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ABSTRACT: Effects of peripheral substituents in the A- and B-rings of the chlorin macrocycle on demetalation of zinc chlorophyll derivatives were examined. The demetalation rate constants of zinc 8-formyl-chlorin (1) were comparable to those of zinc 3-formyl-chlorin (2). Both 1 and 2 exhibited significantly slower demetalation kinetics than the corresponding zinc chlorin 3, which had no formyl group. Comparison of demetalation kinetics between zinc 3-ethyl-chlorin (3) and zinc 3-vinyl-chlorin (4) indicated that the substitution at the 3-position of the chlorin macrocycle



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from a vinyl to an ethyl group accelerated removal of the central zinc. Activation energies of demetalation of zinc chlorins 1, 2, and 5 possessing a formyl group in the A- or B-ring of the chlorin macrocycle were larger than those of zinc chlorins 3 and 4 lacking a formyl group. These results indicate that the formyl groups in the A- or B-ring provide tolerance to demetalation of chlorin molecules due to their electron-withdrawing effects.

INTRODUCTION

Chlorophyll (Chl) molecules are major photosynthetic pigments in nature. The photofunctional cores of Chls are tetrapyrrole macrocycles, and peripheral substituents of the cyclic tetrapyrrole moieties provide their structural diversity.¹ Figure 1A shows molecular structures of naturally occurring Chls in oxygenic photosynthetic organisms. The difference of molecular structures between Chls a and b is the substituent at the 7-position of the chlorin macrocycle: the substituents are methyl and formyl groups, respectively. The 7-formyl group of Chl *b* causes bathochromic and hypsochromic shifts of Soret and Q_{ν} absorption bands, and Chl *b* can capture photons that are scarcely absorbed by Chl a in photosynthetic light-harvesting complexes. Chlorophyll d_i a major photosynthetic pigment in a cyanobacterium Acaryochloris (A.) marina, has a formyl group at the 3-position (Figure 1A), resulting in the large red shift of the Q_{ν} absorption band of Chl *d* compared with that of Chl *a*. Such a spectral property enables A. marina to use longer wavelength light for its photosynthetic activity. Recently, a new Chl possessing a formyl group at the 2-position, which was designated to $\operatorname{Chl} f(\operatorname{Figure 1A})$, was reported by Chen et al.² Q_y absorption and fluorescence emission bands of Chl f are considerably red-shifted compared with other Chls.

Almost all naturally occurring Chls have central magnesium in the cyclic tetrapyrroles,¹ except for zinc bacteriochlorophyll (BChl) *a* in *Acidiphilium rubrum*.^{3–5} Demetalation compounds of Chls and BChls, namely pheophytin(Phe)s and bacteriopheophytin(BPhe)s, respectively, function as the primary electron acceptors in photosystem (PS) II-type reaction centers.^{6–9} Removal of central metals from chlorophyllous pigments is a key reaction in the early process of

Chl degradation¹⁰⁻¹⁶ and formation of (B)Phe molecules that function in PS II-type reaction centers. Therefore, demetalation of chlorophyllous pigments is important, and the physicochemical properties of demetalation of natural Chls and synthetic metallochlorins have been extensively investigated.¹⁷⁻³⁰

Some of these works indicated that natural Chls possessing a formyl group, namely Chl b, Chl d, and BChl e, had tolerance to removal of central magnesium due to the electron-withdrawing effect of the formyl group.^{17–19,23,26–28,30} Recently, we showed that 7-formylated Chl b exhibited higher resistance to demetalation than 3-formylated Chl d.²⁸ Such difference might be rationalized in terms of the π -electron densities of the chlorin macrocycle, which are reflected in the redox potentials of Chls *a*, b, and $d^{23,28,31}$ Chl b, whose oxidation potential is most positive,³² exhibits the slowest demetalation kinetics among the three Chls. In contrast, Chl a, whose oxidation potential is most negative,³² shows the fastest pheophytinization. Systematic studies of metallochlorins possessing a formyl group in the Aand B-rings of the chlorin macrocycle will be necessary to unravel demetalation properties of formylated chlorins thoroughly. Here we examine demetalation kinetics of Zn 8-formyl-chlorin 1 (zinc methyl 8-deethyl-8-formyl-mesopyropheophorbide a) and Zn 3-formyl-chlorin (2) (zinc methyl pyropheophorbide d) as shown in Figure 1B and compare these properties with those of Zn chlorin 3 (zinc methyl mesopyropheophorbide a), as well as Zn chlorins 4 (zinc methyl pyropheophorbide *a*) and 5 (zinc methyl pyropheophorbide b) in our previous report.³⁰ 8-Formyl-chlorins

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Figure 1. Molecular structures of Chls *a*, *b*, *d*, and f(A) and synthetic Zn chlorins 1-5 (B).

have been synthesized as analogues of 7-formyl-BChls such as BChls e and f, and are appropriate to examine the effect of formyl groups in the B-ring on visible absorption spectra and self-aggregation.^{33,34} 3-Formyl- and 7-formyl-chlorins are good model compounds of Chls d and b, respectively. Comparison of Zn chlorins 1 and 2 is appropriate to study the substituent effects of formyl groups conjugated to the A- and B-rings of the chlorin macrocycle on demetalation in detail without other effects, since both are regioisomers (1, 3-ethyl-8-formyl; 2, 3-formyl-8-ethyl). In the present study, we targeted demetalation properties of these chlorins to investigate the effects of formyl groups directly conjugated to the A- and B-rings of the central zinc metal.

EXPERIMENTAL SECTION

Apparatus. Visible absorption spectra were measured using a Shimadzu UV-2450 spectrophotometer, where the reaction temperatures were regulated with a Shimadzu thermoelectric temperature-controlled cell holder TCC-240A. High-performance liquid chromatography (HPLC) was carried out using a Shimadzu LC-20AT pump and SPD-M20A or SPD-20A detectors.

Materials. Zn chlorins 1–3 were synthesized according to previous works^{33–38} and were purified on a reverse-phase HPLC column 5C₁₈-AR-II (10 mm i.d. × 250 mm, Nacalai Tesque) with methanol at a flow rate of 1.0 mL min⁻¹.

Measurements of Demetalation Processes. Demetalation reaction was done in essentially the same procedure as previous works.^{22–30} Soret peak absorbances of Zn chlorins 1–3 were adjusted to 1.0 in 3.0 mL of acetone and the solution was mixed with 975 μ L of distilled water. Twenty-five μ L of 6.0 M aqueous hydrochloric acid was added to the solution and mixed immediately, and Soret peak absorbance was monitored in measurements of demetalation kinetics. Spectral changes of 3 (Figure 2C) were measured by changing the concentration of the aqueous hydrochloric acid from 6.0 to 1.0 M.

RESULTS

Spectral Changes through Demetalation. Figure 2 shows spectral changes through demetalation of Zn chlorins 1-3 in



Figure 2. Spectral changes of Zn chlorins 1 (A), 2 (B), and 3 (C) in acetone/water (3/1, vol/vol) at the proton concentrations of 3.75×10^{-2} , 3.75×10^{-2} , and 6.25×10^{-3} M at 25 °C. 1: Spectra from 0 to 16 h at 30 min intervals. 2: Spectra from 0 to 15 h at 30 min intervals. 3: Spectra from 0 to 150 min at 5 min intervals. Arrows show the direction of the spectral changes.

acetone/water (3/1, vol/vol) at the proton concentration of 3.75 $\times 10^{-2}$, 3.75×10^{-2} , and 6.25×10^{-3} M, respectively, at 25 °C. Zn chlorin 1 exhibited intense Soret and Qy bands at 450 and 660 nm, respectively, before demetalation. The Soret absorption band gradually disappeared under the acidic condition and a new peak characteristic of the free-base form of 1 appeared at 432 nm. Small absorption bands of 1 in the range between 500 and 600 nm were also gradually changed, and the isosbestic points could be observed at 441, 478, 543, 586, 595, 608, 658, and 662 nm. The Q_{y} peak position of 1 was not shifted, and the Q_{y} bandwidth became sharp in the demetalation process. In demetalation of Zn chlorin 2, Soret and Q_y absorption bands at 450 and 688 nm, respectively, decreased with an appearance of new bands of the free-base form at 427 and 692 nm. The isosbestic points were also observed at 439, 479, and 572 nm through the demetalation reaction of 2. In the case of demetalation of Zn chlorin 3, Soret and Q_y absorption bands at 424 and 648 nm, respectively, decreased with the appearance of new bands at 407 and 655 nm, which were ascribed to a demetalation product of 3. The isosbestic points were positioned at 416, 447, 551, and 655 nm through the demetalation process.

Demetalation Kinetics. Soret peak positions of Zn chlorins 1-3 were clearly different from those of the corresponding freebase forms. Therefore, demetalation kinetics of these Zn chlorins



Figure 3. Kinetic plots for demetalation of Zn chlorins 1 (open circles), 2 (open triangles), and 3 (open squares) in acetone/water (3/1, vol/ vol) at the proton concentration of 3.75×10^{-2} M at 25 °C. Absorbance changes were monitored at 450, 450, and 424 nm for 1, 2, and 3, respectively. A_0 , A, and A_{∞} are Soret absorbances of 1-3 at the onset of measurement, at time t, at the complete demetalation, respectively. Inset: magnified kinetic plot of 3.

can be quantitatively analyzed by monitoring absorbance changes at Soret peak positions of the Zn complexes. Figure 3 depicts the time dependency of Soret peak absorbances of 1-3 through demetalation at 25 °C. The logarithms of the Soret absorbances of 1-3 exhibited linear time dependence, indicating that the demetalation reactions of 1-3 could be regarded as pseudo-first-order reactions. This is in line with the reaction conditions under which the proton concentration $(3.75 \times 10^{-2} \text{ M})$ was much higher than that of Zn chlorins $(<10^{-5} \text{ M}).^{22-30}$ Figure 3 clearly demonstrates that removal of central zinc from 1 and 2, which possessed the 8-formyl and the 3-formyl group, respectively, was significantly slower than that from 3 possessing no formyl group.

Demetalation rate constants, *k*, were obtained by fitting the time courses of Soret peak absorbances as shown in Figure 3 to the following kinetic equation

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

where A_0 , A, and A_∞ are Soret peak absorbances of Zn chlorins 1–3 at the onset of measurements, at time t, and the complete demetalation, respectively.^{22–30} The k-values of 1–3 were determined to be 3.2×10^{-3} , 3.8×10^{-3} , and 2.2 min^{-1} , respectively, in aqueous acetone at the proton concentration of 3.75×10^{-2} M at 25 °C. These values were the averages of more than three independent measurements, and the standard deviations were 3.0, 1.6, and 2.9% of the average values for 1–3, respectively.

The averages of k-values of 1 and 2 were 690- and 580-fold smaller than that of 3, respectively. Comparison of the k-values between regioisomers 1 and 2 indicated that the 8-formyl group provided slightly higher resistance to demetalation than did the 3-formyl group.

Temperature Dependence of Demetalation Rate Constants. Demetalation rate constants of Zn chlorins 1-3 were measured at 15, 25, and 35 °C, where measurements at each temperature were carried out more than three times. Demetalation rate constants of 1-3 became small with a decrease of the reaction temperature. Figure 4 shows Arrhenius plots of the averaged *k*-values of 1-3 in the temperature range between 15 and 35 °C. Activation energies, *E*, of demetalation were estimated by fitting the temperature-dependent *k*-values to the following Arrhenius equation



Figure 4. Arrhenius plots over temperature range of 15–35 °C for demetalation reaction of Zn chlorins 1 (open circles), 2 (open triangles), and 3 (open squares) in acetone/water (3/1, vol/vol) at the proton concentration of 3.75×10^{-2} M.

$$\ln k = -(E/RT) + B \tag{2}$$

where *R* is the gas constant. The Arrhenius plots in Figure 4 gave *E*-values of 99, 99, and 81 kJ mol⁻¹ for 1–3, respectively. The difference of the activation energies between 1 and 3 was 18 kJ mol⁻¹, which was the same as that between 2 and 3. These indicated that Zn chlorins possessing a formyl group in the A- or B-ring had higher activation energies of demetalation reaction than that of the compound lacking a formyl group.

DISCUSSION

The present study indicates that regioisomeric Zn chlorins **1** and **2** possessing a formyl group at the 3- or 8-position, respectively, exhibited significantly slower demetalation kinetics than the reference compound **3** lacking a formyl group. Effects of substituents in the A- and B-rings of the chlorin macrocycle on removal of central zinc are discussed as follows, based on the present results and the previous report on **4** (zinc methyl pyropheophorbide *a*) and **5** (zinc methyl pyropheophorbide *b*).³⁰

Effect of 3-Ethyl and 3-Vinyl Groups. First, the 3-ethyl and vinyl effects on demetalation kinetics should be discussed to clearly understand substitution effects of formyl groups in chlorin molecules. Comparison of demetalation kinetics between Zn chlorins 3 and 4 revealed that transformation of the 3-ethyl to 3-vinyl group decreased the demetalation rate constants (k(4)/ $k(3) = 1.5 \times 10^{-1}$ at 25 °C). This would originate from the difference of electronegativity between a vinyl group and an ethyl group.³⁹ The effect of the 3-vinyl group on demetalation of chlorophyllous pigments was first clarified in the present study, and should be taken into account for unraveling demetalation properties of cyclic tetrapyrrole molecules including natural Chls. We previously reported that demetalation kinetics of Chl bpossessing the 7-formyl group was slower than that of Chl dpossessing the 3-formyl group $(k(\operatorname{Chl} b)/k(\operatorname{Chl} d) = 1.8 \times 10^{-1}$ at the proton concentration of 1.2×10^{-3} M at 25 °C), and this difference was interpreted to be mainly due to the substitution

compd	R ₃	R ₇	R ₈	rate const at 25 °C k/\min^{-1}	activation energy $E/kJ \text{ mol}^{-1}$	
1	CH ₂ CH ₃	CH ₃	СНО	$3.2 imes 10^{-3}$	99	this work
2	СНО	CH_3	CH ₂ CH ₃	3.8×10^{-3}	99	this work
3	CH ₂ CH ₃	CH ₃	CH ₂ CH ₃	2.2	81	this work
4	CH=CH ₂	CH ₃	CH ₂ CH ₃	3.4×10^{-1}	84	ref 30
5	CH=CH ₂	СНО	CH ₂ CH ₃	$7.9 imes 10^{-4}$	96	ref 30

Table 1. Summary of Demetalation Properties of Zn Chlorins 1–5 in Acetone/Water (3/1, vol/vol) at the Proton Concentration of 3.75×10^{-2} M

effects of the 3-formyl and 7-formyl groups in the chlorin macrocycle.²⁸ However, the effect of the 3-vinyl group of Chl *b* cannot be ruled out in discussing the difference of demetalation properties between Chls *b* and *d*. The ratio of demetalation constants of Chl *b* to Chl *d*, k(Chl b)/k(Chl d), was close to that of 4 to 3, k(4)/k(3), suggesting that the 3-vinyl effect was concerned in slower demetalation kinetics of Chl *b* than Chl *d*.

Effect of 7-Formyl and 8-Formyl Groups. Our present and previous studies^{26–28,30} indicate that both the 7- and 8-formyl groups provide resistance to demetalation of chlorin molecules. The ratio of demetalation rate constants of 7-formyl-chlorin 5 to 7-methyl-chlorin 4, k(5)/k(4), is 2.3×10^{-3} , whereas the ratio of 8-formyl-chlorin 1 to 8-ethyl-chlorin 3, k(1)/k(3), is 1.5×10^{-3} . The activation energy of demetalation of 1 (99 kJ mol⁻¹) was similar to the reported value of 5 (96 kJ mol⁻¹).³⁰ These suggest that effects of the 8-formyl group on demetalation properties are analogous to those of the 7-formyl groups in the B-ring would result in resistance of demetalation of chlorin molecules in a similar manner.

Effect of Formyl Groups in the A- and B-Rings. Formyl groups directly linked to the A- and B-rings are responsible for spectral and physicochemical features of natural Chls d and b. Knowledge of the substitution effects of these formyl groups on demetalation will be useful to elucidate the Chl degradation mechanism. From this viewpoint, demetalation kinetics of natural Chls possessing a formyl group has been studied.^{17–19,23,26–28} However, comparison of demetalation properties between Chls b and d in the previous works would include other effects such as that of the 3-vinyl group described above. In contrast, regioisomeric Zn chlorins 1 and 2 in the present study are a good pair to examine substitution effects of formyl groups in the A- and B-rings on demetalation properties without other factors (Table 1).

The demetalation rate constants of 1 possessing the 8-formyl group were on a level similar to those of 2 possessing the 3-formyl group in the temperature range between 15 and 35 °C, although slightly larger *k*-values of 2 than 1 (k(2)/k(1) = 1.2 in 15–35 °C) might be attributable to an inherent difference of formyl groups conjugated to the A- and B-rings. The activation energies of demetalation of 1 and 2 were the same under the present conditions. These results suggest that the formyl group in the A-ring of the chlorin macrocycle has similar effects on demetalation properties of metallochlorins to those in the B-ring.

CONCLUSION

The present study revealed the substitution effects of formyl groups in the A- and B-rings of the chlorin macrocycle on demetalation properties of metallochlorins. Additionally, this study also indicated that the 3-vinyl group made demetalation kinetics slow compared with an ethyl group. The electronwithdrawing 3-, 7-, and 8-formyl groups of chlorin molecules had essentially similar effects on removal of central metal from the chlorin macrocycle, although the formyl groups in the B-ring might have a slightly larger effect than that in the A-ring.

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