Evolution of a New Chlorophyll Metabolic Pathway Driven by the Dynamic Changes in Enzyme Promiscuous Activity

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Organisms generate an enormous number of metabolites; however, the mechanisms by which a new metabolic pathway is acquired are unknown. To elucidate the importance of promiscuous enzyme activity for pathway evolution, the catalytic and substrate specificities of Chl biosynthetic enzymes were examined. In green plants, Chl a and Chl b are interconverted by the Chl cycle: Chl a is hydroxylated to 7-hydroxymethyl chlorophyll a followed by the conversion to Chl b, and both reactions are catalyzed by chlorophyllide a oxygenase. Chl b is reduced to 7-hydroxymethyl chlorophyll a by Chl b reductase and then converted to Chl a by 7-hydroxymethyl chlorophyll a reductase (HCAR). A phylogenetic analysis indicated that HCAR evolved from cyanobacterial 3,8-divinyl chlorophyllide reductase (DVR), which is responsible for the reduction of an 8-vinyl group in the Chl biosynthetic pathway. In addition to vinyl reductase activity, cyanobacterial DVR also has Chl b reductase and HCAR activities; consequently, three of the four reactions of the Chl cycle already existed in cyanobacteria, the progenitor of the chloroplast. During the evolution of cyanobacterial DVR to HCAR, the HCAR activity, a promiscuous reaction of cyanobacterial DVR, became the primary reaction. Moreover, the primary reaction (vinyl reductase activity) and some disadvantageous reactions were lost, but the neutral promiscuous reaction (NADH dehydrogenase) was retained in both DVR and HCAR. We also show that a portion of the Chl c biosynthetic pathway already existed in cyanobacteria. We discuss the importance of dynamic changes in promiscuous activity and of the latent pathways for metabolic evolution.

Keywords: Chlorophyll biosynthesis • Divinyl chlorophyll • Pathway evolution • Promiscuous activity • Synechocystis.

Abbreviations: CAO, chlorophyllide a oxygenase; Chlide, chlorophyllide; CBR, chlorophyll b reductase; DV, divinyl; DVR, 3-divinyl chlorophyllide reductase; Fd, ferredoxin; FNR, ferredoxin-NADP(+) (oxidoreductase; HCAR, 7-hydroxymethyl chlorophyll a reductase; HMChl a, 7-hydroxymethyl chlorophyll a; HMChlade a, 7-hydroxymethyl chlorophyllide a; MV, monovinyl; PChlide, protochlorophyllide; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase.

Introduction

Metabolism plays an essential role in biological activities by producing various molecules, reducing power and energy. Several hundred thousand secondary metabolites are produced in plants and are involved in protection against environmental stress, such as pathogens, herbivory and ultraviolet radiation (Dixon 2001, Neillson et al. 2013). Indeed, organisms have developed metabolic pathways to produce an enormous number of metabolites (Petersen et al. 2009, Kliebenstein and Osbourn 2012). Although the mechanisms by which a new metabolic pathway is acquired remain unknown, several hypotheses on the evolution of metabolic pathways have been proposed (Fani and Fondi 2009), such as the retrograde hypothesis (Horowitz 1945), the Granick hypothesis and the patchwork hypothesis (Jensen 1976).

The patchwork hypothesis is based on gene duplication and the assumption of a broad substrate specificity of the ancestral enzyme and the catalysis of many different but similar reactions. In fact, it has been revealed that, in addition to the primary activity, enzymes have promiscuous and minor activities that are not involved in any cellular processes (O’Brien and Herschlag 1998, Rison and Thornton 2002, Copley 2003, Khersonsky and Tawfik 2010). Such promiscuous enzymes can catalyze the same reaction using a similar substrate and can also catalyze multiple chemical transformations that are classified as different reactions. This idea is supported by the analysis of enzyme superfamilies, showing that 20% are able to catalyze the reaction of a different EC class (Schmidt et al. 2003, Galperin and Koonin 2012). During evolution, one of these promiscuous activities may become valuable under certain environmental conditions, and selection will favor an increase in the level of this activity. This hypothesis based on enzyme promiscuity may solve Ohno’s dilemma that a duplicated gene must be free of inactivating lesions long enough to acquire a new function (Berghorsson et al. 2007). Nonetheless, the manner in which the primary reaction is substituted by the promiscuous reaction and which environmental and cellular states affect the evolutionary fate of a promiscuous activity are almost completely unknown.
Chl has an essential role in photosynthesis by harvesting light energy and driving electron transfer (Nelson and Yocum 2006). Anoxygenic photosynthetic bacteria contain bacteriochlorophyll a, b, c, d, e and g (Chew and Bryant 2007), and oxygenic phototrophs use Chl a, b, c, d and f (Chen and Blankenship 2011) for photosynthesis. Contributing to the photosynthetic characteristics of each organism, these Chl species have been acquired by different lineages by adding several steps to the latter part of the core pathway of Chl biosynthesis. For example, the interconversion pathway of Chl a and Chl b, designated as the Chl cycle (Ito et al. 1996), was acquired by the lineage of green plants (Meguro et al. 2011) via the addition of reactions at the last step of the core pathway, whereas Chl c species appeared after secondary endosymbiosis (Sanchez-Puerta et al. 2007) via the addition of several steps at divinyl (DV)-protochlorophyllide (PChlide) a. Chl metabolism is a good model to study pathway evolution because the Chl biosynthetic pathway has been extensively studied and all the enzymes responsible for Chl a and Chl b biosynthesis in vascular plants and cyanobacteria have been identified (Beale 2005, Nagata et al. 2005, Kato et al. 2010). This knowledge enables the investigation of the evolutionary process of the Chl biosynthetic pathway based on enzymatic and phylogenetic analyses.

Accordingly, to elucidate the dynamic changes in promiscuous activity during enzyme evolution and to evaluate the contribution of promiscuous activity to pathway evolution, we examined the catalytic and substrate specificity of the enzymes of the latter stage of Chl biosynthesis, which contribute to the diversity of Chl species. We found that cyanobacterial 3,8-divinyl chlorophyllide reductase (DVR), a Chl biosynthetic enzyme, has many promiscuous activities. One of these promiscuous reactions (7-hydroxymethyl chlorophyll a reductase activity) has become the primary reaction of the enzyme and participates in the Chl cycle in green plants; other promiscuous activities have been lost or retained during evolution. We propose that enzymes retain a broad catalytic and substrate specificity if these reactions are not disadvantageous and that this broad specificity of the enzyme is a driving force of pathway evolution.

Results

Reductant and substrate specificity of DVRs

Fig. 1 shows the Chl biosynthetic pathways of oxygenic photosynthetic organisms. At the later steps of Chl biosynthesis, DV-chlorophyllide (Chlide) a is converted to monovinyl (MV)-Chlide a by DVR. Two different DVRs have been identified in oxygenic photosynthetic organisms, one of which was initially identified in Arabidopsis (Nagata et al. 2005) and is found among most euKaryotic photosynthetic organisms and in a small group of the genus Synechococcus. Enzymatic experiments have clarified that Arabidopsis DVR uses NADPH as reductant. The other group was identified in Synechocystis sp. PCC 6803 (referred to hereafter as Synechocystis) (Slr1923) (Islam et al. 2008, Ito et al. 2008) and green sulfur bacteria (Beil) (Liu and Bryant 2011, Saunders et al. 2013), and its ortholog is found among most cyanobacteria. To examine the properties of these two DVRs, we prepared recombinant proteins of Arabidopsis DVR (AT5G18660) and cyanobacterial DVR (Slr1923) and determined DVR activity in the presence of NADPH or reduced ferredoxin (Fd) prepared with NADPH, ferredoxin-NADP+ (oxido)reductase (FNR) and Fd (Fig. 2).

Arabidopsis DVR showed high activity when NADPH was used as the reductant (sample 5), whereas recombinant Slr1923 required Fd (sample 4). Based on these enzymatic properties, we refer to the Arabidopsis-type DVR as N-DVR (NADPH-dependent DVR) and to the Slr1923-type as F-DVR (Fd-dependent DVR).

We next examined the substrate specificity of Arabidopsis N-DVR and Synechocystis F-DVR (Fig. 2). As we reported previously (Nagata et al. 2005), Arabidopsis N-DVR catalyzes a reaction using DV-Chlide a (sample 5), but the activity is very low when DV-Chl a is used as a substrate (sample 9). Although we detected no activity when DV-PChlide was used (sample 13), it was reported that Arabidopsis DVR reduced a small amount of DV-PChlide after a 10 h incubation (Wang et al. 2013), a discrepancy that may be due to the different experimental conditions. In contrast, cyanobacterial F-DVR exhibited a broad substrate specificity and converted DV-PChlide a (sample 12), DV-Chlide a (sample 4) and DV-Chl a (sample 8) to the respective monovinyl molecules. These results indicate that F-DVR has a broad substrate specificity, whereas N-DVR has a high specificity for DV-Chlide a.

A portion of the reactions of the Chl cycle evolved from a promiscuous activity of F-DVR

Eukaryotic green plants contain Chl b as a photosynthetic pigment, which is synthesized from Chl a via 7-hydroxymethyl chlorophyll (HMChl) a by Chlide a oxygenase (CAO) (Tanaka et al. 1998, Tomitani et al. 1999). Chl b is reduced to HMChl a by Chl b reductase (CBR) (Ito et al. 1996, Kusaba et al. 2007) and then converted to Chl a by HMChl a reductase (HCAR) (Meguro et al. 2011). In this process, HCAR catalyzes the dehydration of the -CH2OH at the C7 position to -CH3. This interconversion pathway of Chl a and Chl b is referred to as the Chl cycle, which plays a crucial role in Chl degradation during senescence and in light environment acclimation (Tanaka and Tanaka 2011). The Chl cycle exists in the chloroplasts of green algae and vascular plants, but HCAR of the Chl cycle shows high sequence identity to cyanobacterial F-DVR (Supplementary Fig. S1). A phylogenetic analysis showed that HCAR branched off within the F-DVR cluster (Fig. 3), indicating that HCAR evolved from F-DVR (Meguro et al. 2011). However, the catalytic reactions of these two enzymes are greatly different. Thus, to elucidate how F-DVR has evolved to HCAR, we examined the enzymatic activity of F-DVR and HCAR, including their promiscuous activities. When 7-hydroxymethyl chlorophyllide (HMChlide) a was incubated with F-DVR, Chlide a was
generated (Fig. 4A, sample 4; Fig. 4B), indicating that F-DVR has HCAR activity as a promiscuous activity. However, this promiscuous activity (HCAR activity) was extremely low compared with the primary catalytic activity (DVR activity) and with the HCAR activity of Arabidopsis HCAR. To verify the promiscuous activity of F-DVR, a lysate of Escherichia coli expressing recombinant F-DVR was used for the enzymatic analysis (Fig. 4C, sample 4). Interestingly, in addition to Chlide a,
a low but significant level of Chlide b was found in the reaction mixture (Fig. 4A, sample 4; Fig. 4C, sample 4), indicating that F-DVR can convert HMChlide a to Chlide b, the reverse reaction of Chlide b reductase. When HMChlide a was incubated with F-DVR without a reductant, the level of Chlide a became very low, and Chlide b increased (Fig. 4C, sample 8). This result is reasonable because the conversion of HMChlide a to Chlide a is a reductive reaction and the conversion of HMChlide a to Chlide b is an oxidative reaction. When Chlide b was incubated with the E. coli lysate containing F-DVR, HMChlide a and Chlide a accumulated (Fig. 4C, sample 6). The extracts from E. coli had no promotive effect on F-DVR activities (Supplementary Fig. S2). F-DVR activities might be partly lost during purification. In contrast, F-DVR did not catalyze the conversion of Chlide a to HMChlide a in the Chl cycle (Supplementary Fig. S3). These observations indicate that F-DVR can catalyze the interconversion of Chlide b and HMChlide a and the conversion of HMChlide a to Chlide a; therefore, three of the four reactions of the Chl cycle can be catalyzed by cyanobacterial F-DVR (Table 1). In contrast, HCAR has no DVR activity (Supplementary Fig. S4a, sample 1). This result is supported by the previous observation that the Arabidopsis dvr mutant accumulated only DV-Chl, in spite of the presence of HCAR (Nagata et al. 2005). HCAR also has no catalytic activity to convert HMChlide a to Chlide b or Chlide b to HMChlide a (Supplementary Fig. S4a, samples 2 and 3). Thus, HCAR evolved from F-DVR via an increase in its promiscuous HCAR activity and a decrease in its DVR and other promiscuous activities.

During the course of these experiments, we identified an NADH dehydrogenase activity of F-DVR when ferricyanide was used as an oxidant. The $K_m$ values of purified F-DVR and HCAR for the oxidation of NADH were calculated from double reciprocal plots of three independent experiments as 127 ± 35 and 148 ± 70 $\mu$M, respectively. However, NADPH dehydrogenase activity was not detected under any other conditions tested. This promiscuous activity has not changed much during the evolution of F-DVR to HCAR. The deduced amino acid sequence of F-DVR shows homology to that of FpoF protein of the NDH1 complex, which is involved in the oxidation of $F_{420}H_2$ (Islam et al. 2008). Thus, it might be possible that F-DVR can utilize NADH instead of $F_{420}H_2$ (Prommeenate et al. 2004). However, Synechocystis F-DVR did not use NADH as a reductant for the reduction of DV-Chlide a (Supplementary Fig. S5).

The Chl $c_2$ to Chl $c_1$ conversion was pre-existent in cyanobacteria as a promiscuous activity of F-DVR

Chromists and dinoflagellates use Chl $c$ as a major light-harvesting pigment; however, the enzymatic mechanism of Chl $c$ biosynthesis remains completely unknown, and no experimental evidence has been reported for the pathway of Chl $c$ biosynthesis (Green 2011). The possible pathway is that Chl $c_2$ is
synthesized from DV-PChlide \( \alpha \) by the dehydrogenation of 17-propionate to an acrylic moiety and that Chl \( \epsilon_1 \) is synthesized by the reduction of the 8-vinyl group of Chl \( \epsilon_2 \) or Chl \( \epsilon_1 \), which are synthesized from MV-PChlide by the dehydrogenase (Fig. 1), which suggests the involvement of DVR in Chl \( \epsilon \) biosynthesis. Either F-DVR or N-DVR is found in most photosynthetic organisms, yet both F-DVR and N-DVR exist in the genome of diatoms that contain Chl \( \epsilon \), which suggests the involvement of DVR in Chl \( \epsilon \) biosynthesis.

Fig. 3 Phylogenetic tree of F-DVR and HCAR. A phylogenetic maximum likelihood tree was constructed with the translated sequence of HCAR (accession No. XP_001699546); Chlamydomonas reinhardtii (accession No. XP_001699546); Chlorophytoxenon thalassium ATCC 35110 (accession No. YP_001966119); Gloeobacter violaceus PCC 7421 (accession No. NP_923824); Methanosaeta thermophila PT (accession No. YP_842613); Phaeodactylum tricornutum CCAP 1055/1 (accession No. XP_000186080); Physcomitrella patens subsp. patens (accession No. XP_000186080); Synechocystis sp. PCC 6803 (accession No. NP_441896); Thalassiosira pseudonana CCMP1335 (accession No. XP_002288079).

**Discussion**

**Pre-existence of the partial Chl cycle and Chl \( \epsilon_1 \) biosynthesis pathway as promiscuous reactions in cyanobacteria**

Chl \( \beta \) is a photosynthetic pigment of the peripheral antenna systems of green algae and plants, and Chl \( \alpha \) and Chl \( \beta \) are interconverted by the Chl cycle. HCAR in the Chl cycle shows a high sequence homology to F-DVR, and our phylogenetic tree clearly shows that HCAR evolved from F-DVR. However, the catalytic properties of these enzymes are greatly different. Cytanbacterial F-DVR has a broad substrate specificity; in addition to a Chl activity (reduction of \( -CH=CH_2 \) to \( -CH_2CH_3 \)), the enzyme also has HCAR activity (dehydration of \( -CH_2OH \) to \( -CH_3 \)), though the activity is low (Fig. 4). Interestingly, F-DVR has other catalytic activities, such as the interconversion of HMChlide \( \alpha \) and Chlide \( b \) and conversion of HMChlide \( a \) to Chlide \( a \) (Fig. 4), indicating that the Chl cycle partially exists in cyanobacteria. It cannot be excluded that CAO and F-DVR tentatively formed the Chl cycle in the early phase of evolution, with F-DVR then being substituted by CBR and HCAR to generate the present Chl cycle.

Most photosynthetic organisms contain either N-DVR or F-DVR. One exception is the diatom, which contains both N-DVR and F-DVR. Based on the enzymatic experiments using diatom N-DVR and cyanobacterial F-DVR, Chl biosynthesis in diatoms can be proposed as follows. First, an unidentified dehydrogenase converts DV-PChlide \( \alpha \) to Chl \( \epsilon_2 \) and an 8-vinyl group of Chl \( \epsilon_2 \) is reduced to Chl \( \epsilon_1 \) by F-DVR. An alternative pathway is the reduction of DV-PChlide \( a \) to MV-PChlide \( b \) by F-DVR followed by the conversion to Chl \( \epsilon_1 \). For the synthesis of...
Fig. 4 Various activities of Synechocystis F-DVR. (A) Various activities of Synechocystis F-DVR (SyF-DVR) analyzed using purified recombinant SyF-DVR. After incubation, the pigment compositions were analyzed by HPLC. The absorption spectra corresponding to peak 1 and peak 2 are shown in B. 1, Chlide \( \text{a} \); 2, Chlide \( \text{b} \); 3, HMChlide \( \text{a} \); 4, HMChlide \( \text{b} \); 5, Chlide \( \text{a} \) and Chlide \( \text{b} \); 6, HMChlide \( \text{a} \) and Chlide \( \text{b} \); 7, HMChlide \( \text{a} \) and the culture lysate containing empty vector incubated with NADPH, FNR and Fd; 8, HMChlide \( \text{a} \) and the culture lysate containing empty vector incubated with NADPH, FNR and Fd; 9, Chlide \( \text{b} \) and the culture lysate containing empty vector incubated with NADPH, FNR and Fd.

Chl \( \text{a} \), DV-PChlide is converted to DV-Chlide \( \text{a} \) by PChlide oxidoreductase, and a vinyl group of DV-Chlide \( \text{a} \) or DV-Chlide \( \text{b} \) is then reduced to MV-Chlide \( \text{a} \) or MV-Chlide \( \text{b} \) by N-DVR. Arguably, it is reasonable for diatoms to possess two different DVRs because these organisms must regulate Chl \( \text{c} \_1 \) and Chl \( \text{a} \) synthesis differently. Chl \( \text{c} \_1 \) synthesis might be regulated by F-DVR and that of Chl \( \text{a} \) by N-DVR, enabling the fine regulation of the Chl \( \text{c} \_1 \)/Chl \( \text{c} \_2 \) ratio, regardless of the Chl \( \text{a} \) synthesis rate.

Our enzymatic experiments of the Chl cycle and Chl \( \text{c} \) biosynthesis indicated that three of the four reactions of the Chl cycle and Chl \( \text{c} \_1 \) to Chl \( \text{c} \_2 \) conversion activity already exist in cyanobacteria, the progenitor of the chloroplast. It should be noted that, although these metabolic pathways potentially existed in the progenitor, the pathways are not functional due to a lack of substrate molecules. However, the pre-existence of a latent metabolic pathway is a strong driving force of metabolic evolution. When the progenitor acquired Chl \( \text{b} \), the Chl cycle was immediately formed because of the presence of F-DVR. When the unidentified dehydrogenase was obtained, both Chl \( \text{c} \_1 \) and Chl \( \text{c} \_2 \) could be synthesized using the pre-existing F-DVR. This idea is consistent with the report that, when Coptis japonica scoulerine-9-O-methyltransferase was introduced to Eschscholzia californica, the reaction product of this enzyme was synthesized and was further converted to novel molecules by endogenous enzymes (Takemura et al. 2010), indicating a latent metabolic pathway.

**Catalytic promiscuity and enzyme evolution**

It has been suggested that promiscuous enzyme activity plays an essential role in pathway evolution (James and Tawfik 2001, Copley 2012, Nam et al. 2012). Indeed, a promiscuous activity becomes a major activity when the activity plays an important role in a new environment during evolution (Deng et al. 2010, Hackenberg et al. 2011). In contrast, an enzyme may lose its major activity when the activity is not beneficial or becomes
disadvantageous. In addition, some neutral promiscuous activities may be retained for other reasons during the long evolutionary process. Although this idea appears reasonable and can explain how a new pathway appears, there is no report clearly demonstrating the fate of promiscuous enzyme activity during evolution. Nonetheless, our detailed enzymatic study of cyanobacterial F-DVR and Arabidopsis HCAR partly elucidated the dynamic changes in individual promiscuous activities during evolution (Fig. 6).

In addition to DVR activity, cyanobacterial F-DVR has many promiscuous activities, as discussed previously. Except for the major (DVR) and NADH dehydrogenase activities, most of the activities are neutral for cyanobacteria, as the cells lack the substrates, such as Chl b, Chl c2 and HMChl a, for these activities.

The presence of some of these activities (HCAR, Chl c1, synthesis and NADH dehydrogenase activities) in both chloroplasts and cyanobacteria indicates the presence of these promiscuous activities in the common progenitor. Other promiscuous activities might also exist in the progenitor, although we have no direct evidence for this. Therefore, the question arises as to why cyanobacterial F-DVR has retained so many non-functional activities over such long evolutionary time. One possible explanation is that the increase in catalytic specificity resulted in a decrease in the catalytic rate of the primary activity (van Loo et al. 2010), which is well characterized for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco catalyzes the incorporation of CO2 into ribulose 1,5-bisphosphate, the first step in the production of carbohydrates by plants. However, Rubisco cannot completely discriminate between O2 and CO2 due to their electrostatic similarity, resulting in the unwanted oxygenation of ribulose 1,5-bisphosphate (photorespiration). Enzymatic studies of various Rubisco enzymes have shown that higher specificities for CO2 over O2 are accompanied by lower carboxylation rates (Whitney et al. 2011). Alkaline phosphates and arylsulfatas are evolutionarily related, and E. coli alkaline phosphatase was found to have a low level of sulfatase activity (O’Brien and Herschlag 1998, O’Brien and Herschlag 1999), and amino acid substitution of the phosphatase decreased both the sulfatase and phosphatase activities. These results indicate that an increase in catalytic specificity is occasionally accompanied by a decrease in the catalytic rate of the primary reaction, a situation that can enable the promiscuous activity to remain over a long period of time.

In the next evolutionary stage, the DVR activity of F-DVR disappeared, and the HCAR activity increased, resulting in the appearance of HCAR. However, it is unclear why HCAR needed to lose its DVR activity. Modern green plants employ N-DVR for the reduction of the 8-vinyl group. Additionally, it is well known that Chl biosynthesis must be strictly regulated to avoid photo-damage. However, Chl biosynthesis would not be finely controlled if two different DVR enzymes were present because DVR and HCAR are regulated differently: N-DVR must be functional to prevent damage. However, Chl biosynthesis would not be finely controlled if two different DVR enzymes were present because DVR and HCAR are regulated differently: N-DVR must be functional to prevent damage. However, Chl biosynthesis would not be finely controlled if two different DVR enzymes were present because DVR and HCAR are regulated differently: N-DVR must be functional to prevent damage. However, Chl biosynthesis would not be finely controlled if two different DVR enzymes were present because DVR and HCAR are regulated differently: N-DVR must be functional to prevent damage. However, Chl biosynthesis would not be finely controlled if two different DVR enzymes were present because DVR and HCAR are regulated differently: N-DVR must be functional to prevent damage.

### Table 1 Summary of various DVR properties

<table>
<thead>
<tr>
<th>Reductant</th>
<th>NADPH</th>
<th>Fd</th>
<th>DV-Chl(ide)</th>
<th>DV-PChlide</th>
<th>Chl c2</th>
<th>HMChl</th>
<th>Chlide</th>
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<tbody>
<tr>
<td>SyF-DVR</td>
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<td>O</td>
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<td>X</td>
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<tr>
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<td>O</td>
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<td>PhaeN-DVR</td>
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<td>O</td>
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<tr>
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The properties of purified recombinant Synechocystis F-DVR (SyF-DVR), Arabidopsis N-DVR (AtN-DVR), Phaeodactylum N-DVR (PhaeN-DVR) and Arabidopsis HCAR are summarized.

a Meguro et al. (2011)

ND, not determined.
activity, a notion that is supported by the fact that most photosynthetic organisms contain only one type of DVR. The minor promiscuous activity of F-DVR, the interconversion of Chl $b$ and HMChl $a$, has also disappeared (Supplementary Fig. S4). CBR is responsible for Chl $b$ to HMChl $a$ conversion in modern green plants, and the interconversion of Chl $b$ and HMChl $a$ by HCAR would disturb the Chl cycle. Furthermore, it should be noted that all the enzymes of the Chl biosynthetic pathway are irreversible, which might be important for the fine regulation of Chl biosynthesis. In this sense, the involvement of a reversible enzyme (F-DVR) in the Chl cycle must be avoided. The question arises as to why F-DVR has not evolved to CBR. One possible reason for it is that F-DVR could not acquire a sufficient catalytic activity compared with NYC1 and NOL. Another reason is that present CBRs (NOL and NYC1) have other important functions besides CBR activities, such as the direct interaction with the light-harvesting Chl–protein complex (Shimoda et al. 2012), which could not be achieved by F-DVR.

Conversely, NADH dehydrogenase activity has been retained during the evolution from F-DVR to HCAR, though it is not clear whether the NADH dehydrogenase activity of F-DVR and HCAR participates in some cellular process. Furthermore, the NADH dehydrogenase activity of these enzymes is not related to Chl metabolism, which might be the reason why the NADH dehydrogenase activity has been retained over such long evolutionary time during which a new Chl metabolic pathway appeared. In the present study, we showed that F-DVR has many promiscuous activities involving different chemical reactions and different substrates. Accordingly, it is reasonable to assume that F-DVR has additional promiscuous activities that have not yet been identified. A large number of promiscuous reactions form many latent metabolic pathways, some of which might have become functional during evolution.

In summary, enzymes have many promiscuous activities, and some neutral activities are retained to maintain a high activity of the primary reaction. However, when a promiscuous activity becomes disadvantageous during evolution, the enzyme loses this activity; in contrast, when a promiscuous activity becomes important under certain environmental or cellular conditions, this activity will become the primary activity. These dynamic changes in primary and promiscuous activities drive pathway evolution.

**Materials and Methods**

**Expression and purification of recombinant DVR**

The coding regions of F-DVR derived from *Synechocystis* (Slr1923) and N-DVR derived from *P. tricornutum*
(XP_002184654) were cloned into pET-30a(+) (Novagen) using the NdeI and Xhol sites. The coding region of N-DVR from Arabidopsis (ATSG18660) was cloned into pCold ProS2 (TAKARA) at the NdeI and EcoRI sites. The primers used in the construction are listed in Supplementary Table S1. The expression plasmids were introduced into E. coli BL21 (DE3). DVR cloned into pCold ProS2 was expressed at 15°C for 24 h with 0.4 mM isopropyl-β-D-thiogalactopyranoside. During the induction of Synechocystis F-DVR and Arabidopsis HCAR, 100 μM ammonium ferric citrate was added into the medium. After incubation, the culture was harvested by centrifugation, and the collected cells were resuspended in buffer (25 mM Tris–HCl, pH 7.5, and 150 mM NaCl) and disrupted by sonication. The recombinant protein contained in the soluble fraction was applied to a nickel column (HisTrap HP, GE Healthcare), and the recombinant protein was eluted with a buffer containing 500 mM imidazole. The imidazole was removed from the purified DVR protein using a desalting column (HiTrap Desalting, GE Healthcare). Arabidopsis N-DVR was further purified using an ion exchange column: the recombinant protein was applied to an ion exchange column (HiTrap DEAE FF, GE Healthcare) equilibrated with 25 mM Tris–HCl (pH 7.5) buffer. The column was eluted with a linear gradient of the buffer containing 0.5 M NaCl for 5 min at a flow rate of 1 ml min⁻¹. The column chromatography for protein purification was performed using an ÄKTAprime plus system (GE Healthcare). When the E. coli lysate was used as the source of the enzyme, 30 ml of the culture was resuspended in 1 ml of BugBuster Protein Extraction Reagents (Novagen) containing 1 μl of benzonase. HCAR was prepared as reported previously (Shimoda et al. 2012). The purified protein and E. coli lysate were analyzed by SDS–PAGE (Supplementary Fig. S7) as reported previously (Meguro et al. 2011).

Preparation of Chl derivatives

DV-Chl was extracted from an slr1923-deficient Synechocystis mutant (Ito et al. 2008). HMChl a was obtained via the reduction of Chl b with NaBH₄ as reported previously (Shimoda et al. 2012). Chlde was prepared from Chl through hydrolysis with recombinant chlorophyllase (Tsuchiya et al. 1999), as reported previously (Shimoda et al. 2012). PChlide was synthesized by the chemical oxidation of Chlde with 2,3-dichloro-5,6-dicyanobenzoquinone (Shedalkar et al. 1991). Chdehyde was solubilized in 200 μl of diethyl ether, and 1 μl of 100 mM 2,3-dichloro-5,6-dicyanobenzoquinone solubilized in acetone was added. After 5 min, the reaction was stopped by the addition of 1 ml of water. The diethyl ether phase was washed with water several times to remove the unreacted 2,3-dichloro-5,6-dicyanobenzquinone, and the diethyl ether was evaporated using nitrogen gas. Chl c₂ was prepared from dinoflagellates (a kind gift from Professor T. Horiguchi, Hokkaido University). A Chl c₂ and Chl c₁ mixture was prepared from P. tricornutum, and Chl c was extracted with acetone from the harvested cells. Hexane was added to the acetone mixture to remove the hydrophobic Chl a. The Chl c contained in the aqueous phase was transferred to diethyl ether, and the diethyl ether was evaporated using nitrogen gas.

Enzyme assay

Purified recombinant DVR (10 μg) or a culture lysate containing expressed DVR (10 μl) was suspended in 50 μl of reaction buffer [25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 1 mM NADPH]. For the analysis of Synechocystis F-DVR and HCAR, 1 μl of spinach FNR (0.1 mg ml⁻¹, Sigma-Aldrich) and 1 μl of spinach Fd (1 mg ml⁻¹, Sigma-Aldrich) were added to the reaction buffer. The pigments were solubilized in acetone, and 1 μl of the solution was added to the reaction buffer. PChlide (150 pmol), Chl c₂ (300 pmol) and other Chl derivatives (500 pmol) were used for every reaction. The reaction mixtures were incubated for 15 min at 25°C and reactions were stopped by the addition of 200 μl of acetone. The pigments were analyzed by HPLC using a C8 column, as reported previously (Shimoda et al. 2012). When the culture lysate containing Synechocystis F-DVR was incubated with HMChlide a or Chlide b, the concentration of NADPH, FNR and Fd was four times higher than the standard conditions, and the reaction mixtures were incubated for 60 min.

Sequence analysis and phylogenetic analysis of DVR

A database search was performed using the National Center for Biotechnology Information database. The amino acid sequences were aligned using the ClustalW program (Thompson et al. 1994) in the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). For the phylogenetic analysis, the protein sequences were aligned using MEGA 5 software (Tamura et al. 2011) and the maximum likelihood bootstrap method (1,000 replicates).

NADH dehydrogenase activity assay

The NADH dehydrogenase activities of Synechocystis F-DVR and HCAR were measured in the presence of 1 mM potassium ferricyanide using 10 μg ml⁻¹ purified Synechocystis F-DVR or HCAR. The decrease in absorbance at 340 nm was monitored with a spectrophotometer (U-3310, Hitachi).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

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References


Evolution of a new chlorophyll metabolic pathway


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