Non-enzymatic conversion of chlorophyll-\(a\) into chlorophyll-\(d\) in vitro: A model oxidation pathway for chlorophyll-\(d\) biosynthesis

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**A B S T R A C T**

Chlorophyll-\(a\) (Chl-\(a\)) was readily converted into Chl-\(d\) under mild conditions without any enzymes. Treatment of Chl-\(a\) dissolved in dry tetrahydrofuran (THF) with thiophenol and acetic acid at room temperature successfully produced Chl-\(d\) in 31% yield. During the acidic oxidation, removal of the central magnesium, pheophytinization, was sufficiently suppressed. This mild pathway can give insights into the yet unidentified Chl-\(d\) biosynthesis.

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**1. Introduction**

A series of chlorophylls (Chls) possessing a formyl group, Chl-\(b\), Chl-\(d\), Chl-\(f\) and BChl-\(e\), have been found in photosynthetic organisms to date (Fig. 1) [1–5]. Chl-\(b\) and BChl-\(e\) have a formyl group at the C7-position, and function as an antenna pigment in higher plants and green photosynthetic bacteria, respectively. Chl-\(f\) bearing the C2-formyl group and isolated from stromatolites, has been reported very recently and its function is unknown. Among these chlorophylls, Chl-\(d\), found in the cyanobacterium *Acaryochloris (A.) marina*, is quite unique, as it functions as a major photosynthetic pigment not only in the light-harvesting antennas (LHs) but also in the reaction centers (RCs) [6]. The Chl-\(d\)-containing LHs and RCs show close homology to the conventional Chl-\(a\)-type ones, fine-tuning their excitation energy levels and redox properties due to the structural difference between Chl-\(d\) (3-CHO) and Chl-\(a\) (3-CH=CH\(_2\)). Biosynthesis of such an exotic pigment is of much interest in view of its adaptation and evolution of photosynthetic supramolecular systems.

The pathway and reaction mechanism of Chl-\(d\) biosynthesis have not yet been elucidated. Chl-\(d\) (or chlorophyllide-\(d\)) is thought to be derived from Chl-\(a\) (or chlorophyllide-\(a\)) on the basis of genome analysis of *A. marina* [7]: oxidative cleavage of the C3-vinyl group of the latter to the C3-formyl group of the former. The oxygen atom of the C3-formyl group would come from molecular oxygen [8]. An enzyme for such an oxidation in the biosynthesis of Chl-\(d\) may be P450-type [9], while Chl-\(b\) bearing the C7-formyl group is synthesized from Chl-\(a\) with Chl-\(a\) oxygenase (CAO) [10]. The C7-methyl group of Chl-\(a\) is converted into the formyl group via its 7-hydroxymethyl intermediate. Chl-\(d\) would be synthesized through a pathway different from that of Chl-\(b\), because the C3-formyl group of Chl-\(d\) has to be derived from the C3-vinyl group with a loss of one carbon atom.

We have recently found that methyl pyropheophorbide-\(a\) (PPhe-\(aM\), see Fig. 1) was converted readily into methyl pyropheophorbide-\(d\) (PPhe-\(dM\)) in the presence of a thiol and an acid at room temperature in vitro [11]. This novel one-pot oxidation required neither hazardous reagents such as KMnO\(_4\), OsO\(_4\) and O\(_3\) [5,12], nor any enzymes [13]. Here we report that naturally occurring Chl-\(a\), an acid-sensitive Mg-chlorin, was successfully transformed into Chl-\(d\) in substantial yield under modified conditions. The mild and efficient monooxidation can give insight into the yet unidentified biosynthetic pathway of Chl-\(d\) in *A. marina*. 

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2. Materials and methods

Chl-α was extracted from spinach and purified using a normal-phase HPLC as described previously [14] (Column: Senshu-Pak 5251N (200 × 250 mm), pump: JASCO PU-2089, detector: JASCO MD-2018, eluent: hexane/2-propanol/methanol = 100/8/4, flow rate: 5 mL/min), room temperature). Chl-α (10 μmol) was dissolved in dry tetrahydrofuran (THF, 3 mL) and the solution was chilled in an ice-water bath. Thiophenol (PhSH, 5 equiv.) was added to this solution and stirred for 45 min. Then, acetic acid (AcOH, 1 equiv.) was added to the mixture, and stirred for 3–4 h at room temperature. The reaction mixture was diluted with chloroform, washed with aqueous 5% NaHCO₃ and brine, and dried over Na₂SO₄, followed by evaporation of the solvent in vacuo. The residue was purified by the above normal-phase HPLC and analyzed by ¹H NMR (Varian VNMRS-500) and UV/VIS spectroscopies (JASCO V-550) as well as MALDI-TOF-MS (Bruker autoflex II) and HPLC-MS spectrometries (Column: Cosmosil 5SL-II (3.0 V-550) as well as MALDI-TOF-MS (Bruker autoflex II) and HPLC-MS spectrometries (Column: Cosmosil 5SL-II (3.0 V-550), pump: Shimadzu LC-20AD, detector: Shimadzu LCMS-2010EV and SPD-M20, eluent: hexane/2-propanol/methanol = 100/0.7/0.3, flow rate: 0.5 mL/min, 30 °C). Conversion yields were determined by ¹H NMR measurements of the reaction mixtures. Reagents were purchased from Kanto Chemical Co., Inc.

3. Results and discussion

Naturally occurring pheophytin-α (Pheo-α, see Fig. 1), the primary electron acceptor of RC in photochemical system II, was transformed into Pheo-d according to the reported procedures (68% yield in methanol) [11]. When Pheo-α (containing 10% of the C13³-epimer, Pheo-α’) was reacted with PhSH (5 equiv.) and p-toluenesulfonic acid (TsOH, 5 equiv.) in methanol at room temperature for 15 h, Pheo-d/α’ was obtained successfully in a good yield (74%, as a C3-acetal form that was readily transformed into Pheo-d by an aqueous diluted acid). During the reaction, C13³-epimerization between Pheo-d and Pheo-d’ reached equilibrium (Pheo-d/α’ = 88/12). Since A. marina does not have Pheo-d but Pheo-α as the primary electron acceptor of its photosystem II reaction center [15–17], the above result suggests that the central Mg atom is crucial for the substrate specificity of the enzyme for in vivo conversion of Chl-α into Chl-d.

Oxidation of Chl-α with PhSH (5 equiv.) and TsOH (5 equiv.) in chloroform did not, however, afford the desired Chl-d. Though the C3-vinyl group of Chl-α could be converted into the formyl group, acid-labile magnesium complexes, Chls-α/d, were demetalated in the presence of TsOH to give the corresponding pigments, Pheos-α/d. After stirring for 24 h, Pheo-d (53%) was obtained as the major product without detection of Chl-α/d and Pheo-α. Even in the reduction of TsOH to 1 equiv., no Chl-d was found in the reaction mixture. We then examined a weaker acid, AcOH, using a small amount. The reaction of Chl-α with AcOH (1 equiv.) and PhSH (5 equiv.) in chloroform afforded Pheo-d as well. Change of the solvent to methanol still led to the formation of Pheo-d.

When Chl-α was incubated with PhSH (5 equiv.) and AcOH (1 equiv.) in THF, Chl-d was successfully obtained. The product was subjected to the normal-phase HPLC analysis (Fig. 2), and the peak was detected at the retention time (21 min) of Chl-d, together with the peak at 19 min of Chl-α/d obtained by C13³-epimeric equilibration prior to the injection. HPLC-purified Chl-d gave the Qy and Soret peaks at 696 and 451 nm, respectively (Fig. 3a), characteristic of the visible absorption spectrum of Chl-d. The MS spectrum of the synthetic Chl-d (Fig. 3b) clearly showed its molecular ion at m/z of 895.55 ([M + H⁺]) and a fragment ion at m/z = 617.20 produced by loss of a phytyl chain, which confirmed the assignment of the product as Chl-d bearing a phytyl chain. The ¹H NMR spectrum (Fig. 3c) of the synthetic pigment well matched that of natural Chl-d extracted from A. marina [18]. The yield of Chl-d by this oxidation was 31% as determined by ¹H NMR spectroscopy (Fig. 52). This is the first report that demonstrates in vitro conversion of Chl-α into Chl-d with a substantial yield. It is noted that this oxidation reaction transformed the acid-labile Mg-chlorin without demetallation to Pheos, allomerization to ring E-oxidized products, or hydrolysis of esters to chlorophyllides (or pheophorbides). During the reaction, little change was observed in the region of 500–540 nm where Pheos gave their...
characteristic Qx absorption bands, indicating that demetallation was sufficiently suppressed (Figs. S1–S2). The reaction of Chl-a with excess AcOH (5 equiv.) facilitated both the oxidation and demetallation, while no AcOH greatly decelerated the reaction. Chl-a and Chl-a’ were fully consumed for 4 h, and gave also by-products 1 (t_R = 25 and 26 min, Fig. 2b) and 2 (t_R = 20 min) in 30% and 20% yields, respectively. The formation of by-products 1 and 2 [11] seems to depend on the acidity of the reaction mixture. The control of acidity in the reaction may also play an important role in a possible active site of the enzyme for conversion of Chl-a into Chl-d.

Thus, we have shown, for the first time, that Chl-d can be synthesized in one-step from Chl-a in a substantial yield at room temperature. The reaction conditions were quite mild enough to be applied to Mg-chlors that were readily denatured by acid, base, trace metals, oxygen, and so on. It can be safely said that the simple and mild reaction is a possible model reaction of the Chl-d biosynthesis. Kobayashi’s group recently reported that treatment of Chl-a with papain or an extract from the root of a Japanese radish in aqueous acetone gave Chl-d in 2% and 8%, respectively (see Ref. [13] and also Supplementary data): the reaction mechanisms, however, are not clear. The present yield of Chl-d (31%) was greatly improved. Tomo et al. hypothesized that Chl-d was biosynthesized by oxidation of 3-desvinyl-3-hydroxymethyl-Chl-a, based on reversible spectral changes upon reduction with sodium dithionite or NaBH₄ and oxidation with potassium ferricyanide [19]. It is unlikely that the intermediate C3-hydroxymethyl group is formed directly from the C3-vinyl group, because the C3-formyl group has to be derived from the C3-vinyl group under hard oxidation conditions with the loss of a carbon atom.

We tentatively assume that thyl radical (PhS•) and molecular oxygen add to the C3-vinyl group of chlorophylls followed by

![Scheme 1](attachment:image1.png)

Scheme 1. Proposed synthetic pathway of Chl-a to Chl-d. A thyl radical (PhS•) attacked to the vinyl group of Chl-a, followed by addition of a molecular oxygen. The resulting hydroperoxide oxidatively decomposed into Chl-d and by-product 2.
and P450 utilize molecular oxygen to form reactive hydroperoxy species in the Chl-a biosynthesis. They are abundant reactive species that also function in Chl/porphyrin biosynthetic pathways. CAO carries Tyr radical [20]. Coproporphyrinogen III oxidase (HemN) uses S-adenosylmethionine to form a radical species [21]. Heme oxygenase (HmuO) and P450 utilize molecular oxygen to form reactive hydroperoxy species [22]. Ferrous heme iron of HmuO and P450 bind O2, and the following protonation and one-electron reduction affords ferric peroxide (Fe(III)–OOH). With the aid of an acid, P450 cleaves the O–O bond to give water and oxo ferryl species (Fe(IV)=O) that oxidizes the substrates, while HmuO transfers OH of the hydroperoxide to the substrate. Hydroperoxy species may also be formed in the present oxidation procedures as an intermediate and require an acid for further transformation. The idea is in line with the conversion of Chl-a into Chl-d by H2O2 and peroxidase (see Supplementary data). This study shows that AcOH, and thus Asp or Glu residue, can sufficiently activate the conversion of Chl-a into Chl-d. The P450-family has a wide variety in substrates and reactions including C–C bond cleavage [23]. Our findings are in line with the previous report suggesting that a P450-type oxygenase participates in the Chl-d biosynthesis [9]. Detailed study is now underway.

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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.febslet.2012.05.036.

References