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Effects of molecular structures on reduction properties of formyl groups in chlorophylls and pheophytins prepared from oxygenic photosynthetic organisms

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ABSTRACT

Reduction of the 7-formyl groups in chlorophyll (Chl) *b* and its demetalated compound pheophytin (Phe) *b* was kinetically analyzed by using *tert*-butylamine–borane complex (*t*-BuNH$_2$·BH$_3$), and was compared with that of the 3-formyl groups in Chl *d* and Phe *d*. Reduction kinetics of the 7-formyl group in Chl *b* was similar to that in Phe *b* in dichloromethane containing 5 mM *t*-BuNH$_2$·BH$_3$. Little difference of the reduction kinetics of the 7-formyl groups between Chl *b* and Phe *b* was in sharp contrast to the reduction kinetics of the 3-formyl groups in Chl *d* and Phe *d*: the 3-formyl group in Phe *d* was reduced 5.3-fold faster than that in Chl *d*. The 7-formyl groups in Chl *b* and Phe *b* were reduced more slowly than the 3-formyl groups in Chl *d* and Phe *d*, respectively. The difference of the reactivity between the 3- and 7-formyl groups was in line with ^{13}C NMR measurements of chlorophyllous pigments, in which the chemical shifts of carbon atoms in the 7-formyl groups of Chl *b* and Phe *b* were high-field shifted compared with those in the 3-formyl groups of Chl *d* and Phe *d*, respectively. These indicate that the 7-formyl groups in chlorophyllous pigments were less reactive for reduction to the corresponding hydroxymethyl groups than the 3-formyl groups due to the difference in electronic states of the formyl groups in the A- and B-rings of the chlorin macrocycle.

1. Introduction

Chlorophyll(Chl)s play crucial roles in photosynthesis. Chl molecules have cyclic tetrapyrrole moieties as the photofunctional core, and peripheral substituents of the cyclic tetrapyrroles give their structural diversity.[1] Molecular structures of natural Chls *a*, *b*, and *d* in oxygenic photosynthetic organisms are shown in Figure 1. The structural difference between Chls *a* and *b* is the substituent at the 7-position of the chlorin macrocycle: methyl and formyl groups are occupied at this position in Chls *a* and *b*, respectively. The 7-formyl group of Chl *b* is responsible for the shift of its main absorption bands, namely Soret and Q$_y$ absorption bands, to bathochromic and hypsochromic, respectively, compared with those of Chl *a*. Owing to such spectral properties, Chl *b* can capture photons that are scarcely absorbed by Chl *a* in photosynthetic light-harvesting complexes. Chl *d* is a major photosynthetic pigment in a cyanobacterium *Acaryochloris*,[2,3] and play essential roles in both light-harvesting complexes and reaction centers in this organism.[4–11] Chl *d* has a formyl group at the 3-position of the chlorin macrocycle, and the other substituents are the same as those of Chl *a*. This structural difference results in the large red-shift of the monomeric Q$_y$ absorption band of Chl *d* compared with Chl *a*. Because of such spectral properties of Chl *d*, *Acaryochloris* can use far-red light for the photosynthetic activity.

Transformation of the formyl groups in Chls (or chlorophyllide(Chlide)s) *b* and *d* is key reaction in biosynthesis and biodegradation of the chlorophyllous pigments. Formation and degradation of

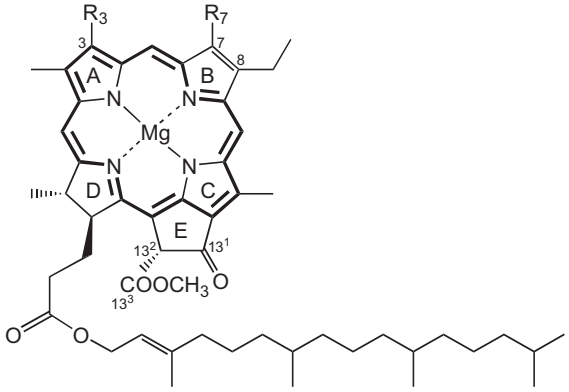

Figure 1. Molecular structures of Chl *a*, Chl *b*, and Chl *d*. Chl *a*: R$_3$ = CHCH$_2$, R$_7$ = CH$_3$. Chl *b*: R$_3$ = CHCH$_2$, R$_7$ = CHO. Chl *d*: R$_3$ = CHO, R$_7$ = CH$_3$. The bold lines show a major 18π-circuit.

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Chl *b* (or Chlide *b*) are based on interconversion between formyl and methyl groups at the 7-position via a hydroxymethyl group, which is called the Chl cycle.[12–15] The 7-formyl group of Chl *b*-type molecules are reduced to the corresponding hydroxymethyl group in the initial step of the Chl *b* degradation by Chl *b* reductase, and this process has been reported to be responsible for degradation of light-harvesting complex II (LHC II) proteins in *Arabidopsis*.[16] In the case of Chl *d*, conversion of the 3-formyl group in bioprocesses has not been unraveled yet. It is worth noting that biological formation of the 3-formyl group in Chl *d* has attracted considerable attention, and the oxygen atom of the 3-formyl group was reported to be derived from molecular oxygen.[17] Therefore, reactivity of the formyl groups conjugated to the chlorin macrocycle will be useful to understand such biologically important processes in oxygenic photosynthetic organisms.

Chemical reduction of the formyl group to the hydroxymethyl group of natural Chls and synthetic chlorins has attracted much attention from the viewpoints of in vivo transformation of formylated Chls described above and synthesis of photofunctional pigments.[18–32] Reduction of the 7-formyl groups in Chl *b*-type pigments (Chl *b*, Phe *b*, and synthetic 7-formyl-chlorins) was first performed by using sodium borohydride ($NaBH_4$).[19–21] A mild reagent, sodium cyanoborohydride ($NaBH_3CN$), was used in place of $NaBH_4$,[22–24] since $NaBH_4$ caused undesired reduction of the carbonyl group at the 13^1-position. A much milder reagent, *tert*-butylamine–borane complex (*t*-$BuNH_2{\cdot}BH_3$), has also been employed for selective reduction of the 7-formyl group.[25–27] In the case of Chl *d*-type pigments (Chl *d*, Phe *d*, and synthetic 3-formyl-chlorins), reagents such as $NaBH_4$ and *t*-$BuNH_2{\cdot}BH_3$ have been used for reduction of the 3-formyl groups.[28–32] However, little information is available, to the best of our knowledge, on physicochemical properties of reduction of the formyl groups in natural Chls and their model chlorins.[30,32] Tamiaki et al. reported comparison of reactivity between the 3- and 8-carbonyl groups in synthetic free-base chlorins.[30] We preliminarily analyzed reduction kinetics of the 3-formyl groups in Chl *d* and Phe *d*.[32] In this study, we report physicochemical properties of reduction of the 7-formyl groups in Chl *b* and Phe *b* by using *t*-$BuNH_2{\cdot}BH_3$, and compare their properties with the 3-formyl groups in Chl *d* and Phe *d*. Additionally, we investigate the electronic states of the formyl groups in the four chlorophyllous pigments prepared from oxygenic photosynthetic organisms by means of ^{13}C NMR measurements. The present analysis enables us to elucidate effects of central magnesium and the substituted positions of the formyl groups on formyl reduction of naturally occurring chlorophyllous pigments.

2. Results and discussion

Spectral changes of Chl *b* and Phe *b* possessing the 7-formyl group by incubation in dichloromethane at the *t*-$BuNH_2{\cdot}BH_3$ concentration of 5 mM at 25 °C are depicted in Figure 2. Chl *b* had Soret and Q_y bands at 458 and 646 nm, respectively, as shown in Figure 2(A). Incubation of Chl *b* in the solution containing *t*-$BuNH_2{\cdot}BH_3$ decreased the Soret absorption band, accompanying with a new absorption band at 434 nm. The 646-nm Q_y absorption band also decreased and a new Q_y band appeared in the longer wavelength region of the Q_y band of Chl *b*. The isosbestic points were present at 442, 602, 628, and 649 nm in this spectral change.

In the case of Phe *b* in Figure 2(B), the 438-nm Soret band of Phe *b* decreased with appearance of a new Soret band at 418 nm in dichloromethane containing *t*-$BuNH_2{\cdot}BH_3$. The 655-nm Q_y absorption band also decreased with increase of a new 661-nm Q_y band. Small bands in the wavelength region between 500 and 600 nm were also varied. This spectral change exhibited the isosbestic points at 427, 494, 517, 604, 636, and 656 nm.

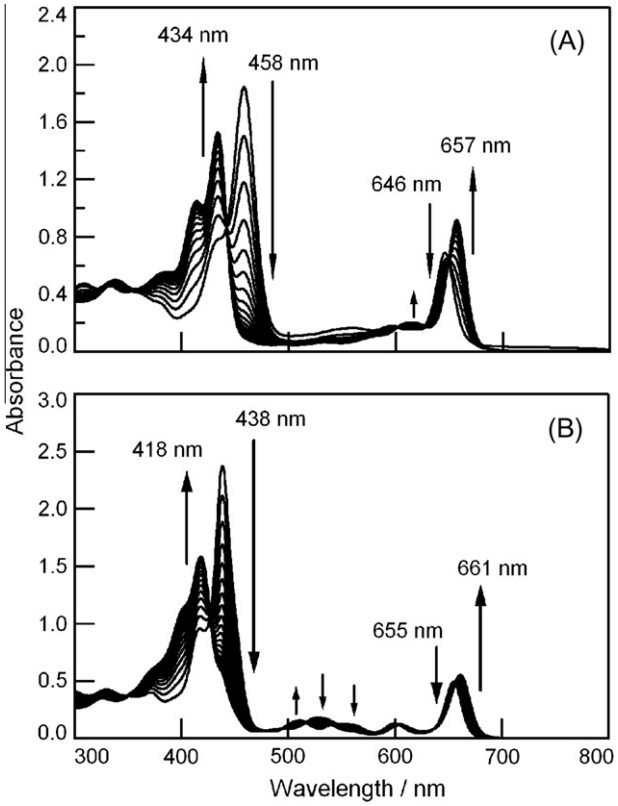

Figure 2. Spectral changes of Chl *b* (A) and Phe *b* (B) in dichloromethane at the concentration of *t*-$BuNH_2{\cdot}BH_3$ of 5 mM at 25 °C. Both spectra were measured from 0 to 1020 min at a 60-min interval. The arrows show the direction of the absorbance changes.

Incubation of Chl *d* and Phe *d* possessing the 3-formyl group with *t*-$BuNH_2{\cdot}BH_3$ showed similar spectral changes.[32] Both Soret and Q_y absorption bands of the 3-formylated chlorophyllous pigments decreased, and new bands appeared in the presence of several isosbestic points. The new Q_y bands in the spectral changes of Chl *d* and Phe *d* were positioned in the shorter wavelength region of the original 3-formylated pigments (Chl *d*: 692→657 nm, Phe *d*: 695→662 nm), which were different from the spectral changes of the 7-formylated pigments described above. The new absorption bands in the spectral changes of Chl *d* and Phe *d* were ascribed to reduction of the 3-formyl groups, namely formation of 3-deformyl-3-hydroxymethyl Chl *d* (3-OH Chl *d*) and 3-deformyl-3-hydroxymethyl Phe *d* (3-OH Phe *d*) from Chl *d* and Phe *d*, respectively.[32]

Reaction products of Chl *b* and Phe *b* by incubation in dichloromethane containing 5 mM *t*-$BuNH_2{\cdot}BH_3$ were analyzed by reverse-phase and normal-phase HPLC, respectively. Figure 3(A) depicts typical HPLC elution patterns of Chl *b* before incubation and its reaction products after incubation. A fraction at 16 min before incubation was assigned to Chl *b*. After incubation for 240 min, a main product was eluted at 13 min, accompanying the unreacted Chl *b* at 16 min. This product at 13 min had Soret and Q_y bands at 437 and 661 nm in this HPLC eluent. FAB-MS analysis of the product eluted at 13 min indicated that this main product gave a molecular ion peak at *m/z* 908.5, which was the same as the calculated value ($[M]^+$ = 908.5) for 7-deformyl-7-hydroxymethyl Chl *b* (7-OH Chl *b*). This proved that the main product from Chl *b* by incubation with *t*-$BuNH_2{\cdot}BH_3$ was 7-OH Chl *b*. Figure 3(B) shows typical chromatograms of Phe *b* before incubation and its reaction products. Phe *b* before incubation was eluted at 12 min. A main product after incubation for 240 min, which was detected at 25 min, possessed the Soret and Q_y bands at 414 and 664 nm in

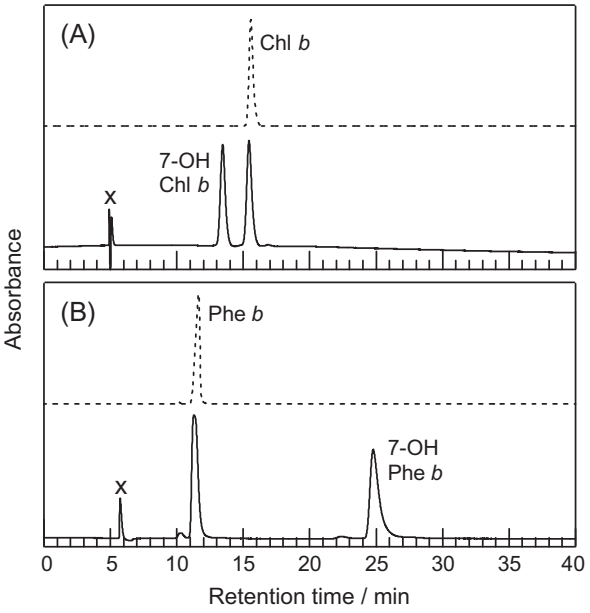

Figure 3. HPLC elution patterns of Chl *b* (A) or Phe *b* (B) before incubation of *t*-$BuNH_2{\cdot}BH_3$ (dotted line) and their reaction products after incubation (solid line) in dichloromethane at the concentration of *t*-$BuNH_2{\cdot}BH_3$ of 5 mM at 25 °C for 240 min. Chls and Phes were eluted on $5C_{18}$-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 before and after incubation of Chl *b* were recorded at 470 and 436 nm, respectively. Chromatograms before and after incubation of Phe *b* were recorded at 434 and 412 nm, respectively. The signals denoted by × were due to the injection of mixed solvents of HPLC eluents and dichloromethane. The procedure of HPLC analysis of chlorophyllous pigments after incubation was described in Section 3.

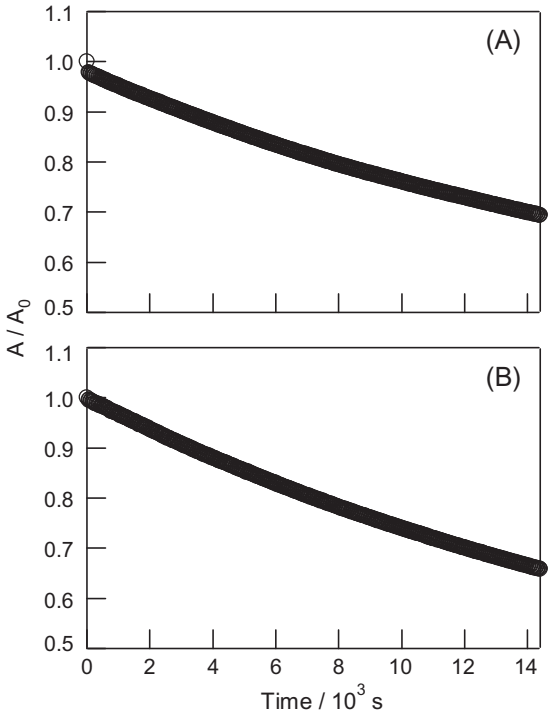

Figure 4. Kinetic plots for reduction of Chl *b* (A) and Phe *b* (B) in dichloromethane at the concentration of *t*-$BuNH_2{\cdot}BH_3$ of 5 mM at 25 °C. Absorbance changes were monitored at 459 and 438 nm for Chl *b* and Phe *b*, respectively. A_0 and *A* are Soret absorbances of chlorophyllous pigments at the onset of measurements and at time *t*, respectively.

the eluent. The unreacted Phe *b* was also detected at 12 min after incubation for 240 min. A molecular ion peak at *m/z* 886.6 of this main product at 25 min in FAB-MS measurements corresponded to the calculated value ($[M]^+ = 886.6$) for 7-deformyl-7-hydroxymethyl Phe *b* (7-OH Phe *b*). Therefore, 7-OH Phe *b* was predominantly formed from Phe *b* by incubation with *t*-$BuNH_2{\cdot}BH_3$. Slight fractions were detected at 17 and 22 min in the chromatograms of reaction products of Chl *b* and Phe *b*, respectively, as shown by solid lines in Figure 3. Visible absorption spectra of these fractions were almost identical to those of the main products. Their absorption spectra and elution patterns indicated that these slight products would be the 13^2-stereoisomers of 7-OH Chl *b* and 7-OH Phe *b*, namely 7-OH Chl *b*′ and 7-OH Phe *b*′, respectively. A small fraction at 10 min in the chromatogram of reaction products of Phe *b* (solid line in Fig. 3(B)) was also assigned to Phe *b*′ by its absorption spectrum and elution pattern. Such slight epimerization was also observed in reduction of Chl *d* and Phe *d* under the same condition.[32] No other reaction products such as a 13^1-hydroxylated compound were observed, indicating that side reaction hardly occurred under the present condition.

Reduction kinetics can be quantitatively analyzed by absorbance changes at the Soret peak positions for Chl *b* and Phe *b*, as judged from their spectral changes in Figure 2. Time courses of Soret absorbances of Chl *b* and Phe *b* incubated with 5 mM *t*-$BuNH_2{\cdot}BH_3$ at 25 °C are shown in Figure 4. The reaction conditions in which the concentration of *t*-$BuNH_2{\cdot}BH_3$ (5 mM) was much higher than the pigment concentration (14 μM) allows us to regard these reactions as pseudo-first-order reactions.[32] The reduction rate constants, *k*s, can be estimated by fitting the logarithms of the time courses of absorbance at Soret peak positions of Chl *b* and Phe *b* for 600 s to the following kinetic equation,

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

where A_0, *A*, and A_∞ are Soret absorbances of Chl *b* and Phe *b* at the onset of measurements, at time *t*, and at the complete reduction, respectively. The rate constants of reduction of the formyl groups in Chl *b* and Phe *b* under the condition in Figure 4 were estimated to be 3.5×10^{-5} and 3.4×10^{-5} s^{-1}, respectively. These values were the averages of more than five independent measurements, where the standard deviations were 14% and 6% of the averaged values for Chl *b* and Phe *b*, respectively. The reduction rate constants of the 7-formyl groups in Chl *b* and Phe *b* were significantly smaller than those of the 3-formyl groups in Chl *d* and Phe *d* (1.2×10^{-4} and 6.3×10^{-4} s^{-1}, respectively) under the same reaction condition. Therefore, the 7-formyl group in the B-ring of the chlorin macrocycle was converted to the hydroxymethyl group more slowly than the 3-formyl group in the A-ring. Lower reduction reactivity of formyl groups attached to the B-ring of the chlorin macrocycle than that in the A-ring was also reported by using synthetic free-base chlorin molecules.[30] The difference of the reactivity between formyl groups in the A-ring and those in the B-ring would be ascribed to electronic properties of these formyl groups. The 3-formyl group is connected to the chlorin 18π-conjugated system, whereas the 7-formyl group is attached to the rather isolated double bond between C7 and C8 atoms. As a result, the 7-formyl group would be more strongly conjugated to the adjacent π-system than the 3-formyl group. Such difference should change the electron densities on the carbon atoms of the 3-formyl and 7-formyl groups in the chlorin macrocycle.

^{13}C NMR spectra of the four formylated Chls and Phes were measured in a mixed solvent of methanol-d_4/chloroform-*d* (1:19, v/v) and chloroform-*d*, respectively, to investigate the electronic states of the formyl groups of these chlorophyllous pigments. ^{13}C NMR signals were assigned by ^1H–^{13}C-HSQC, ^1H–^{13}C-HMBC, and previous reports.[33,34] Table 1 summarizes the chemical shifts δs of carbon atoms in the formyl groups as well as carbonyl carbon

Table 1
Chemical shifts of carbonyl carbon atoms in Chl *b*, Chl *d*, Phe *b*, and Phe *d*

Pigments	δ ($C3^1$)	δ ($C7^1$)	δ ($C13^1$)	δ ($C13^3$)
Chl *b*	—	188.29	189.97	171.99
Chl *d*	189.02	—	189.71	172.29
Phe *b*	—	187.57	189.46	169.26
Phe *d*	188.27	—	189.44	169.31

Chls and Phes are dissolved in methanol-d_4/chloroform-*d* (1:19) and in chloroform-*d*, respectively.

atoms in the E-ring ($C13^1$ and $C13^3$ carbon atoms). The δs of carbon atoms in the formyl groups in Chl *b*, Chl *d*, Phe *b*, and Phe *d* were 188.29, 189.02, 187.57, and 188.27 ppm, respectively. Both the high-field shifts of the δs of the formyl carbon atoms in Chl *d*→Chl *b* (189.02→188.29 ppm) and Phe *d*→Phe *b* (188.27→187.57 ppm) indicated the $C7^1$ atoms of the 7-formylated pigments were less electropositive than the $C3^1$ atoms of the 3-formylated pigments. This is in line with the present results that Chl *b* and Phe *b* are less reactive to a nucleophile than Chl *d* and Phe *d*, respectively. Previous ^{13}C NMR experiments of the synthetic free-base chlorins[30] also support such discussion.

The reduction rate constant of the 7-formyl group in Chl *b* was almost similar to that in Phe *b* under this condition. This is in sharp contrast to reduction of the 3-formyl groups in Chl *d* and Phe *d*: the reduction rate constant of the 3-formyl group in Phe *d* ($k = 6.3 \times 10^{-4}\ s^{-1}$) was about 5.3-times larger than that in Chl *d* ($k = 1.2 \times 10^{-4}\ s^{-1}$) under the same condition. These indicate that central magnesium has smaller effect on reduction kinetics of the 7-formyl group in Chl *b*-type pigments than that of the 3-formyl group in Chl *d*-type pigments. This might originate from possible stronger conjugation of the 7-formyl group to the C7–C8 double bond than the 18π-aromatic circuit. In contrast, the 3-formyl group might be more influenced by the 18π-conjugated system compared with the 7-formyl group. Such difference in connection of the formyl groups with the adjacent π-systems in chlorophyllous pigments is likely to change the effect of central magnesium on reduction of the formyl groups linked to the chlorin macrocycle.

IR spectra of monomeric Chl *b*, Phe *b*, Chl *d*, and Phe *d*[35–38] were compared in order to examine the properties of the formyl groups in natural chlorophyllous pigments. The stretching vibrational bands of the 7-formyl carbonyl groups in Chl *b* and Phe *b* appeared at 1663 and 1667 cm^{-1}.[35] In a while, the stretching vibrational peaks of the 3-formyl carbonyl groups in Chl *d* and Phe *d* were positioned at 1667 and 1677 cm^{-1}.[37,38] Both the lower wavenumber shifts in Chl *d*→Chl *b* (1667→1663 cm^{-1}) and Phe *d*→Phe *b* (1677→1667 cm^{-1}) suggest that the 7-formyl groups in Chl *b* and Phe *b* are more conjugated with the adjacent π-system than the 3-formyl groups in Chl *d* and Phe *d*. Smaller difference of wavenumber shifts of the stretching vibrational peaks of the 7-formyl carbonyl groups between Chl *b* and Phe *b* ($\delta\nu = 4\ cm^{-1}$) than that of the 3-formyl carbonyl groups between Chl *d* and Phe *d* ($\delta\nu = 10\ cm^{-1}$) implies that the 7-formyl groups would be less influenced by central magnesium in the 18π-conjugated system than the 3-formyl groups. These IR spectroscopic data of the four pigments are in line with their reduction properties.

Reduction potentials of Chl *b* and Phe *b* in acetonitrile were reported to be −1.02 and −0.64 V vs. SHE, respectively.[39] These reduction potentials are lower than those of Chl *d* (−0.91 V) and Phe *d* (−0.63 V), respectively,[39] suggesting that the 7-formylated chlorophyllous pigments are less reactive to reduction than the 3-formylated pigments. Such assumption would not contradict the present results on the reactivity of the 3-formyl and 7-formyl groups. However, the order of reduction potentials of the four pigments disagrees with the order of the reactivity of formyl reduction in this study. Further detailed studies will be necessary for understanding the relationship between reactivity of the formyl groups and redox potentials of chlorophyllous pigments.

Little difference of reduction properties of the 7-formyl group between Chl *b* and Phe *b* might be in line with in vivo degradation of Chl *b* in higher plants. In the initial step of Chl *b* degradation, the 7-formyl group in Chl *b* or Chlide *b* is reduced to the hydroxymethyl group, and subsequently converted to the methyl group, followed by removal of central magnesium from the chlorin macrocycle.[12,13] It has been reported that Chl *b*-type molecules possessing the 7-formyl group exhibited higher resistance to demetalation than chlorin molecules possessing the 7-methyl and 7-hydroxymethyl groups.[27,40,41] Such demetalation properties of 7-formyl chlorins suggest potential difficulty of smooth demetalation of Chl *b* or Chlide *b*. In contrast, the present results indicate that possible biochemical reduction of the 7-formyl group would similarly proceed in both Chl *b* and Phe *b*. Therefore, both the reduction properties of the 7-formyl group and the demetalation properties in the 7-formylated chlorophyllous pigments would not contradict the biological process of Chl *b* degradation, where the 7-formyl group is reduced before removal of central magnesium.

In conclusion, we demonstrated that the 7-formyl groups in Chl *b* and Phe *b* were less reactive to the reducing reagent than the 3-formyl groups in Chl *d* and Phe *d*. Moreover, this study also indicates that effect of central magnesium on reduction kinetics in 7-formylated chlorophyllous pigments was small compared with that in 3-formylated chlorin molecules. This work could be useful for elucidation of biochemical conversion of formyl groups in photosynthetic pigments.

3. Experimental

3.1. Apparatus

Visible absorption spectra were measured with a Shimadzu UV-2450 spectrophotometer, where the reaction temperatures were regulated with a Shimadzu thermo-electric temperature-controlled cell holder TCC-240A. High-performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-20AT pump and an SPD-M20A or SPD-20A photodiode array detector. Mass spectra were measured with a JEOL JMS700 spectrometer; *m*-nitrobenzyl alcohol was used as a matrix. ^1H and ^{13}C NMR spectra were measured with a JEOL JNM-ECA500 or JNM-ECA700 NMR spectrometer; chemical shifts of ^1H and ^{13}C were expressed (in ppm) relative to chloroform (7.26 and 77.0 ppm, respectively) as internal references.

3.2. Materials

Chls *b* and *d* were extracted from spinach leaves and harvested cell pellets of *Acaryochloris marina* MBIC11017, respectively, with acetone/methanol (1:1, v/v).[32,41] The extracted solutions were diluted with diethyl ether and washed with NaCl–saturated water (neutral pH) to remove water-soluble components, and then were concentrated under reduced pressure. Chlorophyllous pigments were extracted again from the concentrated solution with dichloromethane to remove residual water, and were evaporated under reduced pressure and dried with nitrogen gas. Chls *b* and *d* were finally purified on a reverse-phase HPLC column $5C_{18}$-AR-II (10 mm internal diameter (i.d.) × 250 mm, Nacalai Tesque).

Phes *b* and *d* were prepared from Chls *b* and *d*, respectively, by treatment with aqueous HCl in dichloromethane and were purified on a normal-phase HPLC column 5SL-II (6 mm i.d. × 250 mm, Nacalai Tesque). Purified Chls and Phes were stored in the dark at −20 °C, and re-purified by HPLC just before measurements.

3.3. Measurements of reduction kinetics

Dichloromethane solution of the purified pigment (28 μM), whose concentration was the same as that of Chl *d* and Phe *d* in the previous report,[32] was mixed with the same amount of dichloromethane solution of *t*-$BuNH_2{\cdot}BH_3$ (10 mM), and their visible absorption spectra were measured at 25 °C.

3.4. HPLC analysis before and after reduction

The purity of Chls and Phes before measurements of reduction kinetics was checked on a reverse-phase HPLC column $5C_{18}$-AR-II (6 mm i.d. × 250 mm, Nacalai Tesque) with methanol and a normal-phase HPLC column 5SL-II (6 mm i.d. × 250 mm, Nacalai Tesque) with hexane/2-propanol (97:3, v/v), respectively, at a flow rate of 1.0 mL min^{-1}. After measurements of reduction kinetics, the reaction mixtures were diluted with HPLC eluents, and the solutions were filtrated with 0.45-μm pore size filter. The resulting solutions were analyzed under the same conditions as the analysis of pigments before measurements.

3.5. NMR measurements

^1H and ^{13}C NMR spectra of Chls and Phes were measured in a mixed solvent of methanol-d_4/chloroform-*d* (1:19, v/v) and chloroform-*d*, respectively. Chl *b*, Chl *d*, Phe *b*, and Phe *d* concentrations were 15, 11, 15, and 14 mM, respectively, in NMR measurements.

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