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Reconstitution of a sequential reaction of two nitrogenase-like enzymes in the bacteriochlorophyll biosynthetic pathway of *Rhodobacter capsulatus*

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ABSTRACT

The parental structure of bacteriochlorophyll *a*, bacteriochlorin, is formed by a sequential operation of two nitrogenase-like enzymes, dark-operative protochlorophyllide oxidoreductase (DPOR) and chlorophyllide *a* oxidoreductase (COR). Both DPOR and COR consist of two components, Fe protein and MoFe protein cognates. Here we determined kinetic parameters of COR and established the reconstitution system for the formation of bacteriochlorin (3-vinyl bacteriochlorophyllide *a*) from porphyrin (protochlorophyllide) with purified components of DPOR and COR from *Rhodobacter capsulatus*. This reconstitution system confirmed the recent finding that COR catalyzes 8-vinyl reduction of 8-vinyl chlorophyllide *a* in addition to the known activity of C7=C8 double bond reduction, and provides a promising model to investigate how two nitrogenase-like enzymes are coordinated in bacteriochlorophyll biosynthesis.

1. Introduction

Chlorophylls (Chls) are tetrapyrrole pigments essential for photosynthesis. There are more than 100 different structures in various photosynthetic organisms. These various Chls are categorized into three types according to their basic skeletal structure: porphyrin, chlorin, and bacteriochlorin [1]. Chl *a* is a chlorin-type Chl with an absorbance maximum at 661 nm (Q_y band in ether) that enables oxygenic photosynthesis using red light in plants and cyanobacteria. Bacteriochlorin-type Chls include bacteriochlorophyll *a* (Bchl *a*), which is distributed among anoxygenic photosynthetic bacteria. The absorbance maximum of the Q_y band of Bchl *a* is 773 nm (in ether). This light-absorbing property of Bchl *a* enables the use of a longer-wavelength light than that used by Chl-*a*-containing photosynthetic organisms. In addition to some substitutions of side chain groups, such as the C-7 formyl group in Chl *b* and C-3 acetyl group in Bchl *a*, the basic skeletal structures exert a decisive influence on the absorption properties of Chl pigments.

In the biosynthesis of Bchl *a*, the bacteriochlorin structure is formed by a sequential operation of two nitrogenase-like enzymes. The first enzyme is dark-operative protochlorophyllide (Pchlide) oxidoreductase (DPOR) that converts a porphyrin-type precursor, Pchlide, to a chlorin-type precursor, chlorophyllide *a* (Chlide) by C17=C18 double bond reduction (Fig. 1) [2]. The second enzyme is Chlide oxidoreductase (COR) that reduces the C7=C8 double bond to produce the bacteriochlorin-type precursor, 3-vinyl bacteriochlorophyllide *a* (MV-Bchlide) (Fig. 1) [3]. The Q_y band shifts from 625 nm (Pchlide) to 745 nm (MV-Bchlide) via 661 nm (Chlide) by the sequential reaction.

DPOR consists of two separable components, L-protein (a BchL dimer) as a reductase component and NB-protein (a BchN–BchB heterotetramer) as a catalytic component, which are structurally related to nitrogenase Fe protein and MoFe protein, respectively [2]. DPOR was the first enzyme whose reaction mechanism was proposed on the basis of X-ray crystal structures in (B)Chl biosynthesis [4]. L-Protein transfers electrons to NB-protein coupled with ATP hydrolysis, similar to the electron transfer by Fe protein of nitrogenase [5]. The electron transfer is mediated by a unique Fe–S cluster, NB-cluster, to reach the Pchlide molecules in the catalytic site of NB-protein [6,7]. Although there have been extensive studies of DPOR from the purple bacterium *Rhodobacter capsulatus*

Abbreviations: Bchl, bacteriochlorophyll; Chl, chlorophyll *a*; Chlide, chlorophyllide *a*; COR, chlorophyllide *a* oxidoreductase; DPOR, dark-operative protochlorophyllide oxidoreductase; DV, 3,8-divinyl; MV, 3-vinyl; Pchlide, protochlorophyllide; MV-Bchlide, 3-vinyl bacteriochlorophyllide *a*.

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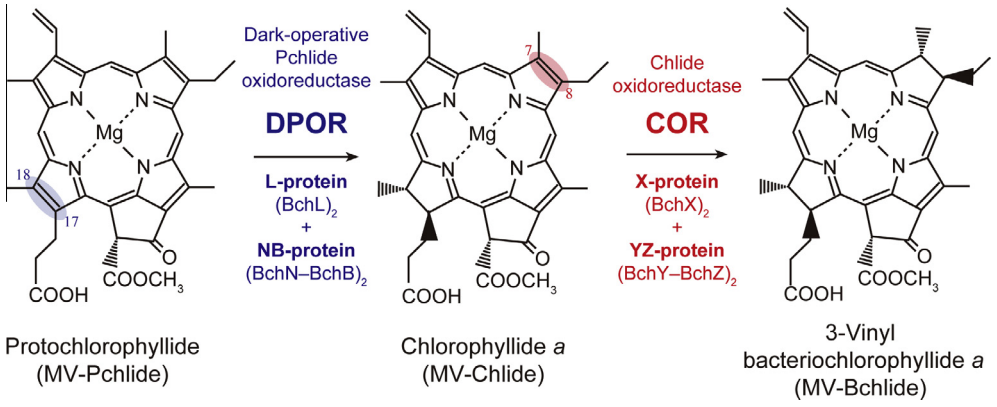

Fig. 1. The sequential operation of DPOR and COR in the conversion of Pchlide to MV-Bchlide. DPOR reduces the C17=C18 double bond (blue) of Pchlide to produce Chlide, and then COR reduces the C7=C8 double bond (red) of Chlide to produce MV-Bchlide. In this figure, Pchlide and Chlide are shown as the MV-forms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[2,4–8] and from a few other species [9–13], little is known about the second nitrogenase-like enzyme, COR. *In vitro* reconstitution using purified components indicated that COR also consists of two components, X-protein (a BchX dimer) and YZ-protein (a BchY–BchZ heterotetramer), which are cognates of L-protein and NB-protein, respectively, and that COR requires dithionite (reduced ferredoxin *in vivo*) and ATP for the catalysis, as do DPOR and nitrogenase [3]. Tsukatani et al. demonstrated that variants of COR are able to catalyze the formation of C8-ethylidene groups by reduction of the C7 and $C8^2$ carbons of 3,8-divinyl Chlide (DV-Chlide) in the biosynthetic pathways of Bchls *b* and *g* [14,15], and that COR from *R. capsulatus* has the additional ability to reduce the $C8^1$=$C8^2$ double bond of C8 vinyl group of DV-Chlide forming to 3-vinyl Chlide (MV-Chlide) [14].

We report the determination of kinetic parameters of COR and reconstitution of conversion of Pchlide (a porphyrin) to MV-Bchlide (a bacteriochlorin) by the sequential reaction of DPOR and COR. COR from *R. capsulatus* shows a significantly lower V_{max} value than that of DPOR, which may be partly compensated by the lower K_m value than that of DPOR. In addition, detailed analysis of the sequential reaction confirmed the 8-vinyl reductase activity of COR in addition to the C7=C8 double bond reduction. We also discuss the plasticity of COR and the redundant role of the 8-vinyl reduction activity in Bchl biosynthesis in *R. capsulatus*.

2. Materials and methods

2.1. Strains, culture conditions and purification of the four components

Escherichia coli JM105 and the transformants harboring pHAL1 [16], pHANB1 [16], pJN1X [14] and pJN1YZ [14] were used to overexpress four components: L-protein, NB-protein, X-protein, and YZ-protein, respectively. Details are described in Supplementary materials and methods. The Bradford method (Protein Assay; Bio-Rad) was used to determine protein concentrations, with bovine serum albumin as the standard. Protein purity was monitored using SDS–PAGE.

2.2. Preparation of Pchlide and Chlide

Pchlide was prepared from the spent medium of a *bchL*-lacking mutant ZY5 of *R. capsulatus* as previously described [2] and we confirmed that the Pchlide preparation was a mixture of 3,8-divinyl Pchlide (DV-Pchlide) and 3-vinyl Pchlide (MV-Pchlide) as previously reported [17] by HPLC as shown below. MV-Pchlide was prepared from the spent medium of dark-grown *chlL*-lacking mutant YFC2 of the cyanobacterium *Leptolyngbya boryana* [18]. 3-Vinyl Chlide (MV-Chlide) was also prepared from the spent medium of CB1200 of *R. capsulatus* [3,19]. To maximize the yield of MV-Chlide, Tween 20 was added to the RCV-2/3PY medium at a final concentration of 0.2% [20]. Müller et al. [20] originally reported that Tween 80 was effective for the overproduction of Chlide, but we found that Tween 20 had a similar effect on MV-Chlide production.

2.3. COR assay and sequential reaction of DPOR and COR

Assays of COR activity were performed essentially as previously described [3,14]. The reaction mixtures (250 μl) contained 100 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM ATP, 20 mM creatine phosphate, 21 units μl^{-1} creatine phosphokinase, 0.7 mM sodium dithionite, various amounts of MV-Chlide, purified X-protein and purified YZ-protein. The reaction mixtures were incubated in anaerobic conditions in the dark for 10 min at 34 °C. To stop the reactions, 200 μl of the assay mixture was mixed with 800 μl of acetone. After centrifugation (15,000 rpm, 10 min), the supernatant (800 μl) was phase-partitioned with 700 μl of hexane, and absorption spectra of the lower acetone phase were recorded with a spectrophotometer (model V550, Jasco, Hachioji, Japan). The amount of formed MV-Bchlide was estimated on the basis of the 734-nm absorption peak in the absorption spectra, as previously described [3,8]. The K_m for MV-Chlide and V_{max} values were estimated from linear regression analysis of a Lineweaver–Burk plot.

For the sequential reaction of DPOR and COR, the assay contained the four purified components and a mixture of MV-Pchlide and DV-Pchlide as the substrate as shown above for the COR assay. The amount of MV-Bchlide was estimated as above. The amounts of MV-Pchlide, DV-Pchlide, MV-Chlide, and DV-Chlide were determined as described in Supplementary materials and methods.

3. Results

3.1. Determination of kinetic parameters of COR with MV-Chlide

The two components of COR, X-protein and YZ-protein, were purified from *E. coli* JM105 (Fig. 2A, inset, lanes 3 and 4). As previously shown [3], X-protein is an ATP-dependent electron donor to YZ-protein. YZ-protein serves as a catalytic component providing the active site for Chlide reduction. In another nitrogenase-like enzyme, DPOR, NB-protein shows its maximal activity in the presence of a 3-fold molar excess of L-protein. To estimate the molar

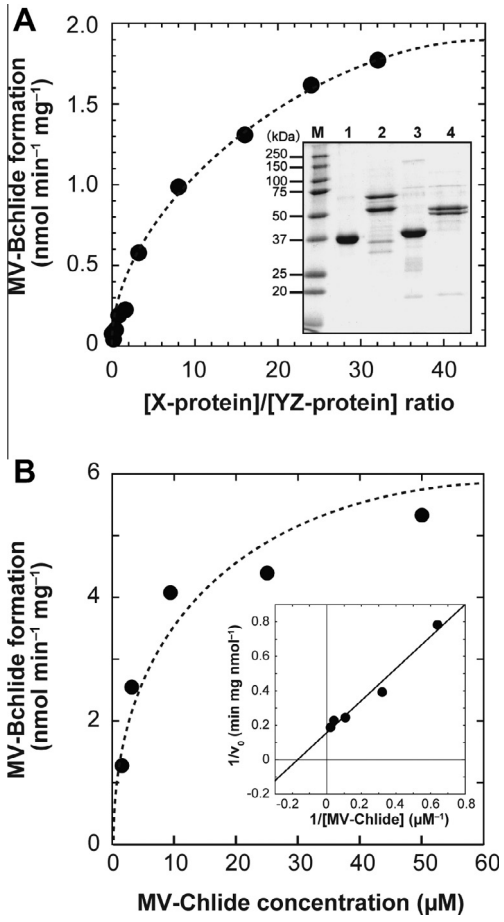

Fig. 2. (A) Titration curve of X-protein with respect to YZ-protein concentration. YZ-protein was fixed (16 μg ml^{-1}, 80 nM) and the amounts of X-protein were increased. (inset) SDS–PAGE profiles of purified X-protein (lane 3) and YZ-protein (lane 4) are shown together with those of the purified L-protein (lane 1) and NB-protein (lane 2) that were used in the sequential reaction. M is a molecular mass marker. (B) Michaelis–Menten plot of the COR assay with X-protein (28 μg) and YZ-protein (10 μg). The MV-Bchlide formation rates were calculated from the absorption spectra. The X-protein/YZ-protein ratio was 4.1. (inset) Lineweaver–Burk plot to determine the K_m and V_{max} of COR.

ratio of X-protein/YZ-protein required for maximal activity, a fixed amount of purified YZ-protein was titrated with X-protein (Fig. 2A). The rate of MV-Bchlide formation increased as the X-protein concentration increased. When the ratio of X-protein/YZ-protein was >40, the activity of YZ-protein almost plateaued. Furthermore, to determine the kinetic parameters of COR, a variety of MV-Chlide concentrations were used to assay the MV-Bchlide formation activity (Fig. 2B). On the basis of the Lineweaver–Burk plot (Fig. 2B, inset), K_m and V_{max} were estimated to be 5.9 μM and 6.3 nmol$_{MV\text{-}Bchlide}$ min^{-1} mg^{-1}, respectively.

3.2. Reconstitution of the DPOR–COR sequential reaction

To reconstitute the conversion of Pchlide to MV-Bchlide, the two components of DPOR, L-protein and NB-protein, were purified from *E. coli* JM105 (Fig. 2A, inset, lanes 1 and 2), along with the components of COR. The four components and the substrate (a mixture of DV-Pchlide and MV-Pchlide) were then mixed, and the reactions were monitored by absorption spectra (Fig. 3A). Chlide (not specified either MV or DV) formed by the activity of DPOR was observed after 3 min and the Chlide peak increased until 7 min. The formation of MV-Bchlide was detected at 10 min with the appearance of a 734-nm peak, and the peak became obvious at 20 min. This spectral change indicated that the Chlide formation by DPOR was followed by MV-Bchlide formation by COR.

Because the DV and MV forms of Pchlide show almost identical absorption spectra with a slight shift of Soret peaks, the change in absorption spectra did not resolve the difference in the 8-vinyl/ethyl group of Pchlide. This was also the case for the DV and MV forms of Chlide. For detailed analysis, the assay mixtures at various time points were analyzed using HPLC (Fig. 3B). In the HPLC conditions, DV-Pchlide, MV-Pchlide, DV-Chlide, and MV-Chlide were resolved to four distinct peaks eluting from 6 to 8 min (Fig. 3B, upper panel). The formation of MV-Bchlide was detected as a single peak at 6.8 min in the elution profiles of 730 nm (Fig. 3B, lower panel). As shown in the chromatogram at 0 min, the Pchlide preparation used in the assay was a mixture of DV-Pchlide and MV-Pchlide in a ratio of approximately 9:11. After 3 min, peaks for both DV- and MV-Chlide appeared, and the amount of DV-Chlide was significantly higher than that of MV-Chlide. DV-Chlide reached a peak after 7 min and decreased to be a trace level after 20 min. In contrast, MV-Chlide increased until 10 min followed by a slow decrease. MV-Bchlide increased gradually until 10 min and became evident after 20 min (Fig. 3C). The time courses of the three pigments, DV-Chlide, MV-Chlide, and MV-Bchlide are shown in Fig. 3C. The profile of these product pigments suggested that DPOR converts DV-Pchlide and MV-Pchlide to the respective Chlide, and that COR converts DV-Chlide to MV-Chlide by the 8-vinyl reductase activity and MV-Chlide to MV-Bchlide by the conventional C7=C8 reduction activity (Fig. 4).

4. Discussion

The basic skeletal structure of Bchl *a* is formed from two reduction reactions: one on the D-ring (C17=C18), catalyzed by DPOR, and the other on the B-ring (C7=C8), catalyzed by COR. Both nitrogenase-like enzymes require ATP and dithionite for catalysis, allowing reconstitution of the sequential reactions by addition of the purified enzyme subunits and the substrate Pchlide to reaction mixtures containing ATP, dithionite, and the ATP-regeneration system [2,3].

In this study, the basic enzymatic parameters of COR for MV-Chlide were determined. The V_{max} value of COR (6.3 nmol min^{-1} mg$^{-1}_{YZ\text{-}protein}$ at an X/YZ ratio of 4) was much lower than that of DPOR (26.2 nmol min^{-1} mg$^{-1}_{NB\text{-}protein}$; [6]); in contrast, the K_m value (5.7 μM) was significantly lower than that of DPOR (12.1 μM) [8], which may partly compensate the low V_{max} value. One characteristic feature of COR is the requirement of the reductase component (X-protein) in much higher amount (>40) than that of DPOR (L-protein; >3) to reach the maximal activity [6]. Although the X/YZ ratio in cells of *R. capsulatus* is unknown, it is unlikely that the amount of BchX is much higher than those of BchY and BchZ to achieve an X/YZ ratio of 40. Thus, it is suggested that COR is the rate-limiting enzyme in the sequential reaction of DPOR and COR.

To avoid detrimental accumulation of precursor molecules during Chl and Bchl biosynthesis, a direct interaction between contiguous enzymes and the presence of metabolic channeling has been proposed [21]. Therefore, there may be a direct interaction between DPOR and COR that allows effective channeling of Chlide molecules from NB-protein to YZ-protein. However, since the protein–protein interaction has not been exemplified by any conventional methods, such as pull-down assay, the interaction between DPOR and COR, if any, may be very weak.

HPLC analysis of the sequential reactions revealed that both DV- and MV-forms of Pchlide are converted to DV- and MV-forms of Chlide, respectively, by the action of DPOR, and then the DV- and MV-forms of Chlide are converged to MV-Bchlide by the actions of COR. The time course of the two Chlide forms suggested that

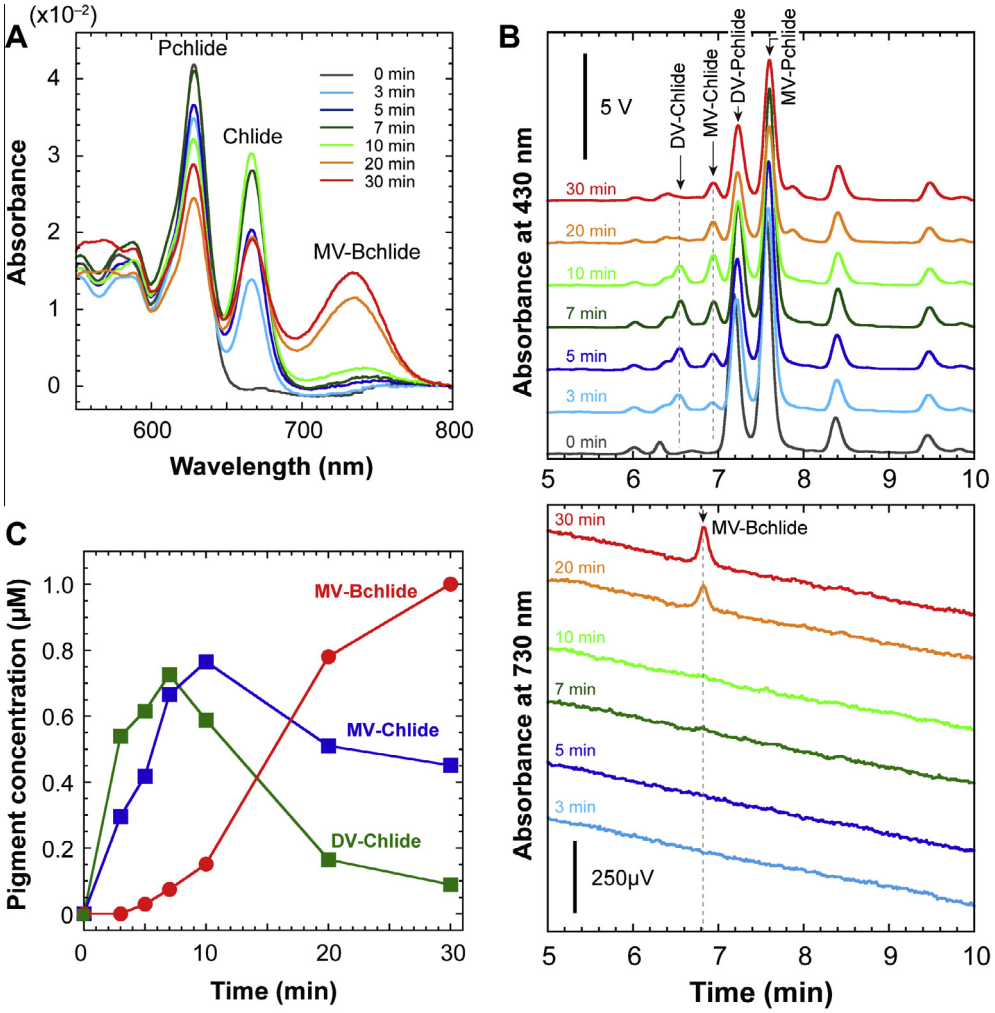

Fig. 3. (A) Time course of the sequential reaction of DPOR and COR. Reaction monitoring by absorption spectra of acetone extracts from the reaction mixtures (250 μl), which contained L-protein (1.6 μg), NB-protein (14 μg), X-protein (24 μg), and YZ-protein (7.0 μg). The molar ratios of L-protein/NB-protein and X-protein/YZ-protein are 0.35 and 10.2, respectively. The ratio of NB-protein/YZ-protein is 2.0. Pchlide concentration is 10 μM (DV-Pchlide, 4.5 μM and MV-Pchlide, 5.5 μM). (B) HPLC profiles of the respective reaction time. Aliquots of the assay mixture (20 μl) are loaded onto the HPLC column, and the elution is monitored by 430-nm (upper panel, a UV/VIS detector) and 730-nm (lower panel, a PDA detector) absorption. (C) The time courses of the formation of DV-Chlide (green squares), MV-Chlide (blue squares), and MV-Bchlide (red circles) are shown. The amounts of DV-Chlide and MV-Chlide are estimated from the HPLC profile, and the MV-Bchlide amounts are estimated from the 734-nm peak of the absorption spectra (A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the 8-vinyl reduction is catalyzed by COR just before the B-ring reduction. There is still another possibility that COR has the ability to catalyze the B-ring reduction of DV-Chlide to produce 3,8-divinyl Bchlide (DV-Bchlide) in addition to MV-Bchlide. Since no standard pigment of DV-Bchlide is available, it is difficult to completely exclude the possibility. However, we think that DV-Bchlide was not generated in the sequential reaction for the following reasons: (1) in the HPLC conditions where DV and MV forms of Pchlide and Chlide are resolved, a Bchlide derivative was detected as a single peak at 6.8 min (Fig. 3B, lower panel), and (2) the absorption peak value 734 nm which shows the generation of a Bchlide derivative in the sequential reaction (Fig. 3A) is identical to that of the product peak value (P734) in the single COR reaction in the previous report [3]. P734 was identified MV-Bchlide by an absorption spectrum and mass spectrometry [3]. In addition, Tsukatani et al. [14] showed that COR from *R. capsulatus* exhibited the 8-vinyl reduction activity in a reaction with DV-Chlide as the substrate. Thus, we propose that the sequential reaction consists of four reactions (Fig. 4): (1) conversion of DV-Pchlide to DV-Chlide by DPOR, (2) conversion of MV-Pchlide to MV-Chlide by DPOR, (3) the conversion of DV-Chlide to MV-Chlide by COR, and (4) conversion of MV-Chlide to MV-Bchlide by COR. In the future, the enzymatic parameters of the 8-vinyl reduction activity of COR with DV-Chlide as the substrate should be determined.

The Pchlide sample used in the assays showed a DV-Pchlide:MV-Pchlide ratio of approximately 9:11. Both Pchlide forms were converted to the corresponding Chlide forms by DPOR at initial rates of 0.18 $\mu M_{DV\text{-}Chlide}\,min^{-1}$ and 0.10 $\mu M_{MV\text{-}Chlide}\,min^{-1}$, which were roughly estimated from Fig. 3C. This suggests that DPOR from *R. capsulatus* has a slightly higher activity for DV-Pchlide than for MV-Pchlide. The next issue of DPOR is the determination of enzymatic parameters for the respective Pchlide.

In (bacterio)chlorophyll biosynthesis, two types of 8-vinyl reductase, BciA (DVR) and BciB (CVR), have been identified so far [22–26]. In *Rhodobacter sphaeroides*, a mutant lacking the *bciA* ortholog still retained the activity to reduce the 8-vinyl group of Bchl, which suggested the presence of a third type of 8-vinyl reductase [26]. The discovery of COR 8-vinyl reductase activity indicates that COR is the third 8-vinyl reductase [14]. Because *R. capsulatus* and *R. sphaeroides* have the *bciA* gene [25,26], the 8-vinyl reduction activity of COR appears to be redundant with BciA activity. BciA probably catalyzes the 8-vinyl reduction of DV-Pchlide to form

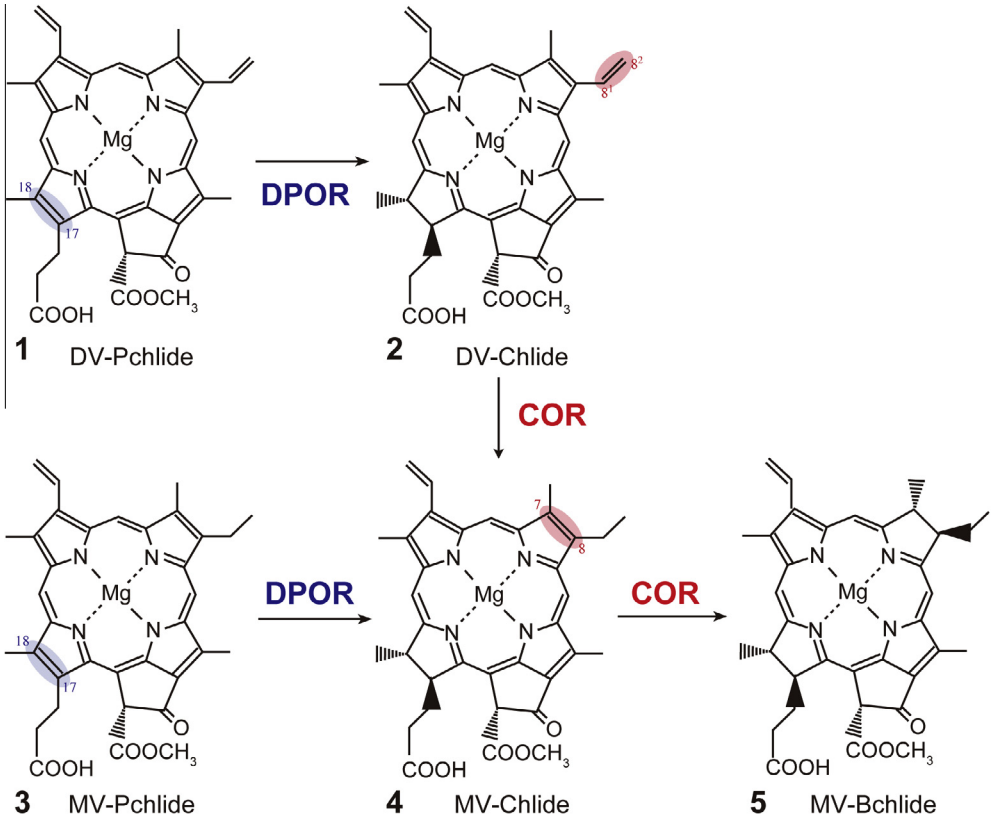

Fig. 4. Steps of the conversion of Pchlide to MV-Bchlide. The substrates are DV-Pchlide (1) and MV-Pchlide (3). DPOR catalyzes the C17=C18 double bond of DV-Pchlide and MV-Pchlide to form DV-Chlide (2) and MV-Chlide (4), respectively. DV-Chlide is converted to MV-Chlide by the additional 8-vinyl reductase activity of COR; finally, MV-Bchlide (5) is formed by the C7=C8 reduction of MV-Chlide. The parental structures of Pchlide, Chlide, and MV-Bchlide are porphyrin, chlorin and bacteriochlorin, respectively.

MV-Pchlide. If BciA worked in full to convert DV-Pchlide, the substrate of COR would be only MV-Chlide, and there would be no need for the alternative 8-vinyl reductase activity of COR. However, the accumulation of Pchlide as a mixture of DV-Pchlide and MV-Pchlide in the mutant ZY5 (Δ*bchL*) of *R. capsulatus* suggests that the BciA activity is not high enough to catalyze all DV-Pchlide molecules *in vivo*. Thus, it is suggested that a dual operation of the two 8-vinyl reductases, BciA and COR, are required for the full conversion of the 8-vinyl group to the 8-ethyl group in *R. capsulatus*.

Nitrogenase and nitrogenase-like enzymes have a wide variety of catalytic activities. Nitrogenase catalyzes not only dinitrogen reduction but also reduction of acetylene, cyanide, and azide [27]. Recently, nitrogenase was shown to be involved in a series of novel reactions in the formation of hydrocarbon compounds; methane, ethane, propyne, and propane, from CO [28–30] and CO_2 [31]. A nitrogenase-like enzyme, NifEN, catalyzes the conversion of a Fe–S cluster (L-cluster) to FeMo-co (M-cluster) [32]. Other nitrogenase-like enzymes, DPOR and COR, catalyze D-ring and B-ring reduction reactions, respectively, in Bchl biosynthesis. Although sequence similarities between the three subunits of CORs from *R. capsulatus* and *Blastochloris viridis* are very high (66–85% identity [14]), CORs from *R. capsulatus* and *B. viridis* catalyze the two different reductions: namely reduction of $C8^1$=$C8^2$ and C7=C8 double bonds and the ethylidene formation by C7 and $C8^2$ reduction, respectively. This variety of reaction exemplifies the high plasticity of nitrogenase-like enzymes.

Nitrogenase performs an 8-electron reduction reaction to produce ammonia from nitrogen accompanied by hydrogen production [33]. The unique metallocenters, FeMo-co and P-clusters in MoFe protein, provide the structural basis that enables a multielectron reduction. In contrast, the DPOR NB-protein containing a simple [4Fe–4S] cluster (NB-cluster) performs only a two-electron reduction. The COR YZ-protein was also believed to catalyze a two-electron reduction with a simple conventional [4Fe–4S] cluster. If the two reactions of 8-vinyl reduction and C7=C8 reduction are tightly coupled on the state of the enzyme–substrate bound form, COR could perform a 4-electron reduction. Such a tightly coupled 4-electron reduction is found in the PcyA catalysis of the conversion of biliverdin IXα to phycocyanobilin, although PcyA itself is not an Fe–S enzyme and the electrons are supplied from ferredoxin [34]. However, the detection of the intermediate MV-Chlide on a minute scale during the 4-electron reaction from DV-Chldie to MV-Bchlide suggested that this reaction consists of the following two independent 2-electron reactions rather than tightly coupled sequential reactions: COR can only catalyze the C7=C8 reduction for MV-Chlide and only performs the 8-vinyl reduction for DV-Chlide. It is of interest to understand how the two reductions are coordinated dependent on the C8 substituent. Further biochemical analysis is needed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.087.

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