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Pheophytinization of Eight Chlorophyll Derivatives in Aqueous Acetone

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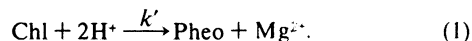
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Synopsis. A significant difference in the pheophytinization rates among four C13²-epimer pairs of chlorophylls is explained in terms of the inductive effects of ring substituents, and/or a strain at the macrocycle core due to the C13² stereochemistry. The rate-determining step appears to involve two protons at high [H⁺] and one proton at low [H⁺].

In a chlorophyll (Chl) molecule, replacement of the central Mg²⁺ ion by two protons gives a pheophytin (Pheo),



Since this reaction is caused by an electrophilic attack of proton on the nitrogen atom(s) coordinating to Mg²⁺, study of the pheophytinization rate provides an insight into the charge distribution and rigidity at the core part of the macrocycle. Pheophytinization is also of major concern in ensuring molecular integrity of Chls submitted to physicochemical characterization or model experiments *in vitro*.

Previously we measured the pheophytinization rates of Chl *a* and its C13²-epimer,¹⁾ Chl *a'*, in aqueous acetone at proton concentrations [H⁺] from 3×10⁻⁴ to 2×10⁻³ M (M=mol dm⁻³).²⁾ As an extension of this, we compare here the pheophytinization rates among eight chlorophylls, namely Chl *a/a'*, Chl *b/b'*, C20-chlorinated Chl (Cl-Chl) *a/a'*, and C13²-hydroxylated Chl (OH-Chl) *a/a'* in a much wider range of [H⁺] (4×10⁻⁵–6×10⁻³ M). The behavior of the primed species (C13²-epimers) is of much interest in view of our recent discovery of two Chl *a'* molecules at the photosystem I core of oxygenic photosynthesis.³⁾ Ring-modified pigments (Cl-Chl *a/a'* and OH-Chl *a/a'*) were examined because they are possibly involved in the metabolic pathway of Chl *a* *in vivo*.^{4,5)}

Experimental

Chl *a* and Chl *b* were extracted from *Chlorella* and were then partially converted into the C13²-epimers (Chl *a'* and Chl *b'*) by treating them with 0.1 M triethylamine in diethyl ether.⁶⁾ Cl-Chl *a* and *a'* were prepared as before.⁴⁾ OH-Chl *a* and *a'* were synthesized, according to Schaber et al.,⁷⁾ by treating Chl *a* with LiBr-saturated tetrahydrofuran for 6 h. All of these products were finally purified by preparative-scale HPLC⁸⁾ to obtain samples of epimeric purity better than 98%. An acetone solution of a given pigment at a concentration of about 10⁻⁶ M was mixed, at a volume ratio of 3:1, with water containing a prescribed amount of HCl to initiate pheophytinization. The proton concentration [H⁺] was calculated as before²⁾ assuming complete dissociation of HCl in aqueous acetone. The

progress of the reaction (formation of a Pheo at the expense of a Chl) was monitored spectrophotometrically at 25°C as in a previous work.²⁾

Results and Discussion

For each pigment the time course of pheophytinization obeyed the first-order kinetics:

$$[\text{Chl}]_t = [\text{Chl}]_0 \exp(-kt) \quad (2)$$

This is as expected because the proton concentration [H⁺] was well in excess of the pigment concentration. The experimentally determined quantity is the pseudo first-order rate constant *k* which may take a form *k'*[H⁺]^{*n*}, where *n* denotes the number of proton(s) involved in the rate-determining step.

In Fig. 1 are plotted the rate constants *k* against [H⁺]

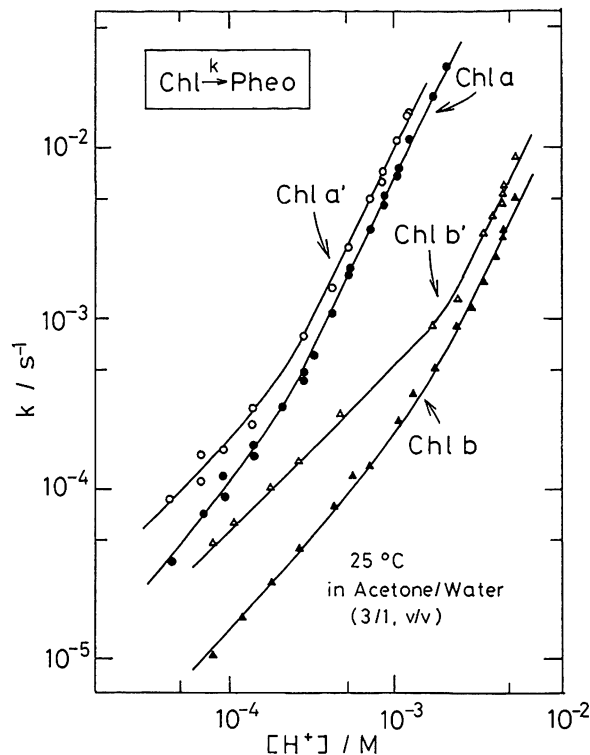


Fig. 1. Pheophytinization rate constants for Chl *a* (●), Chl *a'* (○), Chl *b* (▲), and Chl *b'* (△) as a function of proton concentration [H⁺] in an acetone/water (3/1, v/v) mixture at 25°C.

for the four pigments, Chl *a*, *a'*, *b*, and *b'*. The data for Chl *a* and Chl *a'* in a range $[H^+] = 3 \times 10^{-4} - 2 \times 10^{-3}$ M are essentially the same as those found previously.²⁾ Besides this, the following three features are clearly noted in this Figure: (i) the rate constant *k* for *a*-type pigments is significantly larger than that for *b*-type pigments; (ii) a primed species is more susceptible to pheophytinization than a non-primed species, and this difference becomes more conspicuous at lower $[H^+]$; and (iii) the reaction order of proton (*n* in the $k'[H^+]^n$ above) is strictly 2.0 at higher $[H^+]$ (>0.2 mM for Chl *a/a'* and >2 mM for Chl *b/b'*), but tends to 1.0 with a decrease in $[H^+]$.

Feature (i) is easily rationalized in terms of inductive effects of the substituent on carbon 7. The C7-substituent is electron-donating CH_3 (Hammett meta-substituent constant $\sigma_m = -0.069$) in *a*-type pigments, and is electron-withdrawing CHO ($\sigma_m = 0.355$) in *b*-type pigments. The higher π -electron density in the chlorin macrocycle, and hence on the nitrogen atom, may assist an electrophilic attack of proton resulting in faster pheophytinization of Chl *a/a'* as compared to Chl *b/b'*. A difference in the π -electron density is also reflected in the redox potentials of chlorophyllous pigments.⁹⁾ An exactly inverse relationship holds for the rate of base-catalyzed epimerization ($a \rightleftharpoons a'$ and $b \rightleftharpoons b'$ interconversion),¹⁰⁾ which is initiated by a nucleophilic attack of a base on carbon 13².

Feature (ii) is understood by invoking an enhanced steric hindrance between the C13² methoxycarbonyl group and the C17 propionic acid phytyl ester group protruding to the same side of the molecular plane in the primed pigments.¹¹⁾ The hindrance may generate, to some extent, a strain in the whole macrocycle to weaken the central Mg-N bonds.

Feature (iii) obviously derives from a difference in the rate-determining step as a function of proton concentration. At higher $[H^+]$, two protons are involved there, well in line with scheme (1). At sufficiently low $[H^+]$, in contrast, attack of just one proton must be a slow process and this probably generates an unstable transition state, which then accepts another proton to release the Mg^{2+} ion rapidly. Although the underlying molecular mechanism is still to be elucidated, no report has ever appeared, to our knowledge, on such a second-to-first-order crossover of the initiation mechanism.

Figure 2 displays the *k* values for C20-chlorinated Chl (Cl-Chl) *a* and *a'*: The curve for Chl *a* (Fig. 1) is redrawn for comparison. Features (ii) and (iii) are again clearly noted in this case. The effect of C20-chlorination is, however, substantially smaller than that of C7-formylation (in going from Chl *a/a'* to Chl *b/b'*), despite the fact that σ_m of chlorine (0.373) is fairly close to that of CHO mentioned above. This is presumably due to a back donation of lone pair electrons of Cl to the π -electron system of the macrocycle (resonance effect). A similar argument appears to hold also for the effect of C20-chlorination on the epimerization rates.¹⁰⁾

Figure 3 shows the results for C13²-hydroxylated Chl (OH-Chl) *a* and *a'*. The *k* values are roughly threefold smaller than those of parent Chl *a/a'* in a higher $[H^+]$ range. Since carbon 13² is outside the π -conjugated ring system, it is not clear whether this rate reduction

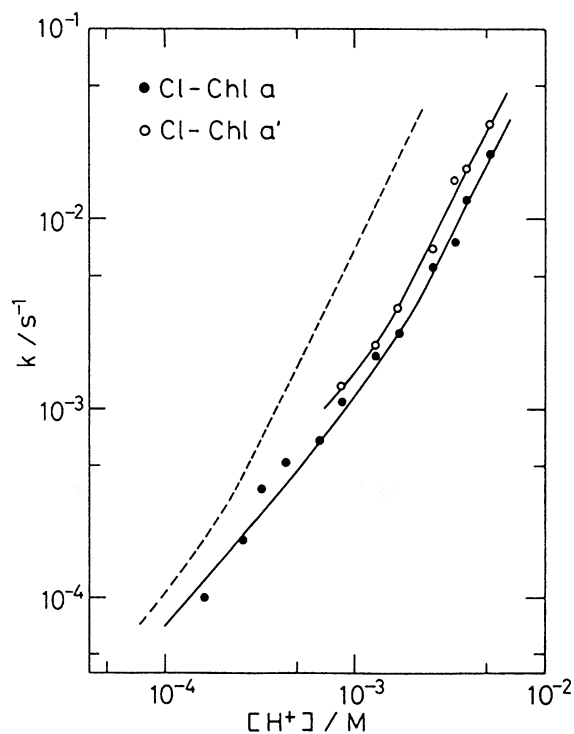


Fig. 2. Same as Fig. 1 but for C20-chlorinated Chl (Cl-Chl) *a* (●) and Cl-Chl *a'* (○). Broken curve is for Chl *a* taken from Fig. 1.

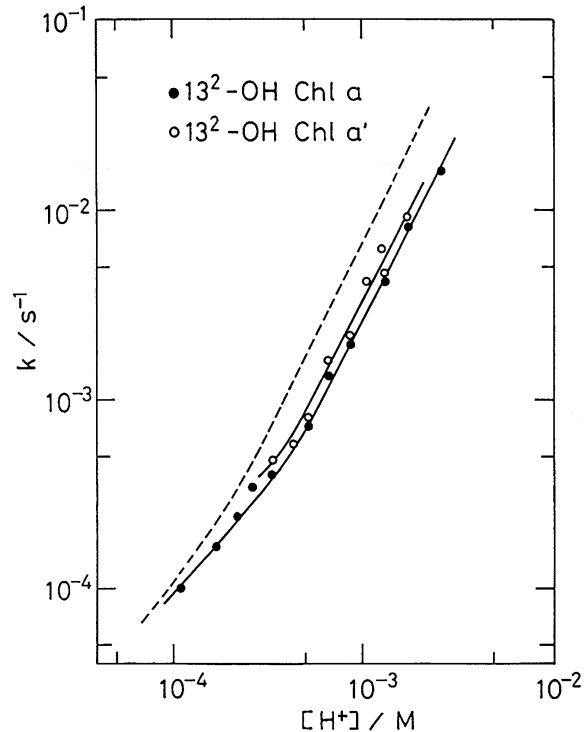


Fig. 3. Same as Fig. 1 but for C13²-hydroxylated Chl (OH-Chl) *a* (●) and OH-Chl *a'* (○). Broken curve is for Chl *a* taken from Fig. 1.

originates in the electron-attracting nature of the OH group ($\sigma_m=0.121$). A large size of the OH moiety may cause a local conformational strain in the cyclopentanone ring. If this strain is transmitted, at least partially, to the whole macrocycle, the k value should be higher than that of a non-hydroxylated pigment. The very small difference in the k value between OH-Chl a and a' suggests that both pigments possess an appreciable steric hindrance of similar degree. However, the steric consideration alone cannot account for the direction of the rate constant shift. Probably the apparent decrease in the k value by C13²-hydroxylation is a compromise between its decrease due to electron attraction by OH and increase due to the Mg-N bond weakening caused by the local steric hindrance.

Previous works on pheophytinization dealt mostly with Chl a and Chl b ,²⁾ because until around 1980 no other pigments were known to exist in vivo. As an example, Haisman and Clarke¹²⁾ report a pseudo first-order rate constant of $55.4 \times 10^{-4} \text{ min}^{-1}$ or $9.2 \times 10^{-5} \text{ s}^{-1}$ for the Chl $a \rightarrow$ Pheo a conversion at 25°C and pH 4. This compares quite well with ours (ca. 10^{-4} s^{-1} at $[\text{H}^+]=10^{-4} \text{ M}$ in Fig. 1). By extrapolation of the linear relationship $k=k'[\text{H}^+]$ found in the present work, we could predict that pure Chl a is converted into Pheo a by about 10% in 30 h even at pH 6. For reliable in vitro characterization of Chl a in aqueous media, the pH value should therefore

be kept above 7 at every stage of handling and measurements.

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