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Note

Docking analysis of models for 4-hydroxy-3-methylbut-2-enyl diphosphate reductase and a ferredoxin from *Botryococcus braunii*, race B

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Abstract The green microalga *Botryococcus braunii* Showa, which produces large amounts of triterpene hydrocarbons, exclusively uses the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprenoid biosyntheses, and the terminal enzyme in this pathway, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), is regarded as a light-dependent key regulatory enzyme. In order to investigate the possible association of HDR and ferredoxin in this organism, we constructed tertiary structure models of *B. braunii* HDR (*Bb*HDR) and one of ferredoxin families in the alga, a photosynthetic electron transport F (*Bb*PETF)-like protein, by using counterparts from *E. coli* and *Chlamydomonas reinhardtii* as templates, respectively, and performed docking analysis of these two proteins. After docked models are superimposed onto their counterpart proteins in a non-photosynthetic organism, *Plasmodium falciparum*, the *Bb*PETF-like protein comes in contact with the backside of *Bb*HDR, which was defined in a previous report (Rekittke et al. 2013), and the distance of the two Fe–S centers is 14.7 Å. This distance is in almost the same level as that for *P. falicarum*, 12.6 Å. To our knowledge, this is the first model suggesting the possible association of HDR with a ferredoxin in O_2-evolving photosynthetic organisms.

Key words: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *Botryococcus braunii*, docking model, ferredoxin, photosynthetic electron transport F-like protein.

The colonial green microalga *Botryococcus braunii* B race can produce large amounts of triterpene hydrocarbons, namely botryococcenes and methylsqualenes. However, key regulatory systems that produce precursors for these compounds are yet to be elucidated. *B. braunii* uses the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway to provide universal precursors of isoprenoids to biosynthesize triterpenes (Sato et al. 2003). Though three isogenes of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), the enzyme catalyzing the initial step in the MEP pathway, have been cloned and enzymatic properties of corresponding recombinant proteins were studied, catalytic efficiencies of these recombinant proteins were not particularly superior to those of DXSs in other organisms (Matsushima et al. 2012). Therefore, further studies on enzymes catalyzing other steps in the MEP pathway are required to better understand the mechanism that supplies isoprenoid precursors to achieve efficient hydrocarbon production by this alga. Figure 1 shows the outline of metabolic pathway of isoprenoids in green algae. Using pyruvate and glyceraldehyde 3-phosphate (GAP), which are provided from Calvin cycle, the universal precursors of all isoprenoids, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are produced in the MEP pathway. 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) catalyzes the terminal reaction of the pathway. In higher plants, upregulation of gene expression for HDR resulted in a higher accumulation of carotenoids (Botella-Pavía et al. 2004). In the green alga *Dunaliella salina*, HDR is known to be a key enzyme for large accumulations of β-carotene in response to environmental cues (Ramos et al. 2009). As such reports, it can be estimated that *B. braunii* HDR (*Bb*HDR) might be involved in the regulation of isoprenoid production, resulting in hydrocarbon yield

Abbreviations: *Bb*, *Botryococcus braunii*; *Cr*, *Chlamydomonas reinhardtii*; *E. coli*, *Escherichia coli*; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; PETF, photosynthetic electron transport F protein; *Pf*, *Plasmodium falciparum*.

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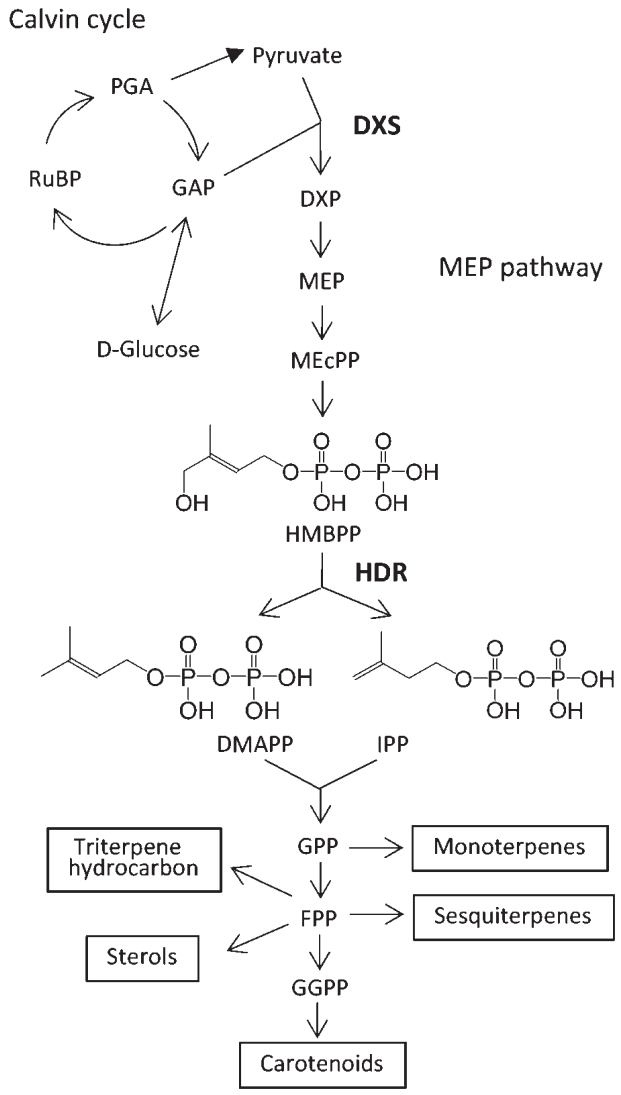

Figure 1. Outline of Calvin cycle and isoprenoid biosynthesis. From Calvin cycle, pyruvate and glyceraldehyde 3-phosphate (GAP) are supplied for 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. Using pyruvate and GAP as substrates, the first enzyme in MEP pathway, 1-deoxy-D-xylulose 5-phophate synthase (DXS), synthesizes 1-deoxy-D-xylulose 5-phophate (DXP). DXP is metabolized into (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) via MEP and then 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP). HMBPP is converted into isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), the last enzyme in the MEP pathway. HDR is reported to be activated by light. From IPP and DMAPP, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are generated sequentially. FPP is the precursors of triterpene hydrocarbon, sterol or sesquiterpene. GGPP is the metabolized into carotenoids.

(Uchida et al. 2018a).

Physiological analyses of MEP pathway enzymes have been well performed in a non-photosynthetic unicellular organism, *Plasmodium falciparum*. This organism is a protozoan parasite to cause malaria in humans. Inactivation of HDR in *P. falciparum* (*Pf*HDR) is expected to alleviate membrane sterol production, which would inhibit cell division. A previous report showed that *Pf*HDR was associated with an electron transport protein, ferredoxin (Röhrich et al. 2005).

Some recent studies have indicated that HDR is activated by light (Ershov 2007) and ferredoxin is transiently bound with photosystem I subunits in algae (Cashman et al. 2014). However, the concrete mechanisms of light regulation of this enzyme in photosynthetic organisms are yet to be revealed. In order to check the possible association of HDR with ferredoxin in *B. braunii*, we made models of *Bb*HDR and one of ferredoxins, photosynthetic electron transport F (PETF)-like protein and performed docking analyses for them. The present study might provide insight to reveal regulation of the biosynthesis of precursors for triterpenes in *B. braunii*.

The *Bb*HDR cDNA sequence information was retrieved from the DDBJ database with an accession number of LC090196. Entire region of *Bb*HDR amino acid sequence possessed similarity and identity to *Plasmodium falciparum* 3D7 sequence (CAD49005.1, 69% and 28%, respectively) and to *Escherichia coli* K12 sequence (P62623.1, 69% and 24%, respectively) (Uchida et al. 2018a). In correspondence to the conserved cysteines *in E. coli* HDR sequence, *Bb*HDR included three cysteines at the 140th, 232th and 363th residues (Uchida et al. 2018a). *Bb*HDR sequence was uploaded into the SWISS-MODEL server (https://swissmodel.expasy.org/) (Biasini et al. 2014), which was accessed on July 27, 2016, and the three-dimensional structure model was built using a crystal structure of HDR from *E. coli* (PDB ID: 3F7T) as a template. Configurations of above-mentioned cysteine residues in *Bb*HDR model and *E. coli* structure were well conserved (Supplemental data). Using PyMOL 1.7.6.0 (https://www.pymol.org/, accessed on July 6, 2016), the tertiary structures were represented.

In order to find a ferredoxin homolog in *Botryococcus braunii* for our docking analysis, a transcriptome database constructed from RNAseq data (Uchida et al. 2015) was mined with TBLASTN using the amino acid sequence of *Chlamydomonas reinhardtii* photosynthetic electron transport F protein (*Cr*PETF, protein ID 147787 in the *Chlamydomonas* genome v4.0) as a query. The reason why the PETF was selected as the representative from various members of ferredoxin is because it is coupled with the photosynthetic system I complex (Knaff and Hirasawa 1991). The highest score hit of a *B. braunii* contig, which encoded a putative protein with 81 amino acid residues, was obtained with an *E*-value of 1e-033. The cDNA nucleotide sequence for this contig was registered in DDBJ with the accession number of LC202849. The encoded protein was referred to as *B. braunii* PETF-like (*Bb*PETF-like) protein.

The *Bb*PETF-like protein was uploaded to SWISS-MODEL and a three-dimensional model was built using

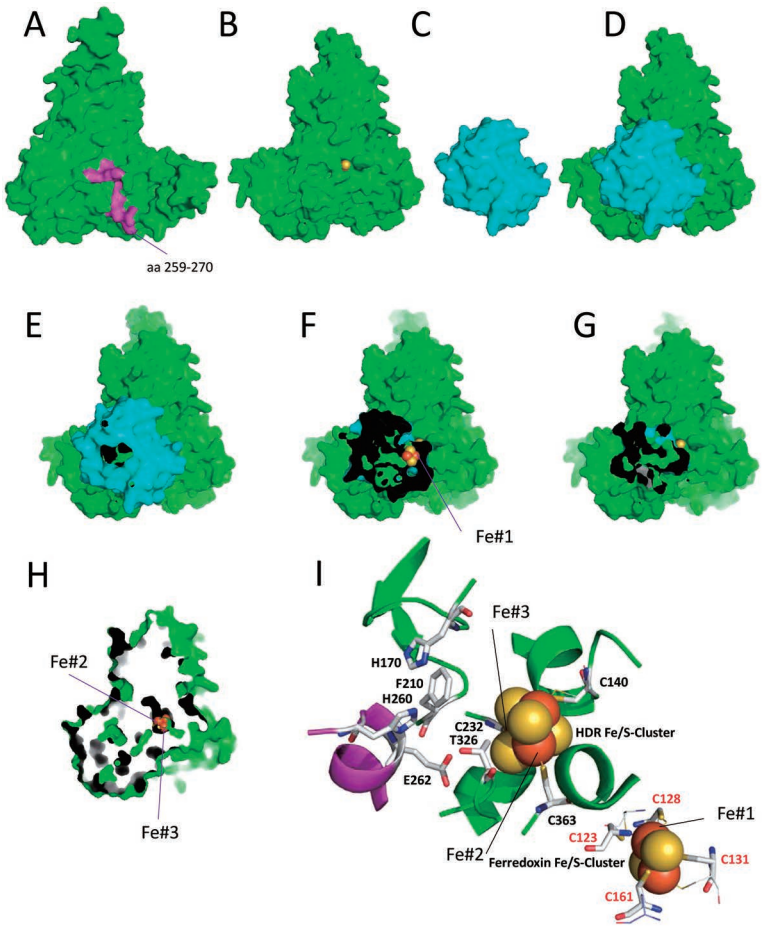

Figure 2. Docking of models of *Bb*HDR and *Bb*PETF-like protein. (A) An image of the surface (green) of the front-side of *Bb*HDR, which was defined by Rekittke et al. (2013), emphasizing the helix-forming region between amino residues 259–270 with cyan color. (B) The image from (A) was turned 180° around the Y axis (green), showing the backside of *Bb*HDR over which the *Pf*HDR-derived S atom (yellow) of the Fe–S was drawn. (C) *Bb*PETF-like protein (cyan). (D) A docked image of *Bb*HDR and *Bb*PETF-like protein analyzed using ZDOCK. (E–H) Image (D) was trimmed successively. Fe atom number 1 (Fe#1, red) was derived from *Pf*FER and was located inside *Bb*PETF-like protein (cyan), while Fe#2 (red) and Fe#3 (red) were derived from *Pf*HDR and were located inside *Bb*HDR (green). Fe#1 is located in closer proximity to HDR Fe–S than the other Fe atom, and is distanced 14.7 Å and 14.8 Å apart from Fe#2 and Fe#3, respectively. (I) An enlarged panel which shows the two Fe–S clusters in a single image. Residues of *Bb*HDR (black letters) and *Bb*PETF-like protein (red letters) are indicated as sticks, while conserved cysteines of *Pf*HDR (PDB: 4N7B) and *Pf*FER (PDB: 1iue) are overlaid as lines. Fe–S clusters of *Pf*HDR and *Pf*FER, which are shown as spheres, are superposed onto the docked *B. braunii* models. T326 in *Bb*HDR is depicted in analogy to Rekittke et al. (2013), while T325 in *Bb*HDR is mentioned in Uchida et al. (2018a) in reference to Gräwert et al. (2009).

*Cr*PETF as a template. The models for *Bb*PETF-like protein and *Bb*HDR were then uploaded to ZDOCK 3.0.2 (http://zdock.umassmed.edu/, which was accessed on December 8, 2016) (Pierce et al. 2011), from which we obtained 10 predicted protein complexes. In these complexes, the root-mean-square deviations (RMSDs) between a complex and *Bb*HDR were all the same. The same result was also obtained in RMSDs between complexes and the *Bb*PETF-like protein. Using the UCSF chimera software (https://www.cgl.ucsf.edu/chimera/, which was accessed on December 6, 2016) (Pettersen et al. 2004), models for *Bb*HDR or *Bb*PETF-like were matched over complexes. *Plasmodium falciparum*-derived HDR (*Pf*HDR) and ferredoxin including their Fe–S centers were further aligned onto each *B braunii* model and the distances between these Fe–S centers were calculated using Pymol.

In six model complexes among the ten that were analyzed in ZDOCK, *Bb*PETF came in contact with the front-side (Rekittke et al. 2013) of *Bb*HDR, where helix 2 in domain 2 was observed (Figure 2A). In these complexes, the distances between the Fe atoms of HDR and ferredoxin ranged from 21.9 to 32.0 Å. In the other four models among the ten complexes, backside of *Bb*HDR (Figure 2B) was docked with *Bb*PETF (Figure 2C) and the distances between the Fe atoms of HDR and ferredoxin were 14.7 Å, 14.8 Å, 16.6 Å, and 18.0 Å, respectively. Using the model complex exhibiting the shortest distance between intra-Fe–S centers with a distance of 14.7 Å (Figure 2D), docking planes in reference to the Fe–S centers were observed in the trimmed models (Figure 2E–H). Two Fe–S centers

for the *Bb*HDR and *Bb*PETF-like protein models that were superimposed with *P. falciparum* structures were observed in the vicinity of the docking points, where marginal projections of the *Bb*PETF-like protein were inserted into *Bb*HDR cavities around the S atom of its Fe–S center (Figure 2G). Interestingly, two Fe atoms (Fe#2 and Fe#3 in Figure 2H, I) of the above-mentioned HDR were situated by an Fe atom in the docked PETF-like protein (Fe#1 in Figure 2F, I) at almost equal distances, at 14.7 Å and 14.8 Å, respectively.

In the apicomplexan *Plasmodium falciparum*, which does not possess a photosynthetic ability, docking analysis of HDR and ferredoxin has previously been reported (Rekittke et al. 2013), and the redox recycling system of HDR using ferredoxin has also been confirmed (Röhrich et al. 2005). Our docking model of *Bb*HDR and the *Bb*PETF-like protein, which were superimposed with corresponding protein structures of *P. falciparum*, revealed that the distance between two Fe–S centers of *Bb*HDR and *Bb*PETF-like protein as a functional ferredoxin was estimated to be 14.7 Å (Figure 2I). To our knowledge, this is the first report of the docking analysis of HDR and PETF-like protein from an O_2-evolving photosynthetic algae, and perhaps even among all O_2-evolving photosynthetic organisms. Our initial docking analysis of *Bb*HDR and *Bb*PETF-like protein could thus serve as key models for designing structure–activity analysis of key regulatory enzymes for terpenoid precursor biosynthesis, and possibly light regulated biosynthesis of various terpenoids. However, expression of *Bb*HDR and *Bb*PETF in bacterial system, purification and co-crystallization of these proteins is indispensable in order to check possible binding of these proteins. This future study may also cast insight to reveal the mechanism of light regulation of isoprenoid biosynthesis, which may determine hydrocarbon yield in *B. braunii*.

Our results coincide with the phylogeny of kinetoplastida including *P. falciparum*, which suggests its ancestral organism might be a photosynthetic alga (Moore et al. 2008). The intra-Fe distance in the complex of HDR and ferredoxin of *P. falciparum* was 12.6 Å (Rekittke et al. 2013). The almost same level of intra-Fe distance observed above-mentioned *B. braunii* models might suggest analogy in the redox recycling of enzymes between protozoan apicoplast and algal chloroplast.

In eukaryotic O_2-evolving organisms, ferredoxin is localized in the chloroplast (Sétif et al. 2002). Colocalization of photosynthetic ferredoxin and MEP pathway enzymes in chloroplast makes the interaction between HDR and ferredoxin reasonable. A previous report (Okada and Hase 2005) indicated that HMBDP synthase ((*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase, HDS), which catalyzes the preceding step of HDR in the MEP pathway (Uchida et al. 2018b), is activated by photoreduction and bind with ferredoxin in a cyanobacterium, *Thermocynechococcus elongatus*. HDS in the higher plant can also be activated by photoreduction (Seemann et al. 2006). These reports suggest that ferredoxin can be involved in the two successive key-regulatory enzymatic reactions in MEP pathway of wide range of photosynthetic organisms. Apicomplexans such as *P. falciparum* possess ferredoxin in the apicoplast. Since these organisms are thought to be derived from photosynthetic alga, apicomplexans might also retain HDR and ferredoxin in the descendent organelle.

Though several enzymes in eukaryotic oxygen-evolving photosynthetic organisms and some bacteria use ferredoxin as a redox partner (Knaff and Hirasawa 1991), this role can be taken by flavodoxin in most bacterial reactions (Rogers 1987). This fact might suggest that HDR can also interact with flavodoxin, though flavodoxin and ferredoxin do not share common tertiary folds (Ullmann et al. 2000). This possibility should be investigated in the future studies.

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