts of chromosomally encoded DPOR. Assayable DPOR activity in wild-type cell extracts appears to be limited by the amount of enzyme in the cells, as only limited activity was observed in extracts overexpressing only one DPOR subunit (Fig. 3, dashed and dotted lines). However, when both crude extracts were added to the assay mixture, synergetic enhancement of DPOR activity was observed (Fig. 3, solid line), indicating that overexpressed L-protein and NB-protein with S-tags were expressed and assembled into active forms. In typical nitrogenase assay systems, the individual activities of Fe protein and MoFe protein are measured in the presence of saturating amounts of the complementary components, not unlike the assay used in this study to measure the activity of DPOR [22]. We used this assay to successfully measure the individual activities of DPOR components by mixing them with complementary crude extracts (Fig. 6).

4.3. Km of DPOR, compared with that of LPOR

We determined that DPOR has a Km for Pchlide of 10.6 μM, as measured with stable crude extracts (Fig. 4). It is interesting to compare the Km value of DPOR with that of LPOR, which is an unrelated enzyme that catalyzes the same Pchlide reduction in a light-dependent manner. Km values for Pchlide reduction by LPOR range from 0.18 to 8.6 μM, depending on the species [6]. LPOR from the cyanobacterium *Synechocystis* sp. PCC6803 has the highest reported Km value (8.6 μM) among the LPORs that have been examined so far [23]. LPORs from eukaryotes, such as green algae and higher plants, have sub-micromolar Km values of 0.19 μM for *Scenedesmus obliquus* [24], 0.18–0.27 μM for *Pisum sativum* [25,26], 0.46 μM for *Hordeum vulgare* [27], and 0.47 μM for *Avena sativa* [28]. The lower eukaryotic Km values suggest that, during the evolution of the photosynthetic eukaryotes, LPOR may have evolved to accommodate a lower Km that is one fiftieth to twentieth of that of cyanobacterial LPOR. The Km value of DPOR from *R. capsulatus* (10.6 μM) is similar to that of LPOR from *Synechocystis* sp. PCC6803 (8.6 μM), indicating that prokaryotic enzymes with totally different molecular mechanisms for Pchlide D-ring reduction have evolved with equivalent affinity for Pchlide. Based on phylogenetic analyses, and the distribution of LPOR and DPOR in phototrophs, it has been suggested that DPOR is an older enzyme, sharing an evolutionary origin with nitrogenase, and that LPOR evolved later in the ancestral cyanobacterium. LPOR is thought to have evolved in response to the rapid rise in atmospheric oxygen levels in the Proterozoic era to compensate for the sensitivity of DPOR to oxygen ([7]; Fujita and Yamazaki, in preparation). Extant cyanobacteria, green algae, and lower land plants such as mosses, ferns and gymnosperms contain both DPOR and LPOR Pchlide reduction systems. It will be interesting to compare the kinetic parameters of various DPORs with those of LPOR from the same species to understand how the two Pchlide reductases in the same cell are differentially utilized in Chl biosynthesis.

4.4. Ferredoxin-dependent DPOR activity

We have provided the first experimental evidence that ferredoxin functions as the electron donor for DPOR.

Although we did not examine whether any ferredoxins from *R. capsulatus* serve as electron donors to DPOR, our observation that electrons from a maize [2Fe–2S]-type ferredoxin can be transferred to DPOR implies that the [2Fe–2S]-type ferredoxin FdxD from *R. capsulatus* could fulfill this role. *R. capsulatus* actually contains three ferredoxins, FdxD, FdxN, and FdxA [29–31], and mutations in any one of these ferredoxins do not affect Pchlide reduction. This indicates that more than one ferredoxin species is capable of DPOR reduction. A variety of reduced ferredoxins can also serve as electron donors to *Rhodobacter* nitrogenase, including a [2Fe–2S] ferredoxin from spinach [30,31]. Electron transfer to DPOR by maize ferredoxin appeared less efficient than electron transfer by dithionite, because the amount of Chlide formed by maize ferredoxin was about 52% of that observed using dithionite (Fig. 5, traces a and b). This is similar to the electron transfer from ferredoxin I (FdxN) to *R. capsulatus* nitrogenase, in which the amount of ethylene formed was only 37% of that observed with dithionite [32]. The efficiency of electron transfer from ferredoxin to nitrogenase is thought to depend on the affinity of the proteins and the redox potential of the ferredoxin [33]. Therefore, we can conclude that the maize ferredoxin can reduce DPOR at least as efficiently as ferredoxin I reduces nitrogenase.

4.5. Molecular mass of L-protein and NB-protein

The elution profile on gel filtration chromatography indicated that L-protein is a homodimer of BchL (Fig. 6A). Nitrogenase Fe protein is a homodimer of NifH protein and a [4Fe–4S] cluster is held by the two protomers. Four Cys residues are conserved in both BchL/ChlL and NifH [7], and two of the four Cys are involved in the chelation of a [4Fe–4S] cluster in Fe protein [34]. The similar positions of the conserved Cys suggests that a [4Fe–4S] cluster is present in the BchL dimer L-protein and that the cluster is most likely coordinated by Cys131 and Cys165. The homodimeric structure of L-protein strongly supports this hypothesis.

The other elution profile indicated a heterotetrameric structure consisting of BchN and BchB for NB-protein (Fig. 6B). Nitrogenase MoFe protein carrying two types of metallocenters, the P cluster and the FeMo-cofactor, is a heterotetramaer of NifD and NifK. In addition to MoFe protein, there is a FeMo-cofactor assembly protein known as NifEN, which is the other protein structurally related to NB-protein. The NifEN complex is also a heterotetramer of NifE and NifN, as well as MoFe protein. NifEN does not have all the Cys required to assemble P clusters and the FeMo-cofactor, but does contain four Cys (three from NifE and one from NifN) that form a [4Fe–4S] cluster between NifE and NifN [35]. Analysis of multiple BchN and BchB sequences indicates that BchN and BchB contain three and one conserved Cys residues, respectively, that are similar to those of NifE and NifN, respectively [7]. The heterotetrameric structure of NB-protein and the conserved Cys arrangement in BchN and BchB imply that NB-protein carries a [4Fe–4S]-type cluster(s) similar to NifEN, rather than like either the P cluster or the FeMo-cofactor in NifDK.

4.6. Perspective

This report provides a framework for the characterization of each component of DPOR in detail and to measure the individual activities of L-protein and NB-protein from photosynthetic bacteria and from a variety of other photosynthetic organisms. In addition to DPOR and nitrogenase, purple non-sulfur bacteria, including *R. capsulatus, Rhodospirillum rubrum*, and *Rhodopseudomonas palustris*, also contain numerous other nitrogenase-like enzymes such as chlorophyllide reductase (COR encoded by *bchXYZ* genes) and other enzymes of unknown function [36]. The overexpression system described here will be useful for the further characterization of this large family of nitrogenase-like enzymes.

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