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Kinetic Analysis of Reduction of Formyl Groups in Chlorophyll *d* and Pheophytin *d*

Kana Sadaoka, Yuki Hirai, Shigenori Kashimura, and Yoshitaka Saga*
Department of Chemistry, Faculty of Science and Engineering, Kinki University, Higashi-Osaka 577-8502

(Received March 16, 2010; CL-100248; E-mail: saga@chem.kindai.ac.jp)

Reduction of formyl groups in natural chlorophyll (Chl) *d* purified from a cyanobacterium, *Acaryochloris marina*, and its demetalation product, pheophytin (Phe) *d*, was kinetically analyzed. Chl *d* exhibited slower reduction kinetics of the formyl group to the hydroxymethyl group by *tert*-butylamine borane complex than Phe *d*.

Chlorophyll (Chl) *d* is a unique photosynthetic pigment in the cyanobacterium *Acaryochloris* (*A.*) *marina*.[1,2] The molecular structure of Chl *d* is depicted in Figure 1A, and is compared with those of Chls *a* and *b* (Figure 1B). Chl *d* possesses a formyl group at the 3-position of the chlorin macrocycle. In contrast, Chl *a*, which is a major chlorophyllous pigment in most oxygenic photosynthetic organisms, has a vinyl group at this position. This structural difference induces the large red-shift of the monomeric Q_y absorption band of Chl *d* compared with that of Chl *a*. Owing to the unique spectral properties of Chl *d*, *Acaryochloris* can use longer wavelength light for photosynthetic activities. Chl *b* is another Chl molecule possessing a formyl group in oxygenic photosynthetic organisms. This pigment has a formyl group at the 7-position, which is occupied by a methyl group in Chls *d* and *a*. The 7-formyl group in Chl *b* is responsible for shifts of the Soret and Q_y absorption bands to bathochromic and hypsochromic, respectively.

Chl *d* is now recognized as playing important roles in photosynthetic reaction centers in *Acaryochloris*,[3–9] although Chl *a*-type molecules were believed to form Chl dimers that function as the primary electron donors in reaction centers in oxygenic photosynthetic organisms until the discovery of *A. marina*. Thus, Chl *d* has recently attracted much attention in photochemistry and photobiology. The primary electron acceptor in photosystem (PS) II in *A. marina* was found to be pheophytin (Phe) *a*, which was a demetalation molecule of Chl *a*, not Phe *d*.[4–9] There would be several possible factors for the selection of Phe molecules in PS II in *A. marina*, such as maching of redox potentials and/or effects of protein matrixes around the Phe-binding sites.

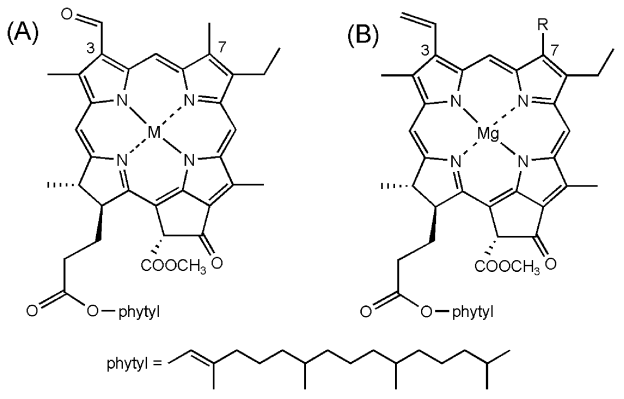

Figure 1. (A) Molecular structures of Chl *d* (M = Mg) and Phe *d* (M = 2H). (B) Molecular structures of Chl *a* (R = CH_3) and Chl *b* (R = CHO).

Biosynthesis and biodegradation of Chl *d* have not been unraveled yet. In contrast, information on such bioprocesses of Chl *b* is now available.[10–16] It is known that interconversion between formyl and methyl groups via a hydroxymethyl group at the 7-position, which is called the Chl cycle, plays a key role in formation and degradation of Chl *b*-type molecules.[10–13] Biosynthetic reduction of the formyl group of Chl *b*-type molecules to the corresponding hydroxymethyl group occurs in the initial step of the Chl *b* degradation by Chl *b* reductase.[14,15] This reaction induces degradation of light-harvesting complex II (LHCII) proteins, which contain both Chls *a* and *b* in *Arabidopsis*.[16] Therefore, reduction of the formyl group in Chl molecules is biologically important in oxygenic photosynthetic organisms.

Recently, in vitro reduction of the 3-formyl group in Chl *d* and its analogs has been reported.[17–19] However, there has been no physicochemical study of the conversion of the 3-formyl to the hydroxymethyl group in Chl *d*. In the present study, we report kinetic analysis of reduction of the 3-formyl groups in Chl *d* and Phe *d* which is a demetalation molecule of Chl *d* as shown in Figure 1A.

Chl *d* was extracted from *A. marina* MBIC11017, and was purified by reverse-phase high-performance liquid chromatography (HPLC) as described.[20] Phe *d* was prepared from isolated Chl *d* by treatment with 2% aqueous HCl, and was purified by normal-phase HPLC. A 1.5 mL of dichloromethane solution of the purified Chl *d* or Phe *d* (2.8×10^{-5} M) was mixed with 1.5 mL of dichloromethane solution of *tert*-butylamine borane complex (*t*-$BuNH_2 \cdot BH_3$, 1.0×10^{-2} M), and visible absorption spectra of the chlorophyllous pigments were measured with a Shimadzu UV-2450 spectrophotometer under the control of reaction temperatures between 15 and 35 °C.

Figure 2 shows spectral changes of Chl *d* and Phe *d* during reduction of 3-formyl groups in dichloromethane at the concentration of *t*-$BuNH_2 \cdot BH_3$ being 5.0×10^{-3} M at 25 °C. Chl *d* exhibited intense Soret and Q_y bands at 450 and 692 nm, respectively. These absorption bands decreased by incubation with *t*-$BuNH_2 \cdot BH_3$, and new Soret and Q_y absorption bands appeared at 429 and 657 nm, respectively. The isosbestic points could be clearly observed at 436 and 671 nm during the reduction.

Similar spectral changes occurred by incubation of Phe *d* with *t*-$BuNH_2 \cdot BH_3$. Intense Soret and Q_y bands at 427 and 695 nm, respectively, of Phe *d* lost their absorbances and new Soret and Q_y bands appeared at 409 and 662 nm, respectively. Small absorption bands in the range between 500 and 600 nm also gradually changed. The isosbestic points could also be observed during the reduction of the 3-formyl group in Phe *d*.

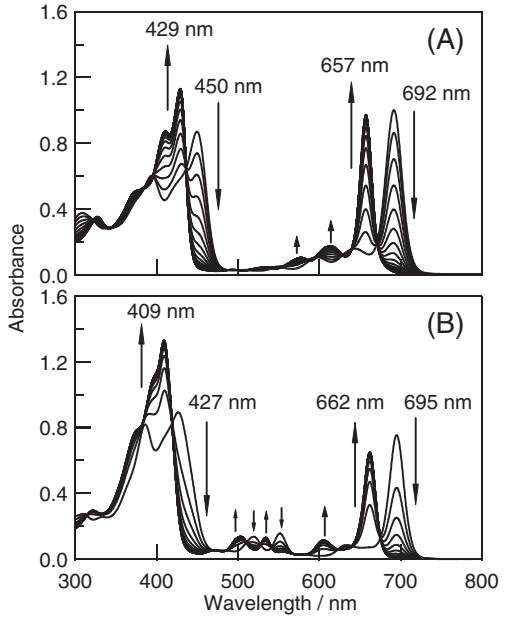

Figure 2. Spectral changes of Chl *d* (A) and Phe *d* (B) in dichloromethane at the concentration of *t*-$BuNH_2 \cdot BH_3$ of 5.0×10^{-3} M at 25 °C. Chl *d*: spectra from 0 to 330 min at a 15-min interval. Phe *d*: spectra from 0 to 270 min at a 15-min interval. Arrows show direction of absorbance changes.

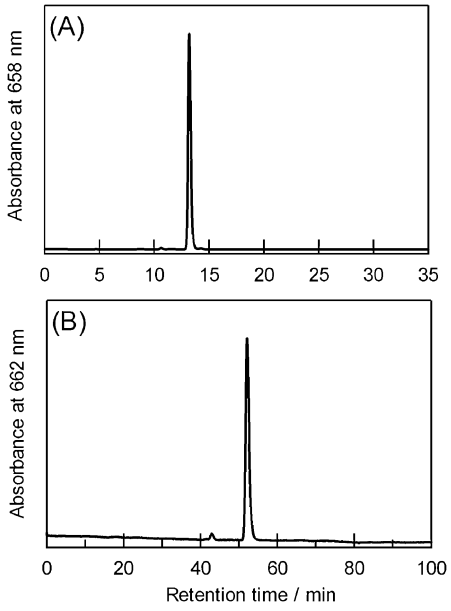

Figure 3. HPLC elution patterns of reaction products of Chl *d* (A) and Phe *d* (B) under conditions in Figure 2 for 330 and 270 min, respectively. Reaction products of Chl *d* and Phe *d* were eluted on 5C18-AR-II (6 mmϕ × 250 mm) with methanol and on 5SL-II (6 mmϕ × 250 mm) with hexane/2-propanol (97/3), respectively, at a flow rate of 1.0 mL min^{-1}. Chromatograms (A) and (B) were recorded at 658 and 662 nm, respectively.

The products after reduction of the 3-formyl groups in Chl *d* and Phe *d* under the conditions in Figure 2 were analyzed by reverse-phase and normal-phase HPLC, respectively, as shown in Figure 3. After the reduction of Chl *d* for 330 min, a main product was detected at 13 min (Figure 3A). Soret and Q_y absorption bands of the main product were positioned at 427 and 658 nm, respectively, in the HPLC eluent. This spectral feature was characteristic of 3-OH Chl *d*. This product gave a molecular ion peak at *m*/*z* 897.5 in LC-MS measurements. These results indicate that the main product after reduction of Chl *d* was 3-OH Chl *d*. A small fraction, which exhibited an almost identical absorption spectrum to the main product at 13 min, appeared at 14 min. This minor product at 14 min was presumed to be the 13^2-stereoisomer of 3-OH Chl *d*, namely 3-OH Chl *d*′.[18] HPLC analysis of reduction products of Chl *d* revealed that little side reaction such as reduction of 13^1-carbonyl group occurred under the conditions in Figure 2. Figure 3B shows an HPLC elution pattern of reduction products of Phe *d* under the conditions in Figure 2 for 270 min. The main product, which was determined to be 3-deformyl-3-hydroxymethyl pheophytin *d* (3-OH Phe *d*) from its visible absorption spectrum and LC-MS analysis, appeared at 52 min. The minor product at 43 min would be surmised to be the 13^2-stereoisomer of 3-OH Phe *d*, 3-OH Phe *d*′.[18] HPLC analysis of reduction products of Phe *d* also indicated little side reaction under the conditions in Figure 2.

Reduction of 3-formyl groups in Chl *d* and Phe *d* was kinetically analyzed by Q_y absorbance changes of these chlorophyllous pigments. Figure 4 depicts time courses of Q_y peak absorbance of Chl *d* and Phe *d* incubated with *t*-$BuNH_2 \cdot BH_3$ (5.0×10^{-3} M) at 25 °C. Figure 4 clearly indicates that reduction of the 3-formyl group in Chl *d* was slower than

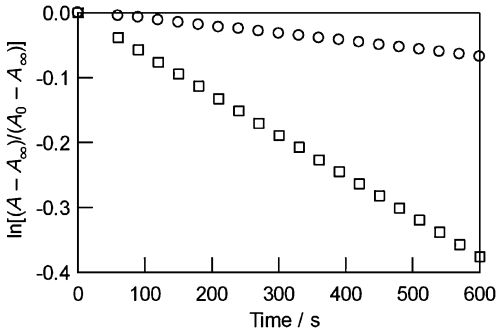

Figure 4. Kinetic plots for reductions of Chl *d* (open circle) and Phe *d* (open square) in dichloromethane at the concentration of *t*-$BuNH_2 \cdot BH_3$ of 5.0×10^{-3} M at 25 °C. Absorbance changes were monitored at 692 and 695 nm for Chl *d* and Phe *d*, respectively. A_0, A, and A_∞ are Q_y absorbances of Chl *d* and Phe *d* at the onset of measurements, at time *t*, and at complete reduction, respectively.

that in Phe *d*. The logarithm of Q_y absorbance of both Chl *d* and Phe *d* through the reduction for 600 s exhibited almost linear time-dependence. Therefore, the reductions were regarded as pseudo-first-order reactions. This is in line with the reaction conditions in which the concentration of *t*-$BuNH_2 \cdot BH_3$ was much higher than the concentration of chlorophyllous pigments.[20–22] In the reduction, therefore, the reaction rate constant, *k*, can be estimated by fitting the time courses of Q_y absorbance of Chl *d* and Phe *d* to the following kinetic equation:

$$\ln[(A - A_\infty)/(A_0 - A_\infty)] = -kt \quad (1)$$

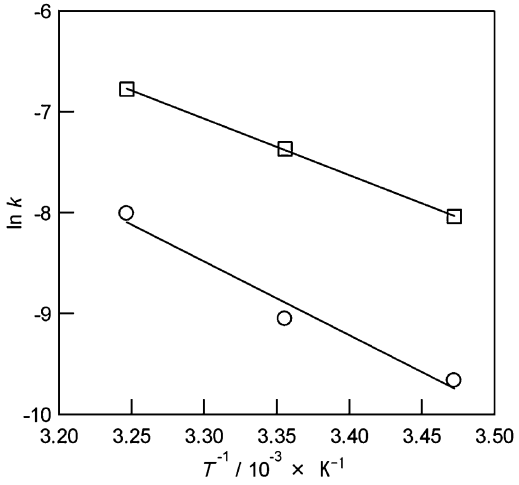

Figure 5. Arrhenius plots over temperature range of 15–35 °C for reduction rate constants of Chl *d* (open circle) and Phe *d* (open square) in dichloromethane at concentration of *t*-$BuNH_2 \cdot BH_3$ of 5.0×10^{-3} M.

where A_0, A, and A_∞ are Q_y absorbances of Chl *d* and Phe *d* at the onset of measurements, at time *t*, and at complete reduction, respectively. As a result, the reduction rate constants of purified Chl *d* and Phe *d* under the conditions in Figure 4 were determined to be 1.2×10^{-4} and $6.3 \times 10^{-4}\,s^{-1}$, respectively. These values were the averages of three independent measurements, and the standard deviations were 11% and 4% of the averages, respectively. The reduction rate constant of the 3-formyl reduction in Phe *d* was 5.3-times larger than that of Chl *d*.

The reduction rate constants of Chl *d* and Phe *d* at 15, 25, and 35 °C were estimated by fitting the time course of Q_y absorbance to the above kinetic equation, since the logarithm of Q_y absorbance almost linearly changed against reaction time in this temperature range.

Figure 5 depicts the Arrhenius plots of the rate constants of the 3-formyl reduction, which were the averages of more than three independent measurements at each temperature for both Chl *d* and Phe *d*. The Arrhenius plots in Figure 5 gave the activation energies of 61 and 47 kJ mol^{-1} for Chl *d* and Phe *d*, respectively. The activation energy of 3-formyl reduction of Chl *d* was 1.3-times larger than that of Phe *d*. Therefore, slower reduction kinetics of the 3-formyl group in Chl *d* than that in Phe *d* was mainly attributed to higher activation energies of 3-formyl reduction of Chl *d* compared to Phe *d*.

The difference of 3-formyl reduction properties between Chl *d* and Phe *d* could be rationalized by invoking their redox potentials. The oxidation potentials of Chl *d* and Phe *d* in acetonitlile were reported to be +0.88 and +1.21 V vs. SHE, respectively.[23] Such difference of oxidation potentials, which are related to the electron densities of the chlorin π-macrocycles, would affect the electron densities of the electron-withdrawing 3-formyl groups in Chl *d* and Phe *d*. Namely, the 3-formyl carbon atom of Phe *d* would be more electropositive than that of Chl *d*, and the formyl group of Phe *d* would be more reactive with a nucleophile than that of Chl *d*. The chemical shifts of the 3-formyl carbon atom in Chl *d* in $CDCl_3/CD_3OD$ (19/1, v/v) and Phe *d* in $CDCl_3$ are 189.02 and 188.27 ppm, respectively. The present ^{13}C NMR measurements could not clearly indicate the difference of the electron densities of 3-formyl groups between Chl *d* and Phe *d*. Further spectroscopic analysis is now in progress.

The present study revealed the physicochemical properties of 3-formyl reduction of Chl *d*, which was the major pigment in *Acaryochloris*, and its demetalation product, Phe *d*. These would be useful for elucidating in vivo conversion of the 3-substituent in chlorophyllous pigments in *Acaryochloris*.

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