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Completion of biosynthetic pathways for bacteriochlorophyll *g* in *Heliobacterium modesticaldum*: The C8-ethylidene group formation

Yusuke Tsukatani [a,b,*], Haruki Yamamoto [c,1], Tadashi Mizoguchi [a], Yuichi Fujita [c], Hitoshi Tamiaki [a,*]

[a] Graduate School of Life Sciences, Ritsumeikan University, Shiga 525-8577, Japan
[b] PRESTO, Japan Science and Technology Agency, Saitama 332-0012, Japan
[c] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

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ABSTRACT

Heliobacteria have the simplest photosynthetic apparatus, i.e., a type-I reaction center lacking a peripheral light-harvesting complex. Bacteriochlorophyll (BChl) *g* molecules are bound to the reaction center complex and work both as special-pair and antenna pigments. The C8-ethylidene group formation for BChl *g* is the last missing link in biosynthetic pathways for bacterial special-pair pigments, which include BChls *a* and *b* as well. Here, we report that chlorophyllide *a* oxidoreductase (COR) of *Heliobacterium modesticaldum* catalyzes the C8-ethylidene formation from 8-vinyl-chlorophyllide *a*, producing bacteriochlorophyllide *g*, the direct precursor for BChl *g* without the farnesyl tail. The finding led to plausible biosynthetic pathways for 8^1-hydroxy-chlorophyll *a*, a primary electron acceptor from the special pair in heliobacterial reaction centers. Proposed catalytic mechanisms on hydrogenation reaction of the ethylidene synthase-type CORs are also discussed.

1. Introduction

Heliobacteria are phototrophic organisms in the phylum *Firmicutes*, one of six phyla that contain microorganisms capable of conducting photosynthesis. It is a characteristic of heliobacteria that they do not have a peripheral light-harvesting complex, unlike phototrophic organisms in the other five phyla. They capture sunlight energy via pigments directly bound to a type-I reaction center (RC) complex. The RC complex of heliobacteria contains 22 ~ 40 bacteriochlorophyll (BChl) *g* and two 8^1-hydroxy-chlorophyll (8^1-OH-Chl) *a* molecules [1–5]. BChl *g* operates as a primary electron donor called a special pair, as well as a light-harvesting antenna pigment [2,4,5]. 8^1-OH-Chl *a* is assumed to function as a primary electron acceptor from the special pair in the RC complex [3–5].

Naturally occurring BChls are grouped into seven species, BChls *a* ~ *g* [6,7]. However, BChls *c* ~ *f* are actually based on chlorin π-systems, not bacteriochlorins. BChls *a*/*b*/*g* are therefore the "true" bacteriochlorin pigments working as the special pair in RCs. Differences in chemical structures of the three bacterial RC pigments (BChls *a*/*b*/*g*) occur at the C3- and C8-substituents, except the C17-esterifying tails (see Supplementary Fig. S1). BChl *b* and BChl *g* have the C8-ethylidene group, whereas BChl *a* has an ethyl group at the C8 position.

Recently, we demonstrated that biosynthetic pathways for BChl *a* and BChl *b* are branched at the step catalyzed by chlorophyllide *a* oxidoreductase (COR) [8]. COR in BChl *a*-producing *Rhodobacter capsulatus* catalyzes the C7═C8 double bond reduction in chlorophyllide (Chlide) *a*, resulting in the formation of a bacteriochlorin ring which has the C8-ethyl group [8,9]. On the other hand, COR in BChl *b*-producing *Blastochloris viridis* reacts with 8-vinyl-chlorophyllide (8V-Chlide) *a* and synthesizes a bacteriochlorin with the C8-ethylidene group [8]. The distinct substrate recognitions and catalytic reactions of two types of CORs correlate with the fact that BChl *b*-producing bacteria lack a conventional 8-vinyl reductase (BciA) and another type of 8-vinyl reductase found in some cyanobacteria (BciB). The substrate for COR in BChl *b*-producing bacteria therefore has an un-reduced C8-vinyl group, which is 8V-Chlide *a*.

The genome sequence of *Heliobacterium modesticaldum* has already been published, and it was reported that this bacterium also lacks a gene for the BciA 8-vinyl reductase [10]. This suggests that COR from heliobacteria also contributes to the conversion of the C8-vinyl to the C8-ethylidene group for BChl *g*. Interestingly, while the CORs of BChl *a*- and *b*-producing bacteria are highly conserved, with up to 89% similarity, heliobacterial COR demonstrates relatively low primary structure similarities with these enzymes [8]. The production of 8^1-OH-Chl *a* in addition to BChl *g* complicates heliobacterial pigment biosynthesis. It is still unknown where biosynthetic pathways for BChl *g* and

Abbreviations: BChl, bacteriochlorophyll; BChlide, bacteriochlorophyllide; BPheoide, Bacteriopheophorbide; 81-OH-Chl, 81-hydroxy-chlorophyll; Chlide, chlorophyllide; COR, chlorophyllide *a* oxidoreductase; Pheoide, pheophorbide; RC, reaction center

* Corresponding authors at: Graduate School of Life Sciences, Ritsumeikan University, Shiga 525-8577, Japan. Fax: +81 77 561 2659.
E-mail addresses: yusuke-t@fc.ritsumei.ac.jp (Y. Tsukatani), tamiaki@fc.ritsumei.ac.jp (H. Tamiaki).
[1] Present address: Institute for Protein Research, Osaka University, Osaka 565-0871, Japan.

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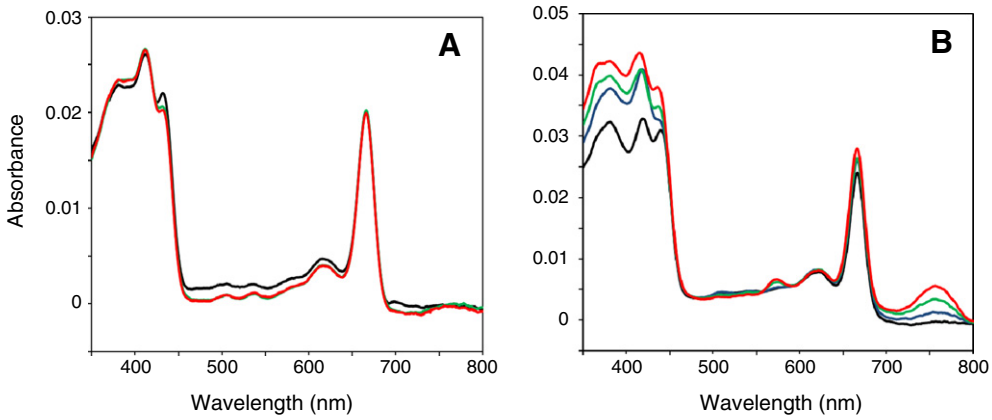

Fig. 1. Absorption spectral changes of enzymatic assay mixtures of *H. modesticaldum* COR with (A) Chlide *a* and (B) 8V-Chlide *a*. Absorption spectra were recorded in 80% acetone (20% aqueous buffer). Absorption spectra at 0, 30, 60, and 90 min are shown in black, blue, green, and red lines, respectively.

8^1-OH-Chl *a* branch from the core biosynthetic pathway conserved in all photosynthetic organisms. In this study, we demonstrate that COR of *H. modesticaldum*, overexpressed and purified from *Escherichia coli*, catalyzes the formation of the 8-ethylidene group from the 8-vinyl group. In addition, we propose plausible biosynthetic pathways for 8^1-OH-Chl *a*.

2. Materials and methods

2.1. Cloning of genes encoding COR from H. modesticaldum and purification of the COR

Cultures of *H. modesticaldum* were kindly provided by Dr. Hirozo Oh-oka (Osaka University). The *bchX* gene for the BchX subunit of COR was amplified from the *H. modesticaldum* genomic DNA by PCR using primers, Hm-X-f1 (5′-ATGGTA*GGTCTC*AGCGCCTCGGAAAAGAA TTTCTACGCCGTC-3′) and Hm-X-r1 (5′-ATGGTA*GGTCTC*ATATCATCGC TGAAACATCTCCTCCGCC-3′; *Bsa*I restriction sites are italicized). The *bchY* and the flanking *bchZ* genes, which encode for the BchY and BchZ subunits of *H. modesticaldum* COR, respectively, were amplified together by PCR using primers Hm-Y-f1 (5′- ATGGTA*GGTCTC*AGCG CCGAACCGATCAAGTCCATCAAGCT-3′) and Hm-Z-r1 (5′- ATGGTA*GG TCTC*ATATCATCGTTTGAACCGGTTGTAGACCT-3′; *Bsa*I restriction sites are italicized). The PCR reactions were performed with KOD-plus polymerase (Toyobo, Osaka, Japan). The amplified DNA fragments were excised from agarose gels, purified using a NucleoSpin Extract II kit (Macherey-Nagel, Duren, Germany), and digested with *Bsa*I. The digested DNA fragments containing *bchX* and *bchY-bchZ* genes were cloned into the *Bsa*I restriction sites of the expression vector

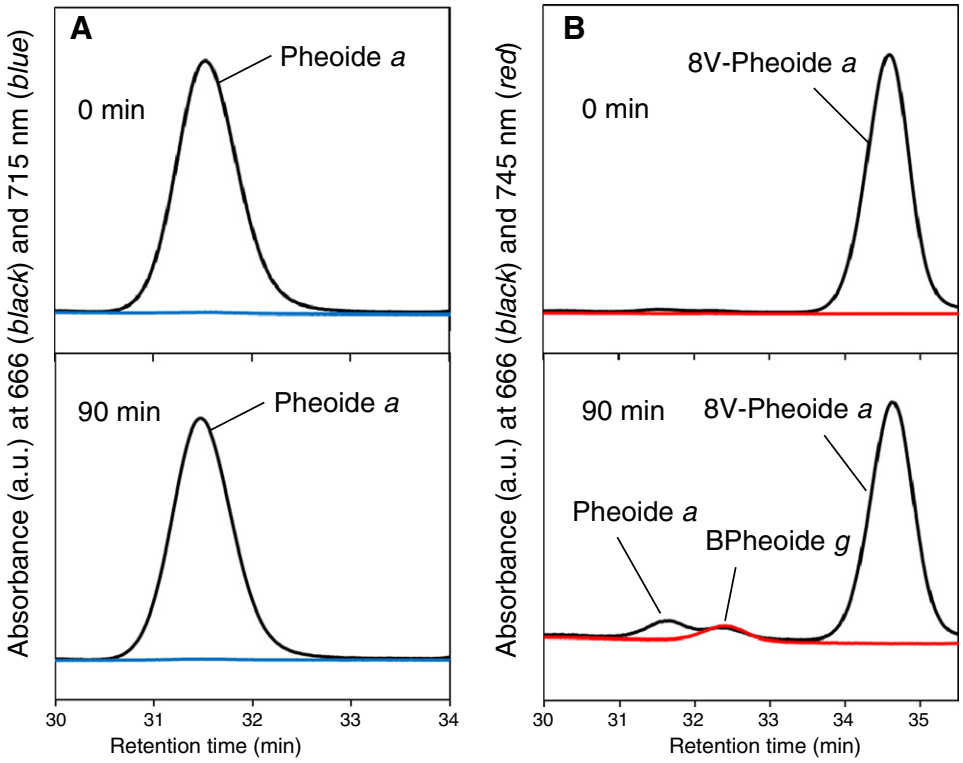

Fig. 2. Reverse-phase HPLC analysis of pigments formed in the COR assays after the treatment with 10% acetic acid (pheophytinization). *H. modesticaldum* COR was mixed with Chlide *a* (A) and 8V-Chlide *a* (B). Elution profiles were measured at 666 nm (black line) to monitor Chlide/Pheoide pigments, and 715 nm (blue line) and 745 nm (red line) to monitor BChlide/BPheoide pigments. Upper and lower panels of columns represent elution profiles before and after 90-min incubation, respectively.

pASK-IBA5plus (IBA, Göttingen, Germany), yielding plasmids pA5-HmX1 and pA5-HmYZ-i1, respectively. Entire sequences of *bchX*, *bchY*, and *bchZ* genes in these plasmids were confirmed by Sanger sequencing. The plasmids were transformed into the *E. coli* strain Rosetta2 (Novagen, Darmstadt, Germany) to overexpress BchX and BchY-BchZ proteins of *H. modesticaldum* as fusion proteins with an affinity tag (Strep-tag II) under the control of the tet promoter. Purification of the proteins from 2.4 L cultures was performed as described previously [8].

2.2. Enzymatic assay for COR and analysis of assay products

Assays for COR activities and measurements of absorption spectra were performed as described in the previous studies [8,9]. Assay mixtures were prepared and incubated at 34 °C in an anaerobic chamber. Substrates for the assays, Chlide *a* and 8V-Chlide *a*, were prepared as described previously [8]. After the incubation, assay products were identified by LC-MS measurements according to the previous study [8] with slight modifications: the Synergi Hydro-RP C18 reverse-phase column (2.5 μm, 2.0 × 100 mm) was used, and the flow rate was 0.125 mL/min.

3. Results and discussion

COR is a member of the nitrogenase-like enzyme family [9,11]. Like other members of the family (nitrogenases and dark-operative protochlorophyllide oxidoreductase), COR is composed of three subunits, BchX, BchY, and BchZ. These three proteins of *H. modesticaldum* were overexpressed in *E. coli* cells and purified by affinity chromatography using Strep-tactin sepharose (Supplementary Fig. S2). As is the case with the CORs previously characterized [8,9], BchY and BchZ from *H. modesticaldum* form a stable YZ-complex component, while BchX was purified as a single polypeptide (X-component, a probable BchX dimer). The two components were mixed with Chlide *a* and 8V-Chlide *a*, and the mixtures were assayed by measuring absorption spectral changes as described previously [8,9]. When COR of *H. modesticaldum* (hereafter called HmCOR) was mixed with Chlide

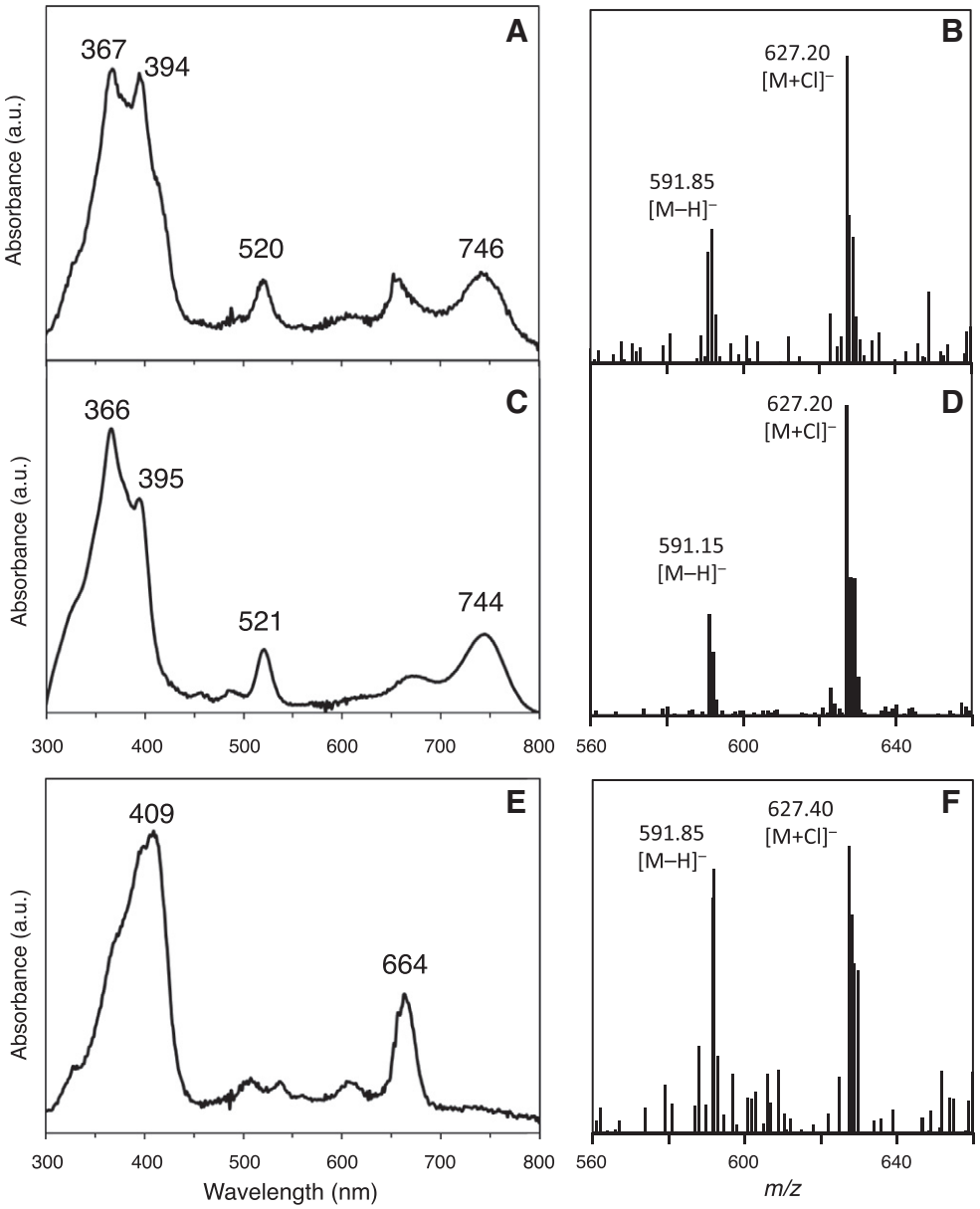

Fig. 3. Identification of products from the assay mixture of HmCOR and 8V-Chlide *a*. In-line absorption (A) and mass spectra (B) of the 32.4-min elution peak in Fig. 2B are shown. Panels (C) and (D) represent in-line absorption and mass spectra of the BPheoide *g* standard, respectively, and Panels (E) and (F) represent in-line absorption and mass spectra of the 31.5-min elution peak in Fig. 2B, respectively.

a, no product peak was observed at the 700–750 nm region after a 90-min incubation (Fig. 1A), suggesting that Chlide *a* is not a suitable substrate for HmCOR. On the other hand, when HmCOR was mixed with 8V-Chlide *a*, a new peak at 756 nm appeared (Fig. 1B). This peak appearance at 756 nm was reminiscent of the phenomenon when COR of BChl *b*-producing *B. viridis* (BvCOR) was mixed with 8V-Chlide *a* [8], suggesting that HmCOR also catalyzed the formation of the C8-ethylidene group on bacteriochlorophyllide (BChlide) *g*. To further identify the product showing the absorption peak at 756 nm, the assay mixtures were analyzed by LC-MS. Prior to the injection to LC-MS, these mixtures were treated with l0% acetic acid in order to increase separation capacities for pigments on the LC-MS measurements and improve the stability of assay products. Therefore, Chlide and BChlide pigments in mixtures were demetallated to the corresponding compounds, pheophorbide (Pheoide) and bacteriopheophorbide (BPheoide), respectively. The assay mixture of HmCOR and Chlide *a* showed only an elution peak of the demetallated substrate, Pheoide *a* even after the 90-min incubation (Fig. 2A). On the other hand, when the assay mixture of HmCOR and 8V-Chlide *a* was analyzed by LC-MS (Fig. 2B), two new peaks were observed at about 31.5 min and 32.4 min in addition to the demetallated substrate (8V-Pheoide *a*) peak at 34.5 min after the 90-min incubation. The in-line absorption spectrum of the new product peak eluting at 32.4 min (Fig. 3A) was almost identical to that of the demetallated BChlide *g* standard (BPheoide *g*; Fig. 3C). In addition, the 32.4-min elution product showed the masses of $m/z = 591.85$ as $[M\text{-}H]^-$ and $m/z = 627.20$ as $[M+Cl]^-$, which were almost identical to those of the BPheoide *g* standard (Fig. 3B vs D). These indicate that the product from the assay mixture of HmCOR and 8V-Chlide *a* was BChlide *g*, and that HmCOR catalyzed the conversion of 8V-Chlide *a* to BChlide *g*, which has the C8-ethylidene group.

The second product peak at 31.5 min (Fig. 2B) eluted at the same time as Pheoide *a* (Fig. 2A) and the in-line absorption spectrum of this product peak (Fig. 3E) is similar to that of a Pheoide-like pigment, but demonstrating a Soret peak at 409 nm, blue-shifted from the 418 nm peak of 8V-Pheoide *a*. The Soret peak of Chlide/Pheoide *a* demonstrates an approximate 10 nm blue-shift when compared to that of 8V-Chlide/8V-Pheoide *a* [12,13]. The Pheoide-like product showed the masses of $m/z = 591.85$ as $[M\text{-}H]^-$ and $m/z = 627.40$ as [M+Cl]~ (Fig. 3F), which were almost identical to those of Pheoide *a*. These established that the fraction eluting at 31.5 nm was Pheoide *a*, demetallated Chlide *a*. One possible explanation for the presence of Chlide *a* in the assay mixture using 8V-Chlide *a* was isomerization from BChlide *g*. It was reported that the isomerization from BChlide *g* to Chlide *a* (isomerization of the C8═C8^1 double bond to the C7═C8 double bond) was relatively easily induced by light, heat, and/or acidic environments [14–16]. We therefore suggest that Chlide *a* in the assay mixture was a by-product from the product BChlide *g*. In the previous study using BvCOR, such a by-product of Chlide *a* was not observed despite the same procedure following the assay [8]. One possibility is that HmCOR might release the product BChlide *g* earlier than BvCOR, in order to enable the enzyme responsible for the formation of 8^1-OH-Chl *a* to access BChlide *g* [Fig. 4, gray arrow (ii)]. Free BChlide *g* in the assay mixture using HmCOR of this study might tend to be isomerized to Chlide *a* during the procedure after the assay.

Although the mechanism for the formation of the C8-hydroxy group of 8^1-OH-Chl *a* is unknown, there are now two possible routes for the biosynthesis of 8^1-OH-Chlide *a*, the precursor of the mature pigment lacking the farnesyl tail (Fig. 4). One leads from 8V-Chlide *a* by Markovnikov-type hydration of the C8-vinyl group [Fig. 4, gray arrow (i)], and the other from BChlide *g* by 7-dehydrogenation and 8^1-hydroxygenation [Fig. 4, gray arrow (ii)]. In BChl *a* biosynthesis,

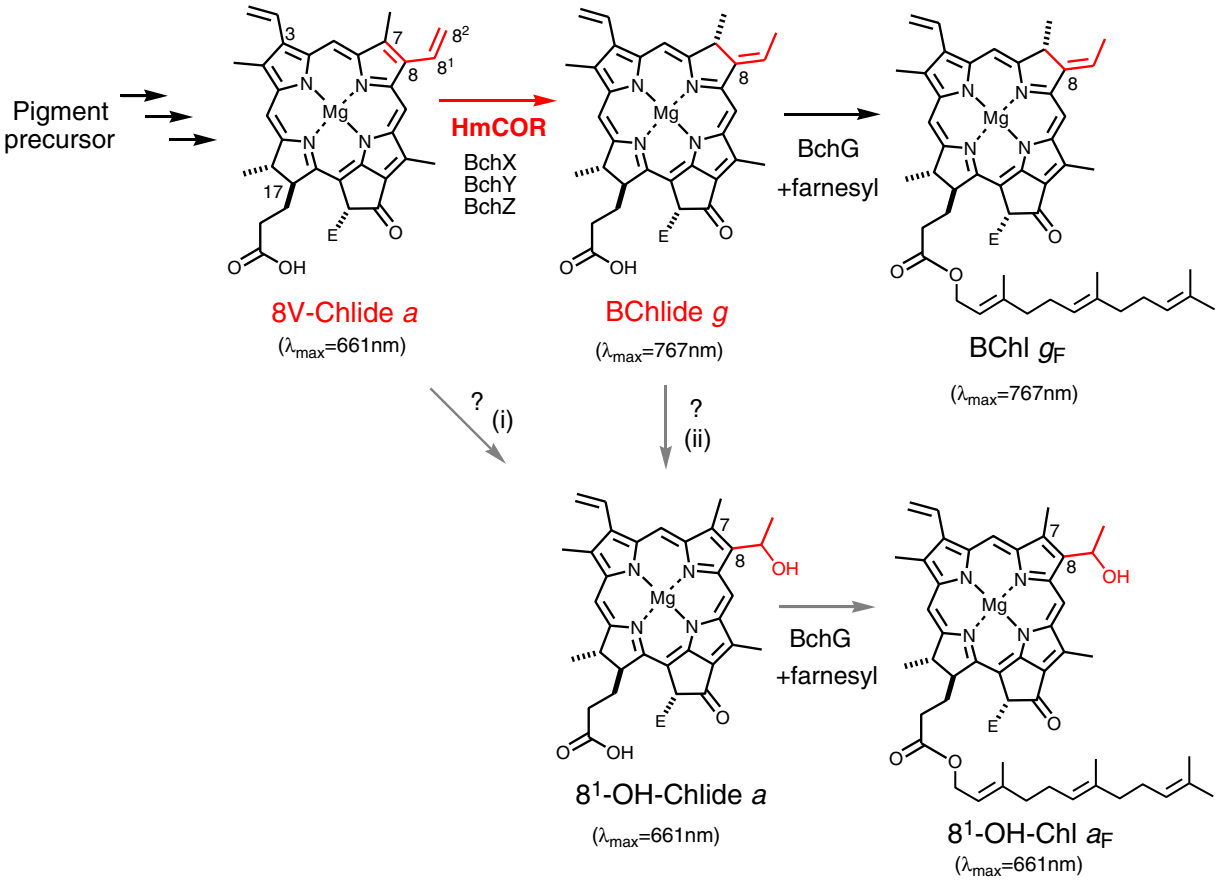

Fig. 4. Late biosynthetic pathways for BChl *g* and possible biosynthetic pathways for 8^1-OH-Chl *a*. The presented λ_{max} peak positions are values obtained in diethyl ether. E═$COOCH_3$.

the C3-(1-hydroxyethyl) group is formed through hydration of the C3-vinyl group by BchF. A BLAST search using BchF (Markovnikov-type C3-vinyl hydratase) of *Rhodobacter sphaeroides* as a query showed that *H. modesticaldum* contained no gene for BchF-type hydratase, even when the *E*-value threshold was set at $<10^{-2}$. Heliobacteria might have a novel C8-vinyl specific hydratase. Otherwise, 8^1-OH-Chlide *a* biosynthesis might branch from BChlide *g*.

Supplementary Fig. S3 shows amino acid sequence alignments of BchX, BchY, and BchZ proteins from *H. modesticaldum* and representative species included in the bacterial phyla *Proteobacteria*, *Chloroflexi*, *Chlorobi*, and *Acidobacteria*. In these bacteria, *H. modesticaldum*, *B. viridis*, and probably also *Thioflavicoccus mobilis* (a BChl *b*-producing proteobacterium) have CORs with the ethylidene synthase-type function [8]. There is no specific amino acid sequence region commonly conserved in any COR subunits of these three bacteria. To convert 8V-Chlide *a* to BChlide *g*, two hydrogen atoms are required for the 1,4-*trans*-hydrogenation at the C7,8^2 positions. Although it is unknown whether both of these hydrogen atoms are provided by amino acids surrounding the catalytic site, amino acid residues potentially donating protons to the carbons of 8V-Chlide *a* might be different from those of COR from BChl *a*-producing bacteria and conserved between BChl *b*-producing proteobacteria and BChl *g*-producing heliobacteria. However, it would be difficult to identify the amino acid residues by the sequence alignments since these bacteria are phylogenetically distantly related. By analogy to other members in the nitrogenase-like enzyme family, BchX is assumed to be the electron-donating component, and the YZ-component consisting of BchY and BchZ would play a role as the catalytic, substrate-binding proteins [9,11,17,18]. However, it is still unknown whether the amino acid residues crucial for the hydrogenation reaction are provided by either BchY or BchZ, or by both of these proteins. Crystal structure studies on CORs of these bacteria and in vitro reconstitution experiments using heterologous BchYZ proteins are in progress to answer this question.

4. Conclusion

All genes encoding enzymes responsible for BChls *a* and *b* biosynthesis have already been assigned [6,8,19,20], although activities of all the enzymes have not been clarified. The C8-ethylidene formation for BChl *g* biosynthesis was the missing link for the biosynthetic pathways for special-pair pigments in bacteria. In this study, we demonstrated that COR catalyzes the formation of the C8-ethylidene group, thus all of the genes encoding enzymes involved in BChl *g* biosynthesis have been assigned.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.06.007.

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