Substrate Recognition of Nitrogenase-like Dark Operative Protochlorophyllide Oxidoreductase from Prochlorococcus marinus*

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Chlorophyll and bacteriochlorophyll biosynthesis requires the two-electron reduction of protochlorophyllide a ring D by a protochlorophyllide oxidoreductase to form chlorophyllide a. A light-dependent (light-dependent Pchlide oxidoreductase (LPOR)) and an unrelated dark enzyme (dark operative Pchlide oxidoreductase (DPOR)) are known. DPOR plays an important role in chlorophyll biosynthesis of gymnosperms, mosses, ferns, algae, and photosynthetic bacteria in the absence of light. Although DPOR shares significant amino acid sequence similarities with nitrogenase, only the initial catalytic steps resemble nitrogenase catalysis. Substrate coordination and subsequent [Fe-S] cluster-dependent catalysis were proposed to be unrelated. Here we characterized the first cyanobacterial DPOR (ChlNB)2. The ChlL2 dimer contains one EPR active [4Fe-4S] cluster, whereas the (ChlNB)2 complex exhibited EPR signals for two [4Fe-4S] clusters with differences in their g values and temperature-dependent relaxation behavior. These findings indicate variations in the geometry of the individual [4Fe-4S] clusters found in (ChlNB)2. For the analysis of DPOR substrate recognition, 11 synthetic derivatives with altered substituents on the four pyrrole rings and the isocyclic ring plus eight chlorophyll biosynthetic intermediates were tested as DPOR substrates. Although DPOR tolerated minor modifications of the ring substituents on rings A–C, the catalytic target ring D was apparently found to be coordinated with high specificity. Furthermore, protochlorophyllide a, the corresponding [8-vinyl]-derivative and protochlorophyllide b were equally utilized as substrates. Distinct differences from substrate binding by LPOR were observed. Alternative biosynthetic routes for cyanobacterial chlorophyll biosynthesis with regard to the reduction of the C8-vinyl group and the interconversion of a chlorophyll a/b type C7 methyl/formyl group were deduced.

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‡ The abbreviations used are: Pchlide, protochlorophyllide; Chlide, chlorophyllide; GST, glutathione S-transferase; Pheide, pheophorbide; Phieide, protoporphorbide; LPOR, light-dependent Pchlide oxidoreductase; DPOR, dark operative Pchlide oxidoreductase; CAO, Chlide a-oxygenase.
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![Diagram showing the reduction of Protochlorophyllide a by DPOR](image)

**FIGURE 1. Reduction of Pchlide a by DPOR.** Ring D is stereo- and regioselectively present in the presence of an electron donor and ATP. Rings A–E and the individual carbon atoms are labeled according to the IUPAC nomenclature (61).

The hydrolysis of the two ATP molecules might result in a decrease in the redox potential of the BchL2-bound [4Fe-4S] cluster allowing for the transfer of one electron onto a second protein complex composed of BchN and BchB. For this complex a heterotetrameric structure was determined and denoted as (BchNB)2 (13, 15, 16). Site-directed mutagenesis experiments of BchN and BchB in combination with kinetic measurements revealed the presence of four cysteine residues crucial for DPOR catalysis. They indicated a significant decrease of the iron and sulfur content of (BchNB)2 modified in residues Cys21, Cys46, and Cys103 of BchN and residue Cys94 of BchB. From these data, two intersubunit [4Fe-4S] redox centers were proposed for (BchNB)2 (13).

This first part of the DPOR catalytic mechanism clearly resembles the initial electron transfer in nitrogenase (13, 17, 18). However, the [4Fe-4S] clusters of (BchNB)2 do not have a direct equivalent in nitrogenase. Instead, nitrogenase contains a complicated [8Fe-7S]-P cluster, which interestingly shares all of the four cysteine ligands that have been identified for C. tepidum DPOR (13, 16). The two additional cysteine residues involved in P cluster coordination have no counterpart in the sequence of BchNB proteins.

The P cluster of nitrogenase is responsible for the electron transfer onto an additional metallo center (1Mo-7Fe-9S-1X-1homocitrate) located on subunit NifD, where the reduction of N2 to NH4+ takes place (19, 20).

However, involvement of such an additional cofactor for DPOR catalysis was clearly ruled out, and a direct electron transfer from the [4Fe-4S] cluster of (BchNB)2 onto the substrate was proposed (13). Obviously, the second part of DPOR catalysis differs distinctly from the nitrogenase system. To date no structural information about (BchNB)2 is available, but it seems reasonable that the active site might be located in close proximity to the [4Fe-4S] cluster of (BchNB)2.

Marine cyanobacteria including *Prochlorococcus marinus* are presumably the most abundant photosynthetic organisms on earth (21). They contribute 30–60% of the oceanic primary production (21–24) and play a significant role in the global carbon cycle.

In comparison with other cyanobacteria, *P. marinus* has an unusual pigment composition (21). Instead of chlorophyll *a* and phycobiliproteins *Prochlorococcus spp.* accumulate [8-vinyl]-chlorophyll *a* and [8-vinyl]-chlorophyll *b* (25). The [8-vinyl]-chlorophylls exhibit a Soret band red shift of about 10 nm, which is favorable for *P. marinus* to better absorb blue light in deep waters down to 200 m (26). The [8-vinyl]-chlorophylls are usually precursors for the synthesis of chlorophylls. The only other organisms where they have been found in significant amounts are chlorophyll biosynthesis mutants of *C. tepidum* (27) and maize (28, 29).

Although LPOR uses both Pchlide *a* and [8-vinyl]-Pchlide *a* as a substrate, with a preference for the latter, there is no study on the substrate specificity of DPOR. To determine the exact location of the [8-vinyl]-reduction step in the biosynthetic pathway, we wanted to see whether DPOR from *P. marinus* is able to reduce Pchlide *a* and [8-vinyl]-Pchlide *a*. Furthermore, it has been shown that the 7-formyl group of chlorophyll *b* is mainly synthesized from chlorophyll *a* by the chlorophyllide *a* oxygenase (CAO) (30), whereas Pchlide *a* is only a poor substrate for CAO. Because of this observation, it was of interest to elucidate a potential role of Pchlide *b* as a substrate for DPOR catalysis.

In the present investigation we established a heterologous expression system for the DPOR enzyme from *P. marinus*, the first cyanobacterial DPOR to be analyzed. Three catalytical [4Fe-4S] clusters were characterized by EPR spectroscopy. To determine the structural elements of Pchlide that are critical for DPOR catalysis, we used chemically modified substrates. The results of these substrate recognition experiments allowed us to propose the location of individual reaction steps in the chlorophyll biosynthetic pathways.

**EXPERIMENTAL PROCEDURES**

*Production and Purification of P. marinus DPOR in Escherichia coli*—The gene *chlN* from *P. marinus* (SS120, Roscoff Culture Collection RCC156) was PCR-amplified using primers CAGCGAATTCTAGAGCGCTCAACG and GCCGTCGAC-CTTAAACAGCTTCTAGG and cloned into the BamHI and Sall sites of pGEX-6P-1 to yield pGEX-chlN. Subsequently, chlB from *P. marinus* was amplified using primers GACGGTCCA-CTCAAATTTCACACAGGAAACAGTATTCCATGGAACACTA- AACACTTTGA and GCTGTATGCGGCGCTTCAAGCTCGGTCGACACAGTGCCCTCAAGCTCGAATTCATGACTACAACCTTAGC and GCCGTCGAC-CTTAAACAGCTTCTAGG and cloned into the BamHI and Sall sites of pGEX-chlN to generate plasmid pGEX-chlNB (The *E. coli*-specific ribosomal binding site implemented upstream of chlB to enhance the protein production is marked in bold letters.) Using primers AGCGGATCCATGACTACAACCTTAGC and TACCGTGCACCCCTGAGTCAAACC and corresponding chlN gene was amplified into the BamHI and NotI sites of pGEX-6P-1 to yield plasmid pGEX-chlN.

Both plasmids were individually transformed into *E. coli* BL21(DE3) Codon Plus RIL cells to produce the ChlNB complex or the ChlI subunit, respectively. The cells were aerobically cultivated at 25 °C in 500 ml of LB medium containing 1 mM Fe(III)-citrate and 1 mM l-cysteine. At an A678 of 0.5, protein production was induced by the addition of 25 μM isopropyl-β-thiogalactoside. After 16 h of cultivation, 1.7 mM dithionite was added, and cultivation was continued without agitation for 3 h at 18 °C in an anaerobic chamber (Coy Laboratories, Grass Lake, MI) to allow [Fe-S] cluster formation. All of the remaining steps were performed under anaerobic condi-
tions (95% N₂, 5% H₂, <1 ppm O₂). The solutions were N₂ saturated prior use. The cells were harvested by centrifugation. The bacterial cell pellet was resuspended in 15 ml of lysis buffer (100 mM Hepes/NaOH, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 10 mM dithiothreitol) and disrupted by a single passage through a French press at 1500 p.s.i. into an anaerobic bottle. Following centrifugation for 60 min at 175,000 × g at 4 °C, the supernatant was applied to 1 ml of glutathione-Sepharose (GE Healthcare) equilibrated with lysis buffer. After washing with 20 ml of phosphate-buffered saline containing 10 mM dithiothreitol (washing buffer), the recombinant fusion protein GST-ChlNB in complex with ChlB or alternatively GST-ChlL alone were eluted using 2 ml of lysis buffer containing 10 mM glutathione in its reduced form. Alternatively, ChlNB or ChlL were liberated from bound GST via PreScission™ protease (GE Healthcare) treatment. Fractions containing the ChlNB complex or ChlL were identified by SDS-PAGE.

Determination of Native Molecular Mass—Analytical gel permeation chromatography was performed using a Superdex 200 HR 10/30 column (GE Healthcare), equilibrated with lysis buffer. The column was calibrated with protein standards (molecular weight marker kit MW-GF 1000; Sigma) at a flow rate of 0.5 ml min⁻¹. A 200-μl sample of purified (ChlNB)_2 or ChlL₂ (~0.6 mg) was run under identical conditions. For preparative gel permeation chromatography up to 5 ml of purified (ChlNB)_2 or ChlL₂ were run at 1.5 ml min⁻¹ on a Superdex 200 26/60 column (GE Healthcare) under strict anaerobic conditions. The eluted proteins were detected at 280 nm and collected in 1.5-ml aliquots.

Determination of Protein Concentration—The BCA protein assay kit (Pierce) was used according to the manufacturer’s instructions with bovine serum albumin as a standard.

N-terminal Amino Acid Sequence Determination—Automated Edman degradation was used to confirm the identity of purified proteins.

UV-visible Light Absorption Spectroscopy—UV-visible light spectra of purified recombinant (ChlNB)_2 complexes and ChlL₂ were recorded using a V-550 spectrometer (Jasco, Gross Umstadt, Germany) under strict anaerobic conditions.

Iron Determination Method—The iron content of the purified (ChlNB)_2 complex and ChlL₂ were confirmed colorimetrically with bathophenanthroline after acid denaturation (31).

Labile Sulfur Determination—To quantify the content of labile sulfur of (ChlNB)_2 and ChlL₂ reactions containing N,N-dimethyl-p-phenylenediamine with FeCl₃ were performed as described before (32, 33).

Preparation of EPR Samples—Sample preparation was carried out in an anaerobic chamber. Purified P. marinus (ChlNB)_2 and ChlL₂ were concentrated to 77 and 50 μM, respectively, using an Amicon stirred ultrafiltration cell (Millipore, Bedford, MA) equipped with a 50,000-Da compound excluding ultrafiltration membrane. Ten μl of a sodium dithionite solution (100 mM) was added to 100 μl of protein solution and incubated for 10–30 min. Where indicated, 100 μl of (ChlNB)_2 were supplemented with 10 μl of ATP (2 mM) and 50 μl of ChlL. Control samples contained protein fractions as purified. The proteins were finally transferred to quartz EPR tubes with 4-mm outer diameters and frozen in liquid nitrogen.

EPR Spectroscopy—9.5-GHz X-Band EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a rectangular microwave cavity in the TE₁₀₂ mode. For temperature control at 10,000, the sample was kept in an Oxford ESR 900 helium flow cryostat with an Oxford ITC4 temperature controller. The microwave frequency was detected with an EIP frequency counter (Microwave Inc., San Jose, CA). The magnetic field was calibrated using a Li/LiF standard with a known g value of 2.002293 ± 0.000002 (34). Base-line corrections, if required, were performed by subtracting a background spectrum, obtained under the same experimental conditions from a sample containing only buffer solution. Simulations of the experimental EPR spectra have been carried out with the program EasySpin (35).

Protochlorophyllide Preparation—Pchlide (substrate 1; see Table 1 and Fig. 4) was isolated from the bchL-deficient R. capsulatus strain ZY-5 (36) and subsequently purified by affinity purification in combination with preparative high performance liquid chromatography according to Ref. 13.

Substrate Analogs—Synthesis and/or isolation of pigments 2-9 and 12-19 is cited in Table 1. Magnesium protoporphyrin IX (substrate 10) and magnesium protoporphyrin IX monomethyl ester (substrate 11) were purchased from Frontier Scientific Inc. (Logan, UT).

Pchlide Reduction Assay—DPOR activity was measured in 125-μl assays containing 100 mM Hepes/NaOH, pH 7.5, 2 mM ATP, 5 mM MgCl₂, 13 μM Pchlide, and 2 mM dithiothreitol as an artificial electron donor. An ATP-regenerating system consisting of 20 mM creatine phosphate and 20 units/assay creatine-phosphokinas was employed. DPOR assays contained 100 pmol of purified (GST-ChlNB)_2 and 200 pmol of purified (GST-ChlL)_2. Standard assays were incubated under strict anaerobic conditions for 5 min up to 70 min at 25 °C in the dark. For the determination of the temperature optimum for P. marinus DPOR, tests were performed at 15–40 °C for 20 min. The reactions were stopped by adding 500 μl of acetone. After centrifugation for 30 min at 12,000 × g, Pchlide a and Chlide a in the supernatant were spectrophotometrically quantified by using an extinction coefficient of ε₆₆₅ = 30.4 mM⁻¹ cm⁻¹ for Pchlide a (16) and ε₆₆₅ = 74.9 mM⁻¹ cm⁻¹ for Chlide a (37). For the analysis of DPOR substrate recognition, the Pchlide reduction assay was performed in the presence of substrate analogs (see Table 1).

DPOR Substrate Competition Assay—All of the substrate analogs showing reduced activity in the Pchlide reduction assay were tested for their ability to inhibit DPOR catalysis. Therefore, Pchlide reduction assays were performed in the presence of 13 μM Pchlide and additionally supplemented with the Pchlide derivatives found not to sustain DPOR activity in concentrations up to 20 μM. Chlide formation was monitored spectrophotometrically.

Substrate Binding Assay—2 mg of purified (GST-ChlNB)_2 were bound to 200 μl of glutathione-Sepharose (GE Healthcare) and incubated with 200 μl of 25 μM Pchlide (or the corresponding substrate analogs) for 10 min. After washing with 5 × 200 μl of lysis buffer (GST-ChlNB)_2, was eluted with 300 μl of 15 mM glutathione in lysis buffer and analyzed for bound pigments by UV-visible spectroscopy.
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RESULTS AND DISCUSSION

Purification and Biochemical Characterization of P. marinus DPOR—GST-ChlN and ChlN each complexed with stoichiometric amounts of untagged ChlB were purified using glutathione-Sepharose (GE Healthcare) (Fig. 2A, lanes 2 and 3). Their identity was confirmed by Edman degradation, further indicating a molar ratio of 1.1/1 for ChlN/ChlB. The relative native molecular mass of the purified ChlN/ChlB complex determined by size exclusion chromatography was 210,000, indicating a tetrameric (ChlN/ChlB)2 complex (ChlN over ChlB) also resulted in the purification of free ChlN in the initial affinity purification step. Obviously, the slightly increased expression level for GST-ChlN and ChlN each complexed with stoichiometric amounts of untagged ChlB were purified using glutathione-Sepharose after PreScission™ Protease cleavage; lane 4, affinity chromatographically purified GST-ChlL complex with ChlB; lane 3, ChlN and ChlB recovered from glutathione-Sepharose after PreScission™ Protease cleavage; lane 4, affinity chromatographically purified GST-ChlL; lane 5, ChlL eluted from glutathione-Sepharose after PreScission™ Protease cleavage. B, UV-visible absorption spectra showing activity of DPOR reconstituted from purified subunits. Assay mixtures composed as described under ‘Experimental Procedures’ were incubated for 20 min at 25 °C, and pigments were extracted with acetone. Trace a, DPOR assay containing 100 pmol of (ChlNB)2 and 200 pmol of ChlL2, traces b–e: extracts of control reactions lacking dithionite (trace b), ATP (trace c), (ChlNB)2 (trace d), or ChlL2 (trace e). C, Michaelis-Menten plot showing the Km values for Pchlide a and zinc Pheide a as P. marinus DPOR substrates. The initial rates of Pchlide a and zinc Pheide a formation were measured at different substrate concentrations, whereas concentrations of dithionite and ATP were kept under saturating conditions. D, DPOR substrate competition assays. DPOR assays were performed in the presence of 13 μM Pchlide, to which increasing concentrations of the substrate derivative chlorophyll c1 were titrated. No competitive inhibition of DPOR activity was observed.

mg of ChlL2 were purified per liter of E. coli culture. From these results we conclude that the cyanobacterial DPOR from P. marinus features the same overall subunit architecture as the C. tepidum and R. capsulatus enzymes (13, 16).

The (ChlNB)2 Complex Coordinates Two Nonidentical [4Fe-4S] Clusters—Anaerobically purified (ChlNB)2 is brownish in color, and the UV-visible spectra revealed an absorption maximum at 428 nm characteristic for [4Fe-4S] clusters. This absorption peak was bleached upon oxygen exposure (24 h) (Fig. 3A) or EDTA treatment (15 mM, 4 h) as described before (38, 39). The determination of iron and sulfur contents of highly purified protein fractions yielded 7.7 mol of iron and 6.9 mol of sulfur/mol of (ChlNB)2. This is in good agreement with 2 [4Fe-4S] clusters/(ChlNB)2 complex (13), and a recent study that revealed a weak [4Fe-4S]+1 EPR signal for the BchNB complex of R. capsulatus after incubation with subunit BchL and ATP (40).

Following reduction of the P. marinus (ChlNB)2 complex with 10 mM dithionite, a complex EPR signal was observed that was tentatively attributed to reduced [4Fe-4S]+1 clusters. Simulation of the obtained EPR spectrum was only possible by a superposition of two clusters (FeS-I and FeS-II) with different g values (Fig. 3C). FeS-II exhibited the typical spectral shape of a [4Fe-4S]+3 cluster, having a rhombic g tensor. The g values deduced for this cluster from the simulation were g1 = 2.13(1), g2 = 2.008(5), and g3 = 1.89(1); the numbers in parentheses are the estimated errors in the last digit. The other cluster, FeS-I, exhibited an almost axial g tensor showing values of g1 = 2.115(5), g2 = 1.935(2), and g3 = 1.917(2). These values are in a range where reduced [4Fe-4S] or [2Fe-2S] clusters could be expected (41, 42). Based on the iron and sulfur analyses, a [4Fe-4S] cluster was deduced. The different g values of the FeS-I and FeS-II clusters indicate a different geometry, possibly because of differences in the coordination between the ChlN and ChlB subunits. No evidence for high spin states greater than S = 1/2 (e.g. S = 3/2, S = 5/2) was obtained. The superimposed EPR spectrum of both clusters changed shape at higher temperatures (T = 20 K), indicating different relaxation behavior for the [4Fe-4S]+1 clusters or magnetic coupling between them. At temperatures above 20 K, the intensity of the EPR signals rapidly declined.

Reducing the (ChlNB)2 complex in the presence of additional ATP and ChlL did not further increase the EPR signal intensity.
The observed EPR signal intensity was in agreement with the rate of reduced (ChlNB)₂ estimated from the bleaching in the UV-visible spectra.

Fractions of concentrated ChlN alone (37 μM) were brownish as well, and UV-visible spectroscopic analysis exhibits an absorption maximum at 415 nm indicative for [3Fe-4S] clusters (data not shown). However, following reduction with 10 mM dithionite, ChlN samples were EPR silent. From these findings we conclude that isolated ChlN still has the ability to coordinate a [Fe-S] center, albeit a residual [3Fe-4S] cluster that did not give rise to an EPR signal.

The properties of these [Fe-S] clusters agree with the results of a previous mutagenesis study for the identification of [4Fe-4S] cluster coordinating amino acid residues. Three cysteinylligands were identified on protein subunit BchN from C. tepidum (13). All three Cys-residues of ChlN are 100% conserved in the known protein sequences from cyanobacterial sources.

The ChlL₂ Complex Coordinates One [4Fe-4S] Cluster—Anaerobically purified ChlL₂ was also brownish in color and revealed an absorption maximum at 428 nm in the UV-visible spectra characteristic for [4Fe-4S] clusters. Upon oxygen exposure (Fig. 3A) or EDTA treatment (15 μM, 4 h), this absorption peak rapidly disappeared. For 1 mol of ChlL₂, 3.6 mol of iron and 3.4 mol of sulfur were determined, suggesting one [4Fe-4S] cluster/dimer. These results were confirmed by the EPR spectrum of reduced ChlL₂ samples. In contrast to the (ChlNB)₂ samples, the spectrum of ChlL₂ can be simulated with only one Fe-S species (Fig. 3D). The deduced g values of \( g_1 = 1.97(1) \), \( g_2 = 1.94(1) \), and \( g_3 = 1.84(2) \) are in the range for a [4Fe-4S]⁺¹ cluster (41, 42).

The results of our new cyanobacterial ChlL protein are in agreement with previous EPR studies for R. capsulatus Bchl (\( g_1 = 2.03 \), \( g_2 = 1.94 \), and \( g_3 = 1.86(1) \) (43). From these experiments and the results of an earlier mutagenesis study (13), we conclude that the DPOR ChlL₂ complex from P. marinus coordinates one intersubunit [4Fe-4S] cluster.

Reconstitution of DPOR Activity from Recombinant Purified (ChlNB)₂ and ChlL₂—The functional DPOR enzyme was reconstituted by supplementing 100 pmol of purified (ChlNB)₂ complex and 200 pmol of ChlL₂ with 13 μM Pchlide a₁, 2 mM dithionite as reductant and 2 mM ATP in combination with an ATP-regenerating system. UV-visible spectroscopic analyses of the acetone-extracted pigments demonstrated the effective reduction of Pchlide a₁ (absorbance maximum, 626 nm) to Chlide a (absorbance maximum, 665 nm) (Fig. 2B, spectrum a). This catalysis was abolished upon exposure to oxygen (data not shown). Likewise, no Chlade a was formed in the absence of either (ChlNB)₂, ChlL₂, dithionite, or ATP (Fig. 2B, spectra b–e). With this standard assay, a temperature optimum of 25 °C was determined for our cyanobacterial DPOR system, which is in agreement with the mesophilic habitat of P. marinus. A specific activity of 910 pmol min⁻¹ mg⁻¹ was determined at 25 °C. In a previous study for the moderate thermophilic C. tepidum DPOR system, a comparable specific activity of 3.12 nmol min⁻¹ mg⁻¹ was obtained (13). The initial velocity of product formation was measured over a broad range of substrate concentrations, whereas concentrations of the cosubstrates ATP and dithionite were kept at saturating concentrations. P. marinus DPOR catalysis followed Michaelis-Menten-type kinetics with a \( K_m \) value of 6.9 μM for Pchlide a₁, which again is comparable with \( K_m = 6.1 \) μM obtained for C. tepidum DPOR (13).
The (ChlNB)₂ Complex Binds the Substrate Pchlide a—Following incubation of purified (ChlNB)₂ bound to glutathione-Sepharose with 25 μM of the substrate Pchlide a, (ChlNB)₂ was eluted with Pchlide a bound to the protein complex. We determined a ratio of 1.4 mol Pchlide/mol (ChlNB)₂. These results are consistent with observations made for R. capsulatus DPOR (40) and the proposal of two active sites/(ChlNB)₂ complex (13). Pchlide a binding was observed without ATP, dithionite, or subunit ChlL being present, suggesting substrate binding by the catalytic (ChlNB)₂ complex to be the initial step in DPOR catalysis.

Zinc Protoporphorhobide a Is an Efficient DPOR Substrate—For the analysis of the substrate specificity of chlorophyll biosynthetic enzymes, the use of zinc derivatives instead of magnesium-containing tetrapyrroles is well established (44–46). When the standard DPOR assay was supplemented with 13 μM zinc Ppheide a, b, a bathochromic absorption shift of 40 nm was observed, analogously to the reaction with the natural magnesium-containing substrate Pchlide a. By measuring the specific absorption of the reaction product zinc Pheide a, the initial velocity of product formation was measured over a broad range of substrate concentrations, whereas concentrations of the cosubstrates were kept at saturating concentrations. Zinc Pheide a formation followed Michaelis-Menten kinetics as described for the natural magnesium-containing substrate Pchlide a with a Kₘ value of 8.5 μM (6.9 μM for Pchlide a) and a specific activity of 730 pmol min⁻¹ mg⁻¹ (910 pmol min⁻¹ mg⁻¹ for Pchlide a). These are only minor differences, indicating that replacement of magnesium by zinc does not affect the reaction significantly. Zinc Ppheide a and the corresponding 10 modified zinc derivatives used in this study are therefore considered suitable as putative substrates for the analysis of DPOR substrate recognition. Furthermore, we made use of eight magnesium-containing chlorophylls and chlorophyll biosynthetic intermediates, which were also tested as substrate analog. The absorption maxima and extinction coefficients of the individual substrates employed in this study are summarized in Table 1. All of the specific activities for the zinc- or magnesium-containing substrates analyzed in this study were related to values obtained for zinc Ppheide a 2 or Pchlide a 1, respectively.

Zinc Protoporphorhobides with Altered Substituents on Ring A—In the first series of potential substrates, the C3 vinyl group on ring A was altered into a more polar formyl group (zinc Ppheide a 3) and into a more bulky acetyl group (zinc [3-acetyl]-Ppheide a 4). The C3 vinyl group was finally modified with a large phenylamino group (zinc 3(1)-phenylamino-Ppheide a 5) (Fig. 4A). The DPOR enzyme tolerated the polar formyl substituent of substrate 3, as indicated by a specific activity of 85% compared with substrate 2. However, compounds 4 and 5 did not result in any detectable DPOR activity. These results indicate the critical involvement of ring A in substrate recognition. Because the 3-formyl group in the accepted substrate 2 is of less importance for substrate recognition than size. In accordance with this hypothesis, compounds 4 and 5 neither functioned as competitive inhibitors in inhibition assays nor did they bind to the purified (ChlNB)₂ complex (Table 1).

Protoporphorhobide and Zinc Protoporphorhobides with Altered Substituents on Ring B—The second set of putative substrates included compounds with alterations of the C7 methyl group into a formyl group (Pchlide b 6; zinc Ppheide b 7) and a phenylamino derivative (zinc 7(1)-phenylamino-Ppheide a 8). The substitution of C8 ethyl group with a vinyl group (zinc 8-vinyl)-Ppheide a 9) was also tested (Fig. 4A).

DPOR from P. marinus efficiently converted Pchlide b (substrate 6) into Chlide b, with a specific activity of 80% compared with substrate 1. It also reduced its zinc derivative zinc Ppheide b 7 to zinc Pheide b with a specific activity of 90% relative to substrate 2. As expected, both compounds were bound by
By contrast, compound 8 with a more bulky phe-nylamino group in C71 position was not a substrate. This indi-cates that also at this position steric hindrance is more impor-tant than polarity for binding and catalytic conversion. This conclusion is confirmed by inhibition experiments in which compound 8 failed to function as a competitive inhibitor for DPOR catalysis. Furthermore, compound 8 did not show detectable affinity for the (ChlNB)2 complex in the substrate binding assay.

Precursor Molecules of Pchlide a Biosynthesis Are Not Substrates for DPOR—Biosynthesis of Pchlide a includes the formation of magnesium protoporphyrin IX 10 and magnesium protoporphyrin IX monomethyl ester 11. Both lack the isocyclic ring E (Fig. 4B) but contain otherwise the same conjugation sys-tem and substituents as Pchlide a, thereby allowing for the analysis of the potential involvement of ring E in substrate recognition. Neither of the two tested substrates lacking this feature allowed for DPOR activ-ity (Table 1), indicating clearly that the reduction of the C17–C18 bond can only take place after the forma-tion of the isocyclic ring in the bio-synthetic pathway.

Zinc Protopheophorbides with Altered Substituents on Ring E—To further specify substrate recogni-tion of ring E by P. marinus DPOR, a series of four substrates modified at ring E were analyzed. First the methoxycarbonyl substituent at C132 was either removed (zinc pyro-Ppheide a 12) or substituted by a hydroxy group (zinc 132OH-pyro-Ppheide a 13). Next zinc 132OMe-Ppheide a was tested. It contains the polar, larger methoxy group instead of the hydrogen at position C132. This substitu-tion stabilizes the stereochemistry at C132; therefore, both enantiomers were tested, with C132 S (substrate 14) and C132 R (substrate 15) configurations (Fig. 4B).

Even though the C132 and hydroxy residue of compound 13 are distinctly smaller than the respective C132 methoxycar-bonyl group of zinc Ppheide a 2, DPOR reduced compound 13 with the same specific activity as zinc Ppheide a 2. Even zinc pyro-Ppheide a 12 carrying no side chain on C132 still resulted
in 30% of DPOR activity, indicating that the intact methyl ester group is not a prerequisite for substrate recognition. However, activity is lost with zinc Ppheide derivatives carrying two C132 side chains, a methoxycarbonyl as well as a methoxy group. In this case, neither the C132 5 (substrate 14) nor the C132 7 (substrate 15) derivatives were accepted as substrates of DPOR. From these data we concluded that only the integrity of the carbon skeleton of ring E is essential, whereas the substituents on position C132 are not determinants for substrate recognition.

Taken together, these findings clearly demonstrate that substrate recognition of DPOR differs considerably from the LPOR system; the latter does not accept compounds 12 and 13 as substrates (47).

Protochlorophylls and Chlorophylls with Altered Substituents on Ring D—Finally, a set of derivatives with modifications on pyrrole ring D were tested as DPOR substrates. Protochlorophylls a 16 and b 17 already contain the C39H39 phytol ester at the propionate side chain at position C17, whereas the C17–C18 double bond is not reduced. Chlorophylls c1 18 and c2 19 contain an acrylic side chain with a C171–C172 double bond instead (Fig. 4C). None of these D ring derivatives allowed for detectable DPOR activity. For the two phytol esters, this may relate to the steric hindrance of the phytol group, to its reduced polarity, or to both. Electronic effects are most likely responsible for the inactivity of DPOR with compounds 18 and 19. The additional C171–C172 double bond is conjugated to the C17–C18 double bond that is reduced during Chlide formation. In agreement with other substrates, inactivity of DPOR seems to be related again to substrate recognition and binding, because the ring D derivatives 16–19 were neither competitive inhibitors of DPOR catalysis, nor did they bind to the purified (ChlNB)2 complex. By contrast chlorophyll c1 was shown to be a competitive inhibitor of LPOR catalysis (48). From these results we conclude a specific interaction with the propionate side chain at ring D during binding to the active site.

Overall Substrate Recognition: A Proposal—From the presented data obtained from the analysis of 19 substrate analogs, a model can be deduced for the overall substrate recognition by DPOR from P. marinus. First, two molecules of Pchlide a are bound by the catalytic (ChlNB)2 complex. During this process, several parts of the Pchlide a molecule are recognized with high specificity. Binding of ring A allowed polarity modifications of the C3 substituent (vinyl or formyl), but bulky ligands were not accepted. The same is true for ring B substituents. Polarity changes (C7-methyl or -formyl, C8-ethyl or -vinyl) were tolerated, whereas larger groups at C7 prevented substrate binding. Furthermore, the overall integrity of the isocyclic ring E is necessary for substrate recognition, whereas individual substituents of ring E are of minor importance as long as only one bulky group is bound at C132. A methoxycarbonyl group on C132 may be removed or altered without detracting recognition of the substrate. However, substrate analogs devoid of ring E or carrying two substituents on C132 were not used as substrates. The least changes were tolerated at the C17 side chain of ring D next to the C17–C18 double bond, which is reduced during DPOR catalysis. Consequently, any modifications on the C17 propionate chain prevented substrate recognition and utilization.

Each of the tested substrate analogs preventing DPOR activity was neither a competitive inhibitor of catalysis nor bound to the (ChlNB)2 complex. These findings suggest steric effects preventing those derivatives from entering the active site. Because all pyrrole rings are involved in substrate recognition, one might propose that the Pchlide molecule is buried in an active site cavity.

Variable Routes of Chlorophyll Biosynthesis Mediated by DPOR Activity—The 8-vinyl variant of Pchlide a has been shown to be a biosynthetic intermediate when chlorophylls and bacteriochlorophylls are synthesized in organisms containing LPOR. For LPOR it was determined that Pchlide a 1 and [8-vinyl]-Pchlide a equally function as substrates (49, 50), indicating that the single divinyl-reductase enzyme is able to also convert [8-vinyl]-Pchlide a as well as [8-vinyl]-Chlide a (26, 51) (Fig. 5A). Investigation of a C. tepidum mutant lacking a 8-vinyl reductase and producing [8-vinyl]-bacteriochlorophylls demonstrated that [8-vinyl]-Pchlide a is also a substrate for DPOR (27).

Even though no gene is found in the genome of P. marinus that encodes an enzyme for the reduction of the 8-vinyl group (52), we found that P. marinus DPOR utilizes Pchlide a 1 and zinc Ppheide a 2 as well as zinc [8-vinyl]-Ppheide a 9 as substrates. These results suggest that in organisms containing 8-ethyl chlorophylls, an 8-vinyl reduction step during chlorophyll synthesis in the dark may likely occur with [8-vinyl]-Pchlide a or [8-vinyl]-Chlide a as a substrate (26) (Fig. 5A). Chlorophyll b is a naturally occurring pigment containing a C7 formyl instead of a C7 methyl group that is found in chlorophyll a. The oxygen at C7 was shown to be introduced into the C7 methyl group of Chlide a by a CAO (53–55). Although the CAO from Chlamydomonas reinhardtii was described to convert chlorophyll a to chlorophyll b (56), no oxygenation of Pchlide a to Pchlide b by CAO was shown to date. The direct function of Pchlide b as a light harvesting pigment in plants is controversially discussed in the literature (57–59). However, the adjustment of the chlorophyll a/b ratio in the “chlorophyll cycle” has been described as an important mechanism for the adaptation to various light conditions (60). With respect to this it is a relevant observation that P. marinus DPOR utilizes Pchlide a (zinc Ppheide a) as well as Pchlide b 6 and its corresponding Zn analog zinc Ppheide b 7. From these results one might conclude that Pchlide b is a natural precursor of b-type chlorophylls.
pigmentsinsomycyanobacteria. A branched pathway via Chl a or via Chl b might then play an important role for the biosynthesis of chlorophyll b in *P. marinus* (Fig. 5B). When Chl b is a natural precursor, it remains open where the biosynthetic route divides into a-type and b-type chlorophyll precursors.

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