Reconstitution of Light-independent Protochlorophyllide Reductase from Purified Bchl and BchN-BchB Subunits

IN VITRO CONFIRMATION OF NITROGENASE-LIKE FEATURES OF A BACTERIOCHLOROPHYLL BIOSYNTHESIS ENZYME

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Protochlorophyllide reductase catalyzes the reductive formation of chlorophyllide from protochlorophyllide during biosynthesis of chlorophylls and bacteriochlorophylls. The light-independent (dark) form of protochlorophyllide reductase plays a key role in the ability of gymnosperms, algae, and photosynthetic bacteria to green (form chlorophyll) in the dark. Genetic and sequence analyses have indicated that dark protochlorophyllide reductase consists of three protein subunits that exhibit significant sequence similarity to the three subunits of nitrogenase, which catalyzes the reductive formation of ammonia from dinitrogen. However, unlike the well-characterized features of nitrogenase, there has been no previous biochemical characterization of dark protochlorophyllide reductase. In this study, we report the first reproducible demonstration of dark protochlorophyllide reductase activity from purified protein subunits that were isolated from the purple nonsulfur photosynthetic bacterium Rhodobacter capsulatus. Two of the three subunits (Bchl and BchN) were expressed in R. capsulatus as S tag fusion proteins that facilitated affinity purification. The third subunit (BchB) was co-purified with the BchN protein indicating that BchN and BchB proteins form a tight complex. Dark protochlorophyllide reductase activity was shown to be dependent on the presence of all three subunits, ATP, and the reductant dithionite. The similarity of dark protochlorophyllide reductase to nitrogenase is discussed.

Protochlorophyllide (Pchlide) is a key intermediate in the biosynthesis of chlorophylls (Chl) and bacteriochlorophylls. Among photosynthetic organisms, there are two different enzymes known to catalyze stereo-specific double-bond reduction of ring-D of Pchlide to form chlorophyllide a (Chlide), which is a direct precursor of Chl a (1–3) (Fig. 1). One enzyme is light-dependent Pchlide oxidoreductase (LPOR, EC 1.3.1.33). LPOR utilizes NADPH to catalyze reduction of Pchlide with the interesting requirement that the substrate (Pchlide) must also absorb light in order for the enzyme to promote double bond reduction. LPORs have been extensively studied for some time, because the requirement for light makes this enzyme a key player in light-dependent greening of flowering plants (angiosperms) (4). The other enzyme that reduces Pchlide is light-independent (dark) Pchlide reductase (DPOR). This enzyme catalyzes Pchlide reduction irrespective of light. Genetic and sequence studies have shown that primitive anoxygenic (non-oxygen evolving) photosynthetic bacteria contain only DPOR (5). In contrast, cyanobacteria, algae, and gymnosperms (non-flowering plants) contain both DPOR and LPOR, whereas angiosperms only contain LPOR (1, 3). Because of the presence of DPOR, photosynthetic bacteria, algae, and gymnosperms are capable of synthesizing bacteriochlorophylls and chlorophylls in the dark, whereas the lack of DPOR in angiosperms makes a requirement for light for Chl synthesis in these cells.

Genetic studies of the purple nonsulfur bacterium Rhodobacter capsulatus indicated that three genes, bchl, bchN, and bchB, are involved in light-independent Pchlide reduction during biosynthesis of bacteriochlorophyll (6–8). Studies with the cyanobacterium Plectonema boryanum (9–11) and the green alga Chlamydomonas reinhardtii (12–15) have also demonstrated that these organisms use similar genes for Pchlide reduction during Chl biosynthesis that are called chlL, chlN, and chlB. Analysis of the deduced amino acid sequences surprisingly showed the presence of significant sequence similarity between the putative Bchl/ChlL, BchN/ChlN, and BchB/ChlB subunits of DPOR with the NifH, NifD, and NifK subunits of nitrogenase, respectively (1, 10, 16, 17). Nitrogenase is a well-characterized enzyme that consists of two separable components, the Fe-protein (also called dinitrogenase reductase) and the MoFe protein complex that catalyzes the reduction of dinitrogen (N2) to form ammonia (2NH3) (18–20). The Fe-protein complex transfers electrons from ferredoxin to the MoFe protein concomitant with Mg-ATP hydrolysis. This complex is comprised of a dimer of NifH proteins that together form a 4Fe:4S redox cluster that is bridged by two Cys from each subunit. The MoFe protein, which serves as the catalytic site for dinitrogen reduction, is comprised of the αβ2 tetramer of the NifD (α) and NifK (β) proteins. The MoFe protein complex contains two types of metallocenters, an 8Fe7S cluster (P cluster) held at the interface between the NifD and NifK proteins, as well as a 1Mo7Fe9S1homocitrate cofactor (FeMo cofactor) that is present in each NifD subunit. The P-cluster is thought to mediate electron transfer from the iron-protein complex to the FeMo cofactor that is the catalytic site for dinitrogen reduction.
The structural similarity between DPOR and nitrogenase is most evident between the Bchl/ChlN and NiFH (dinitrogen reductase) subunits where there is 33% overall identity and 50% similarity (13, 16, 17). Most notable is the fact that a critical feature such as the ATP-binding motif and the two Cys residues that are involved in coordinating the 4Fe:4S cluster are completely conserved among NiFH and Bchl/ChlN proteins (13, 16, 17). This indicates that the Bchl/ChlN proteins might catalyze ATP-dependent transfer of electrons from a reductant, such as ferredoxin, to a catalytic protein complex via the Fe:S center. The amino acid sequences of the N proteins (BchlN and ChlN) and B proteins (BchlB and ChlB) also exhibits similarity to NiFD and NiFK, respectively, (10, 11). Interestingly, only four of the six Cys residues that are involved in forming the 8Fe:7S P cluster in nitrogenase are conserved in the N and B proteins. This implies that the N and B proteins might instead form a 4Fe:4S redox center. There is also no conservation of the residues that are involved in formation of the FeMo cofactor in nitrogenase indicating that the catalytic site, where Pchlide is reduced is highly diverged from the site in nitrogenase where dinitrogen undergoes reduction (1, 10).

Despite the interesting structural similarity between DPOR and nitrogenase, biochemical analysis of DPOR has not yet been undertaken. The absence of biochemical analysis of DPOR can be traced to the fact that there has been no reliable published procedures for assaying DPOR activity in cell-free extracts of photosynthetic cells. Although there have been a few prior reports of DPOR activity in crude cell-free extracts (21–23), there has been no independent confirmation of these reports nor any attempts at purification of the DPOR enzyme from these systems. Heterologous expression of DPOR subunits in Escherichia coli has also not resulted in the generation of extracts that exhibit DPOR activity. Because the purple nonsulfur bacterium R. capsulatus naturally expresses and assembles DPOR in an active form, we believed that this organism would provide an ideal system to overexpress and purify DPOR. To perform this analysis, we constructed two R. capsulatus strains, one that overexpresses an S tag fusion derivative of the BchlN protein and the other that overexpresses an S tag fusion derivative of BchlL. The S tag BchlN protein was purified as a single polypeptide by affinity purification, whereas the S tag BchlL protein was affinity purified as a 1:1 complex with the BchlB protein. DPOR activity was measured in an assay mixture comprised of purified protein fractions, ATP and dithionite. The observed biochemical characteristics of isolated DPOR strongly support "nitrogenase-like" features of this Chl biosynthesis enzyme.

MATERIALS AND METHODS

Construction of Nonreplicable Plasmids—The procedure to construct nonreplicable plasmids, pYCSFXN1 and pYCSFXL3, is summarized in Fig. 2, A and C. A chimeric DNA fragment consisting of the puc promoter (24), S tag (25), and 5’-part of bchN was obtained by an overlap extension method using two-step PCR (26) (Fig. 2A). The puc promoter part (corresponding to –216 to –1, Ref. 24) was amplified with a pair of primers: “pucSNf”, 5’-TGTGAGATTC-GCTGATCGCAGGGTYTTCCTTCTATCAGGGAATCCCTCAAA-3’ and “pucSNr”, 5’-GAAGTCCGAGCACTGGTCTCCGCAATACAGGGATCGTCTTCTATCAGGGAATCCCTCAAA-3’. The observed biochemical characteristics of isolated DPOR strongly support “nitrogenase-like” features of this Chl biosynthesis enzyme.

Light-Independent Pchlide Reductase (DPOR) Light-Independent Pchlide Reductase (DPOR)
Novagen) was added to 80 ml of supernatant and incubated for 1 h with gentle shaking for specific binding of S tag BchN or S tag BchL protein. The S protein-agarose was then washed in 5 ml of the lysis buffer three times by centrifugation and once in 5 ml of factor Xa cleavage/capture buffer (Novagen) containing 1 mM dithiothreitol and 10 mM β-mercaptoethanol. The washed S protein-agarose was suspended in 1 ml of factor Xa cleavage/capture buffer and 88 units of factor Xa (Novagen). After incubation at room temperature with gentle shaking for 16 h, the target protein was then recovered in the supernatant. Factor Xa was removed from the sample by Xarrest-agarose (1.5 ml slurry) according to the manufacturer's protocol (Novagen). Phenylmethylsulfonyl fluoride at a final concentration of 1 mM was added to the sample to inactivate residual activity of factor Xa. Protein was quantified using a dye-binding assay with Coomassie Brilliant Blue G-250 (Bio-Rad).

SDS-PAGE and Amino-terminal Sequence Analysis—Soluble crude fractions and purified proteins were electrophoresed on a 12% acrylamide gel that was stained with Coomassie Brilliant Blue R-250. For the amino-terminal sequence analysis, a total of 4 mg of purified BchN and co-purified BchB proteins (about 2 mg each) were loaded onto a 12% acrylamide gel with 1.5-mm thickness. After electrophoresis, the proteins were electrically transferred onto a piece of polyvinylidene difluoride membrane (Seque-blot, Bio-Rad) using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the instructional manual. Each blot of the BchN and BchB proteins was excised and washed in distilled water. Amino-terminal determination of each protein was carried out with a solid-phase sequencer (Department of Biochemistry, Purdue University).

Preparation of Pchlide—R. capsulatus strain ZY5 (bchL, 6) was grown in RCV-2/3PY medium (125 ml in a 250-ml flask) containing 5 μg/ml kanamycin at 34 °C in the dark with slow shaking at 130 rpm. The culture medium was collected by centrifugation followed by filtration through a 0.4-μm filter. Pchlide in the culture medium was then extracted in one-third volume of ether (Sigma). Water contamination in the ether phase was then removed as ice after cooling the ether on dry ice. Ether was then evaporated to dryness by a stream of nitrogen. The dried Pchlide was dissolved in Me2SO to final concentration of 190 mM. Pchlide concentration was determined in 80% acetone using the millimolar extinction coefficient of 30.4 at 626 nm (30).

Assay of Pchlide Reduction—DPOR assays were carried out in a volume of 1 ml containing 100 mM HEPES-KOH, pH 7.4, 5 mM MgCl2, 5 mM dithiothreitol, 1 mM ATP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 10 mM sodium dithionite, 2 μM Pchlide, and an appropriate amount of purified protein(s). The assay mixtures were incubated in anaerobic conditions in the dark for 1 h at 34 °C. Aliquot (200 μl) of the assay mixture was then mixed with acetone (final 80%), and the absorption spectrum of each sample was recorded on a Beckman DU600 spectrophotometer. Concentration of Chlide a and Pchlide in the acetone solution was estimated by the equation described by Porra (31).

RESULTS

Construction of Strains Overexpressing S Tag-modified BchN and BchL Proteins—The bchN, bchB, and bchL, genes from R. capsulatus have been genetically implicated to code for subunits of DPOR (6–8). Each of these genes is under
the control of a weak promoter (32) (Fig. 2C). To facilitate purification of DPOR, we set up conditions to overexpress affinity tagged (S tag) BchN and BchL genes in *R. capsulatus*. To accomplish this goal, we first constructed the nonreplicable plasmid pYCSFX the details of which are shown in Fig. 2A. This plasmid has a 217-bp DNA segment that contains a strong promoter for the light-harvesting II proteins (*puc* promoter, 24) and the *pucB* translation initiation site fused in frame with 28 amino acid residues that code for an affinity purification S tag amino-terminal sequence and factor Xa cleavage site (Fig. 2).

**Expression of BchN and BchB**

Reconstitution of Protochlorophyllide Reductase

**FIG. 3. SDS-PAGE of the soluble fractions of YCN1 (10 μg, lane 2) and YCL3 (10 μg, lane 4) and purified BchN (1.3 μg, lane 3) and BchL (0.4 μg, lane 5) proteins. A prestained molecular size marker with indicated molecular mass is shown in lane 1.**

In this study, we have reported the first successful isolation of DPOR from a photosynthetic organism. The initial charac-
terization of this enzyme indicates that it contains many features in common with the structurally related enzyme, nitrogenase. Reconstitution of DPOR with purified proteins clearly demonstrates that DPOR consists of two separable components, the BchL protein and the BchN-BchB protein complex (Fig. 5). Activity was shown to be dependent on ATP, an ATP regeneration system, as well as the reductant dithionite. The characteristics of our in vitro DPOR assay system are similar to that of nitrogenase, which also requires ATP and dithionite during in vitro reduction of nitrogen (33). Although the in vivo reductant for DPOR has not been genetically assigned, it is likely that ferredoxin is the most probable candidate given the high degree of structural similarity of BchL to NifH, which is known to obtain electrons from ferredoxin. *R. capsulatus* synthesizes six different ferredoxins with ferredoxin I being a specific electron donor to nitrogenase (34, 35). It is unknown which ferredoxin functions as the specific reductant for DPOR. However, it is possible that more than one ferredoxin can serve as an electron donor, because genetic studies have not yielded examples of mutants that are defective in Pchlide reduction that map to a specific ferredoxin gene.

Besides the unresolved issue of the nature of the electron donor to DPOR, there are several additional questions that can be addressed in future studies with isolated DPOR. For example, sequence similarity between DPOR and nitrogenase subunits suggests that the BchL protein exists in solution as a dimer that transfers electrons from ferredoxin (or dithionite in vitro) to the NB protein complex in an ATP-dependent manner. It also suggests that the NB protein complex serves as the catalytic site for the Pchlide reduction similar to the MoFe protein complex that is the site of nitrogen reduction. The co-purification of the BchN and BchB proteins in an equimolar ratio is consistent with the possibility that the NB protein forms a (BchN)2(BchB)2 heterotetramer similar to the MoFe protein (Fig. 5). Thus, like dimer formation by BchL, the existence of a (BchN)2(BchB)2 heterotetramer warrants future experimentation. The stoichiometry of ATP hydrolysis and donation of electrons in the DPOR reaction also remains to be investigated. The reduction of dinitrogen to two molecules of ammonia by nitrogenase involves hydrolysis of 16 ATP as well as the donation of eight sets of protons and electrons. According to the chemical structure, only six protons and electrons are needed to fully reduce one dinitrogen to two molecules of ammonia with the two extra protons and electrons being used for the evolution of H2. For comparative reasons, it will be interesting to examine the stoichiometry of proton and electron utilization by DPOR that is needed to catalyze double bond

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**FIG. 4.** A, absorption spectra showing DPOR activity with purified protein fractions. (A) Acetone extracts from DPOR assays containing only purified BchN-BchB proteins (trace a), only purified BchL protein (trace b), or both BchL and BchN-BchB protein preparations (trace c). Amounts of protein in the assays were 13 μg in the BchN-BchB proteins and/or 4 μg in the BchL protein. The assay mixtures were incubated for 1 h at 34°C in the dark under the anaerobic condition. B, time course of Chlide production in the DPOR reaction identical to that of trace c in A. C, ATP requirement for DPOR activity. Trace a is of a reaction containing both 1 mM ATP and the ATP regeneration system; trace b is a reaction without ATP; trace c is a reaction without the ATP regeneration system; and trace d is a reaction without ATP and the ATP regeneration system. Assay mixtures contained the same amounts of BchL and BchN-BchB proteins and a similar incubation condition, as in A. D, requirement of dithionite for DPOR activity. Trace e is the complete assay system containing 10 mM dithionite, whereas trace f contains only 340 μM of dithionite derived as carryover from the purified protein fractions. Abs, absorbance.

**FIG. 5.** Comparison of the molecular architecture between nitrogenase (A) and the light-independent Pchlide reductase (B). The model of DPOR is based on the sequence similarity to nitrogenase and the characteristics observed in the in vitro assay.
reduction of Pchlide (Figs. 1 and 5). It will also be interesting to see if H₂ evolution can occur in the DPOR reaction. Nitrogenase is also capable of reducing a variety of substrates other than just dinitrogen, such as acetylene, that used in the conventional in vitro nitrogenase assays as well as other small organic compounds with double or triple bonds such as dinitrogen monoxide, cyanide, azide, acetonitrile, and 1-propyne, etc. (36). It is therefore not surprising that a nitrogenase-like enzyme has evolved that is capable of reducing a double bond in Pchlide. In this regard, it may be interesting to examine whether isolated DPOR is also capable of reducing a variety of different compounds containing double or triple bonds.

Another issue that remains to be resolved is the presence of putative Fe-S clusters or other metallocenter(s) that may be present in the isolated BchL and BchN-BchB proteins. Even though we have not yet isolated sufficient quantities of the DPOR enzyme to perform metal or EPR analysis for the presence of Fe centres, it seems likely they exist (Fig. 5). For example, we have observed that both purified protein fractions exhibit a faint brown color that is constant with known spectral properties of proteins that contain an Fe-S cluster(s). We have also observed that DPOR activity is very sensitive to inhibition by oxygen (data not shown), which is a characteristic of enzymes such as nitrogenase that contain Fe-S centers that are disrupted by oxygen. Assuming that Fe-S center(s) exist, then it remains to be seen what type may be present in DPOR. Sequence analysis indicates that the three Cys residues in NifD and three Cys residues in NifK that together coordinate an 8Fe:7S P cluster are only partially conserved in the N and B proteins (three of the conserved Cys residues are present in the N protein and only one conserved Cys is located in the B protein; 10, 11). Indeed, phylogenetic analysis suggests that DPOR activity is very sensitive to inhibition by oxygen (data not shown), which is a characteristic of enzymes such as nitrogenase that contain Fe-S centers that are disrupted by oxygen. Assuming that Fe-S center(s) exist, then it remains to be seen what type may be present in DPOR. Sequence analysis indicates that the three Cys residues in NifD and three Cys residues in NifK that together coordinate an 8Fe:7S P cluster are only partially conserved in the N and B proteins (three of the conserved Cys residues are present in the N protein and only one conserved Cys is located in the B protein; 10, 11). Indeed, phylogenetic analysis suggests that DPOR may be more closely related to another NifDK-like protein pair known as NifE and NifN. The NifEN complex, which is thought to play a possible redox role in the FeMo cofactor biosynthesis (37), also has a similar partial conservation of the P cluster Cys residues (three in NifE and only one in NifN) (38, 39). Studies have shown that NifEN contains two 4Fe-4S clusters instead of the two 8Fe:7S P clusters that are present in NifDK (37). There is also no conservation of the Cys and His residues that are involved in formation of the active site FeMo cofactor in NifD with B or N protein. Thus, it appears that the DPOR NB protein complex most likely only contains a single pair of 4Fe-4S cluster(s) not unlike that observed for NifEN.

The establishment of a purification and assay system for DPOR should finally allow detailed investigations of the molecular mechanism of dark Pchlide reduction. The continued characterization of the structural and biochemical similarities of DPOR to nitrogenase should lead to new insights into electron transfer events that these classes of proteins undergo. These studies could also provide new clues as to the evolution-ary relationship between (bacterio)chlorophyll biosynthesis and nitrogen fixation that occurred during early evolution of phototrophic organisms.

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