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Self-catalyzed epimerization of chlorophyll *a*/*a*′ in organic solvents *

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Chlorophyll (Chl) *a* and Chl *a*′ in diethyl ether, benzene and hexane underwent epimerization with a rate roughly proportional to the pigment concentration. In contrast, the epimerization rate of pheophytin (Pheo) *a*/*a*′ in diethyl ether remained at a low level over a wide concentration range. A catalytically active site in the Chl *a* or *a*′ molecule was thus assumed to be the central magnesium, as supported by the significant catalytic ability of exogenous magnesium compounds. A magnesium compound presumably interacts with the β-keto ester system of ring V of Chl *a* or *a*′ as an electrophile or an acid catalyst.

Introduction

Chlorophyll (Chl) *a* and its derivatives [1] are the integral molecules driving light energy conversion in photosynthesis of higher plants. These pigments have been extensively studied in vitro for the purpose of elucidating photosynthetic mechanisms or constructing model systems, or from a general viewpoint of complex chemistry. The photosynthetic pigments isolated from plant tissues are, however, susceptible to various transformations, which cause serious alterations in their physicochemical properties. It is hence necessary to quantitate the rates of such molecular transformations under given conditions. Epimerization, or the reversible substituent inversion at C-10 on ring V, is of much practical importance, because it readily occurs in vitro, producing C-10 epimers indistinguishable from each other by their electronic absorption spectra.

In view of this, we attempted to develop a high-resolution separation technique for chlorophylls including the epimeric pair, utilizing a normal-phase silica HPLC [2]. By this method, we have conducted kinetic measurements on Chl *a*/*a*′ epimerization in basic solvents (pyridine and DMF) [3,4] and that catalyzed by coexisting basic compounds [5].

We also observed slow Chl *a*/*a*′ epimerization in neat solvents of negligible basicity. Previous workers described, albeit qualitatively, similar phenomena, but the reported rates exhibit a significant scatter. Katz and co-workers reported an epimerization half-life period of 2 h in tetrahydrofuran and a near-completion period of 24 h in benzene [6], and Hynninen et al. observed the epimer half-lives of 1 h, 24 h and several days in acetone, tetrahydrofuran and benzene, respectively [7]. On the other hand, we [5] noted that Chl *a*′ dissolved at a low concentration in benzene, acetone, diethyl ether, chloroform and hexane was epimerized at rates about two orders of magnitude lower than those described by previous authors.

These observations suggested that Chl *a*/*a*′ epimerization in inert organic solvents proceeds via a mechanism different from usual base-catalysis. In particular, the heavy scatter among the reported epimerization rates appeared to result from a difference in pigment concentration. By carefully studying this aspect, we have demonstrated, to our knowledge for the first time, the occurrence of self-catalyzed epimerization in the Chl *a*/*a*′ system.

Materials and Methods

Chl *a* (epimeric purity 99.1%) and Chl *a*′ (97.0%) were prepared by means of preparative-scale HPLC as described elsewhere [2]. The pigment-containing effluent from the HPLC column was evaporated to dryness in a vacuum line at about 10^{-2} Torr. For comparative measurements, pheophytin (Pheo) *a* (epimeric purity 99.5%) was prepared by acid treatment of Chl *a* followed by HPLC purification.

Diethyl ether, hexane, and benzene of reagent grade

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* Epimerization of chlorophyll derivatives. Part 4 (Part 3: Ref. 5).

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(Wako Pure Chemical Industries) were generally used without further purification. To examine the influence of the stabilizer (3,5-dibutyl-4-hydroxytoluene, < 0.0003%) and residual water, diethyl ether purified by passing through an alumina column and then refluxing over sodium for 4 h followed by fractionation, was also used as a solvent (see below). Benzene was not dried, and hexane was added with 10 vol.% of chloroform (containing 0.5% ethanol as stabilizer) for preventing self-aggregation of the pigment, which is often observed in hydrophobic solvents in the absence of extraneous nucleophiles [8,9]. Other chemicals were methanol of reagent grade (Wako Pure Chemical Industries), and magnesium acetylacetonate ($Mg(acac)_2$, Tokyo Kasei Kogyo) and anhydrous $MgCl_2$ (Junsei Chemical) of extra pure grade, which were used as received.

Each pigment was dissolved in a solvent at a concentration from 0.1 to 2.4 mM, and the solution was kept in darkness under nitrogen atmosphere in a thermostated water-bath at 25°C. The temporal evolution of Chl a/a' and Pheo a/a' composition was measured by means of analytical HPLC [2,10]. The molar fractions of Chl a' or Pheo a' at the onset of measurement, at time t, and at equilibrium are denoted by $[a']_0$, $[a']_t$ and $[a']_\infty$, respectively.

Results

Chl a/a' epimerization in neat solvents

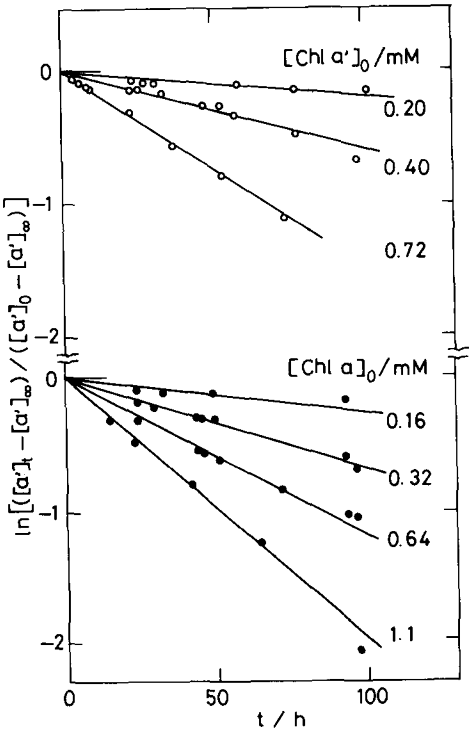

Fig. 2. Kinetic plots for Chl a/a' epimerization according to Eqn. 2 as a function of initial concentration of Chl a or Chl a' ([Chl a]$_0$ or [Chl a']$_0$).

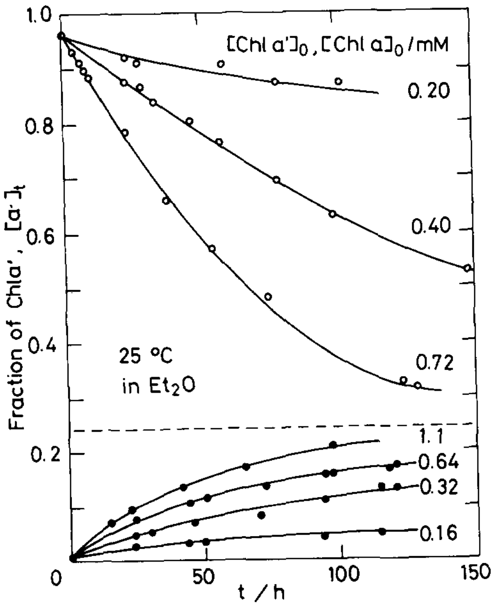

Fig. 1. Temporal evolution of the Chl a/a' composition in neat diethyl ether at various Chl a or Chl a' concentrations at 25°C. Broken line corresponds to the equilibrium composition.

Fig. 1 shows the time-courses of Chl a/a' composition at various pigment concentrations, starting from either Chl a or Chl a' in neat diethyl ether at 25°C. No molecular alterations other than epimerization were noted on HPLC traces. For a 0.72 mM Chl a' solution the equilibrium value was attained in 200 h to be $[a']_\infty = 0.243 \pm 0.005$, which is practically identical with that observed by base catalysis in diethyl ether [5]. The epimerization kinetics were analyzed as in Ref. 5 on the basis of a pseudo-first-order reaction:

$$\text{Chl } a \underset{k'}{\overset{k}{\rightleftharpoons}} \text{Chl } a' \tag{1}$$

$$\ln\{([a']_t - [a']_\infty)/([a']_0 - [a']_\infty)\} = -(k+k')t \tag{2}$$

$$[a']_\infty = k/(k+k') \tag{3}$$

Here k and k' are the apparent pseudo-first-order rate constants. The kinetic plots in Fig. 2 exhibit an approximately linear relationship within 100 h; this justifies the reaction analyses according to formula 1. The rate constants derived from the slope of kinetic plots in Fig. 2 are displayed in Fig. 3 against the initial concentration of Chl a or Chl a'. This concentration is equal to the total concentration of Chl a and Chl a' at a given time. The rough proportionality noted between $k + k'$ and the pigment concentration, irrespective of the starting epimeric composition, indicates that the Chl a or a' molecule itself promoted epimerization in a self-catalytic process. Epimerization of Chl a or Chl a' in purified diethyl ether took place with a $k + k'$ value almost identical to that in untreated diethyl ether at a

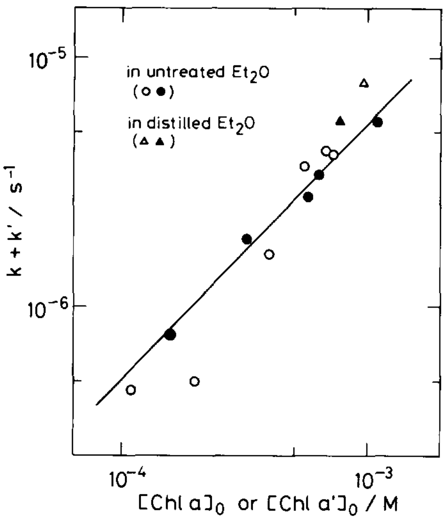

Fig. 3. Dependence of the Chl *a*/*a*′ epimerization rate constant ($k + k'$) on the initial concentration of Chl *a* or Chl *a*′. The starting species is Chl *a* (●, ▲) or Chl *a*′ (○, △).

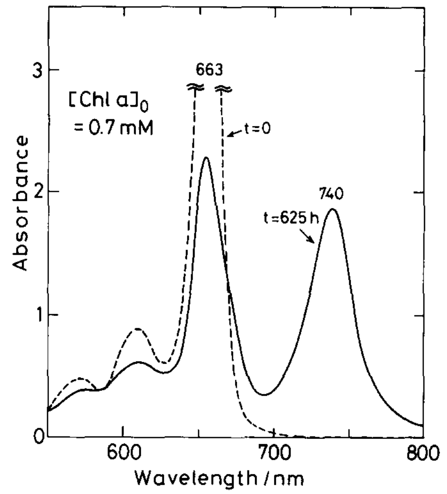

Fig. 5. Absorption spectra of a hexane (+10% chloroform) solution of $[Chl\ a]_0 = 0.7$ mM at the onset of measurement (broken curve) and at 625 h (solid curve). Path length, 1 mm.

common pigment concentration. This indicates that a small amount of coexisting stabilizer or residual water is not involved in the self-catalyzed mechanism in this solvent.

Similar measurements have been done in hexane. In absolute hexane, however, self-aggregation of Chl *a* occurred immediately, thus chloroform (10 vol.%) was added to elevate pigment solubility. In this mixed solvent, a prolonged observation of the Chl *a*/*a*′ epimeric composition revealed the occurrence of an anomalous process depicted in Fig. 4. Initially self-catalyzed epimerization proceeded toward the equilibrium composition, but as time went on, the composition reverted toward pure Chl *a*. The equilibrium epimeric composition $[a']_\infty$ was obtained by base-catalyzed epimerization to be 0.245. Visible spectroscopy showed that this slow process was accompanied by the formation of aggregates, as seen in Fig. 5 (the Chl *a* concentration here is half that for Fig. 4, to record a well-resolved spectrum). The large peak around 740 nm can be assigned to polymeric Chl *a*-water adduct $(Chl\ a\text{-}H_2O)_n$ [11–15] probably due to the action of trace amounts of residual water in this solvent system, and the broadened 663 nm peak at 625 h suggests the existence of self-aggregated species [8,9] besides monomeric Chl *a* and *a*′. At 625 h the solution ($[Chl\ a]_0 = 1.4$ mM; Fig.4) was centrifuged at 10000 × *g* for 5 min, and the supernatant (mainly monomeric Chl *a* and *a*′) and the precipitate (polymeric Chl *a*) were separately analyzed by HPLC. The molar fractions $[a']_t$ were 0.50 in the supernatant, which is in excess of the equilibrium fraction, and 0.00 in the precipitate. The precipitate amounted to 88% of the whole pigments at this time; thus the ratio monomeric Chl *a*:monomeric Chl *a*′:polymeric Chl *a* is 6 : 6 : 88; this is consistent with the data plotted in Fig. 4 ($[a']_t = 0.065$ at 624 h). Based on a reaction scheme depicted in Fig. 4 with pseudo-first-order rate constants k, k' and k_p, the temporal evolution of $[a']_t$ is expressed by the equation:

$$[a']_t = [e^{\alpha t}\{k-(k+k'+\beta)[a']_0\} - e^{\beta t}\{k-(k+k'+\alpha)[a']_0\}] \\ /(\alpha-\beta) \qquad (4)$$

where

$$\alpha = \left[-(k+k'+k_p)+\sqrt{(k+k'+k_p)^2-4k'k_p}\right]/2 \qquad (5)$$

$$\beta = \left[-(k+k'+k_p)-\sqrt{(k+k'+k_p)^2-4k'k_p}\right]/2 \qquad (6)$$

The solid curve in Fig. 4 is the best-fit simulation curve according to Eqn. 4, and demonstrates the validity of this analysis. Such de-epimerization or de-racemization

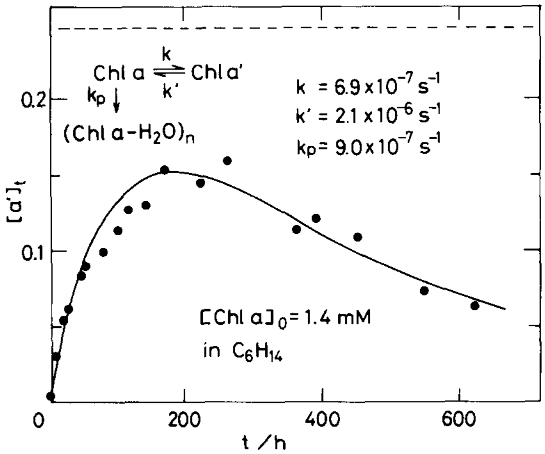

Fig. 4. Self-catalyzed epimerization and de-epimerization of Chl *a*/*a*′ in hexane (+10% chloroform) in prolonged time course.

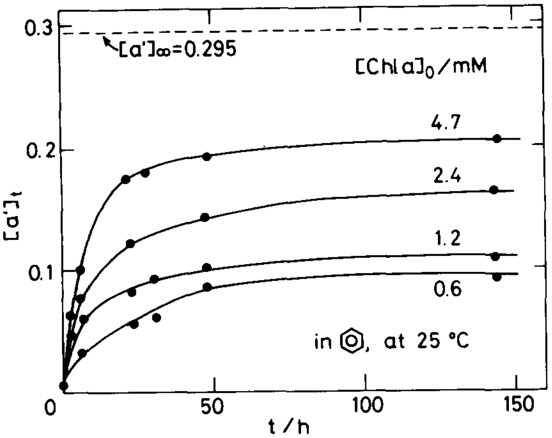

Fig. 6. Temporal evolution of the Chl a/a' composition in neat benzene at various initial concentrations of Chl a at 25°C. Broken line corresponds to the equilibrium composition attained by base-catalysis in this solvent.

through formation of aggregates is known as the asymmetric transformation of second kind [16], of which relatively few examples have been reported to date.

In benzene, Chl a also exhibited an unusual time-course of epimeric composition as shown in Fig. 6, where the epimerization proceeds within 50 h, and then $[a']_t$ value becomes apparently constant at a level lower than the equilibrium composition $[a']_\infty = 0.295$ reached by base catalysis. The visible absorption spectrum of a $[\text{Chl } a]_0 = 0.6$ mM solution at 150 h showed no anomaly such as the formation of self-aggregate reported in benzene [8], but the apparent termination of epimerization suggests the presence of a strong interaction between pigments which would prevent the conformational change at C-10 position. The cause for this unusual phenomenon, however, is not clear at the present stage.

The epimerization rates of Chl a in hexane and benzene were estimated at an earlier reaction stage where the aggregation or anomalous phenomena do not manifest themselves, and are plotted in Fig. 7 against $[\text{Chl } a]_0$ as in Fig. 3. A rough proportionality is again noted between the pigment concentration and $k+k'$ value; this suggests the occurrence of self-catalyzed epimerization also in these solvents. Thus, in inert solvents formula (1) should be converted to the following formula:

$$\text{Chl } a + \underline{\text{Chl } a(a')} \underset{k_t'}{\overset{k_t}{\rightleftharpoons}} \text{Chl } a' + \underline{\text{Chl } a(a')} \tag{7}$$

Here the underlined species denotes Chl a and Chl a' acting as catalysts. The rate constants k_t and k_t' are related to the pseudo-first-order rate constants k and k' by the equations as follows;

$$k + k' = (k_t + k_t') \cdot [\text{Chl } a + \text{Chl } a'] \tag{8}$$

$$k_t/(k_t + k_t') = k/(k+k') = [a']_\infty \tag{9}$$

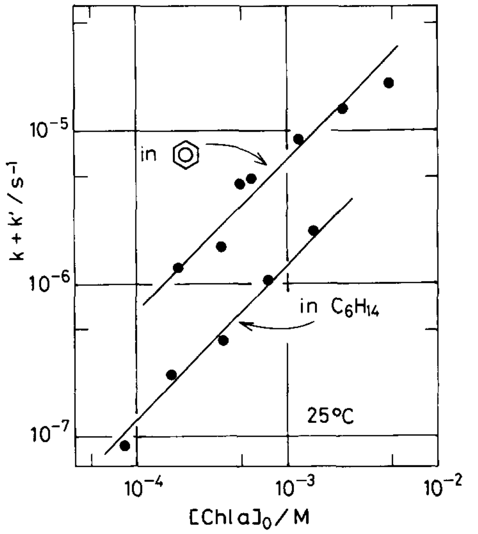

Fig. 7. Dependence of the self-catalyzed Chl a/a' epimerization rate constants ($k + k'$) in benzene and hexane (+10% chloroform) on the initial concentration of Chl a at 25°C. The starting species is Chl a.

where [Chl a + Chl a'] represents the total concentration of Chl a and Chl a' at a given time, which is equal to the initial concentration of the starting species ($[\text{Chl } a]_0$ or $[\text{Chl } a']_0$) and is constant throughout the initial stages of reaction. The $k_t + k_t'$ value was obtained from Fig. 3 or 7 by use of Eqn. 8, and then k_t and k_t' were calculated according to Eqn. 9. The values of k_t, k_t' and $[a']_\infty$ in the three solvents are summarized in Table I.

Pheo a/a′ epimerization in neat diethyl ether

To probe into the molecular mechanism of the Chl a/a' self-catalyzed epimerization, the Pheo a/a' epimerization rate was measured in diethyl ether with Pheo a as the starting species, and the observed first-order rate constants $k + k'$ are plotted in Fig. 8 against the initial concentration of Pheo a ($[\text{Pheo } a]_0$). The equilibrium value $[a']_\infty = 0.176 \pm 0.005$ was determined by base-catalyzed epimerization. As seen, the Pheo a/a' epimerization rate does not depend on the pigment concentration, in spite of the fact that the Pheo a/a' epimerization is base-catalyzed to a greater extent than the Chl a/a' system [5]. This is indicative of the absence of self-catalytic nature in the Pheo a or a' molecule.

TABLE I

Self-catalytic rate constants and equilibrium composition for the Chl a/a′ epimerization in three organic solvents

Solvent	k_t (10^{-3} M^{-1} $\cdot$s^{-1})	k_t' (10^{-3} M^{-1} $\cdot$s^{-1})	$[a']_\infty$
Diethyl ether	1.2	3.9	0.243 ± 0.005
Benzene	1.9	4.4	0.295 ± 0.005
Hexane (+10% chloroform)	0.31	0.98	0.245 ± 0.005

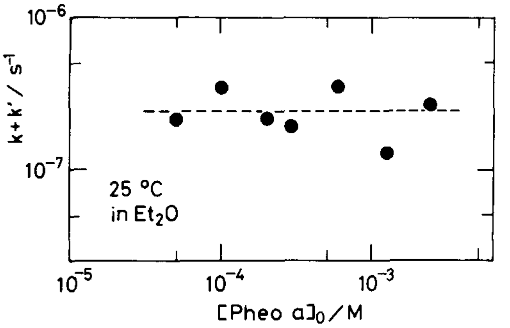

Fig. 8. Dependence of the Pheo a/a' epimerization rate constant $(k + k')$ in diethyl ether on the initial concentration of Pheo a.

Chl a/a′ and Pheo a/a′ epimerization catalyzed by exogenous magnesium compounds

The active site in a Chl a or Chl a' molecule in the Chl a/a' self-catalyzed epimerization was thus suspected to be the central magnesium. In this context the effect of exogenous magnesium compounds in promoting epimerization was examined. Methanol was employed here as a solvent for reasons of solubility of magnesium compounds. To suppress the self-catalyzed epimerization, the pigment concentration here was lowered to 10^{-5} M. Mg(acac)$_2$ indeed accelerated epimerization of both Chl a/a' and Pheo a/a' systems, and the reaction kinetics nicely obeyed Eqn. 2. The values of equilibrium composition $[a']_\infty$ in methanol were 0.17 and 0.13 for Chl a/a' and Pheo a/a', respectively. Allomerization, which generally occurs in methanol [17,18], was not noted up to a period of 6 h. The observed rate constants $(k + k')$ for Chl a/a' and Pheo a/a' at 25°C are plotted in Fig. 9 as a function of Mg(acac)$_2$ concentration. The rate constants are of first order with respect to Mg(acac)$_2$ concentration, and that for Pheo a/a' is about 10-fold larger than that for Chl a/a' under identical conditions. In a separate experiment the effect of the ligand, acetylacetone, was examined by adding it at 0.1 M to Chl a solutions, but no epimerization promotion was noted in this case. The formation constant K_1K_2 of Mg(acac)$_2$ is $10^{6.1}$ in water [19], and is expected to be larger in solvents of lower polarity [20]. Mg(acac)$_2$ is hence considered to exist principally as a neutral molecule in methanol, though the formation constant in methanol is not available in the literature.

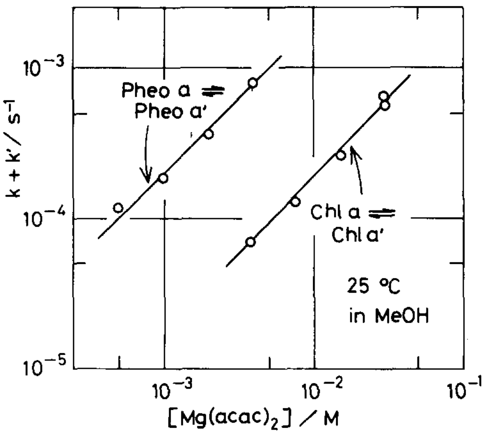

Fig. 9. Dependence of the Chl a/a' and Pheo a/a' epimerization rate constant $(k + k')$ in methanol on the concentration of exogenous Mg(acac)$_2$.

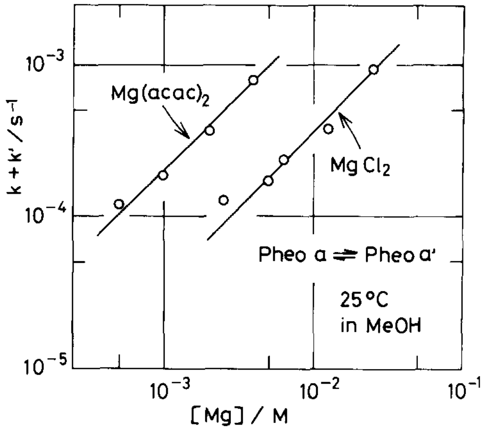

Fig. 10. Dependence of the Pheo a/a' epimerization rate constant $(k + k')$ on the concentrations of exogenous MgCl$_2$ and Mg(acac)$_2$.

On the other hand, anhydrous MgCl$_2$, which is expected to exist in a dissociated state in methanol, promoted epimerization of Pheo a/a' and allomerization of Chl a and a'. The rate constant $k + k'$ for Pheo a/a' epimerization is plotted in Fig. 10 against MgCl$_2$ concentration, in comparison with that catalyzed by Mg(acac)$_2$. Under a given magnesium concentration, the rate constant is larger in Mg(acac)$_2$ catalysis than in MgCl$_2$ catalysis by a factor of 5.8. At $[\mathrm{MgCl_2}] = 50$ mM, where a rapid Pheo a/a' epimerization takes place, the partial formation of a 'peripheral complex', described by Scheer and Katz [21,22], was noticed in the visible absorption spectrum (Fig. 11). Such a peripheral Mg^{2+}-complex of Pheo a enolate ion is supposedly an intermediate state of epimerization in this case. In contrast, no spectroscopic anomaly was noted during epimerization promoted by Mg(acac)$_2$ at the same concentration.

Addition of water (5%, v/v) to the methanolic solution containing a magnesium compound led to an alteration in the rate of Pheo a/a' epimerization. The rate fell back to 23% in the case of MgCl$_2$, whereas it was enhanced 1.7-fold in the case of Mg(acac)$_2$, compared to the rate in absolute methanol. The former may be in line with a previous observation that a small amount of water added to a MgCl$_2$-saturated pyridine solution resulted in disintegration of the Pheo a enol complex [21]. The coordination state of Mg^{2+}, Mg^{2+}(H$_2$O)$_6$, Mg^{2+}(CH$_3$OH)$_6$, or some mixed form thereof, may affect the efficiency of the enol complex formation and thus the epimerization rate. The cause of

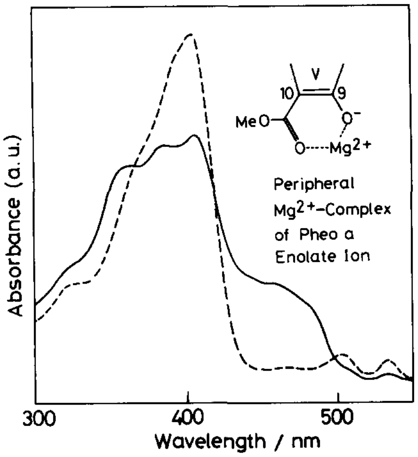

Fig. 11. Absorption spectra of Pheo *a* in neat methanol (broken curve) and in methanol containing 50 mM $MgCl_2$ (solid curve).

the promoting effect of water on the Pheo *a*/*a*′ epimerization catalyzed by $Mg(acac)_2$ is presumably an increase in the solvent polarity.

Allomerization of Chl *a* promoted by $MgCl_2$ in methanol was observed long ago by Johnston and Watson [23]. A preliminary examination in this work suggested that the allomerization kinetics are fairly complicated, but this particular problem is beyond the scope of the present communication.

Discussion

As described above, the Chl *a*/*a*′ epimerization rate in inert organic solvents depends clearly on the pigment concentration. Fairly rapid epimerizations under similar conditions observed by previous authors [6,7] are thus attributed to a relatively high pigment concentration or to contamination with basic compounds. As seen in Fig. 3 nucleophilic water which should have been present in untreated diethyl ether did not affect the self-catalyzed epimerization, but residual water in benzene, and ethanol (stabilizer in chloroform) in hexane, probably played a role in preventing the self-aggregation at an earlier stage of the reaction. The epimerization behavior in benzene needs further investigation, but the feature in Fig. 6 explains well the previously reported low value of $[a']_\infty$ (between 0.15 and 0.20) and a near-completion period as short as 24 h for the Chl *a*/*a*′ epimerization in neat benzene [6]. The results of the present work indicate that enough care has to be taken in handling Chl *a* or other epimerizable pigments, in particular with respect to pigment concentration in organic solvents. Epimers, Chl *a* and Chl *a*′, for instance, are substantially different chemicals, as reflected in differences of CD spectra [2], NMR spectra [6,7,24,25], and pheophytinization rate [26,27].

From the lines in Figs. 3 and 9, the catalytic activity is about 3-times higher for $Mg(acac)_2$ in methanol than the self-catalyzing Chl *a* or *a*′ in diethyl ether at a common magnesium concentration. In view of a 25-fold higher epimerization rate in methanol than in diethyl ether at a common concentration of the catalytic basic compound (unpublished results), however, Chl *a* or *a*′ appears to exhibit a rather strong self-catalytic ability in promoting epimerization, as compared to $Mg(acac)_2$. The catalytic activities of Chl *a* and Chl *a*′ are estimated to be roughly the same in diethyl ether.

A slow epimerization of Pheo *a*/*a*′ in neat diethyl ether independent of the pigment concentration is due probably to the slight electron donating property of the solvent. Such a solvent effect may be involved also in the Chl *a*/*a*′ epimerization, but is practically negligible before the self-catalyzing effect. The solvent effect, which manifests itself as a difference in Chl *a*/*a*′ epimerization rate (cf. Figs. 3, 7 and Table I), may originate from the difference in polarity which should be a crucial factor for reactions involving ionic intermediates.

The formation of a Pheo *a* enol complex with Mg^{2+} in methanol is enlightening to understand the epimerization mechanism. In this case, it is considered that an interaction between Mg^{2+} and the β-keto ester system of ring V of Pheo *a* or *a*′ dissociates the C-10 proton, followed by recombination of the proton to disintegrate the enol complex. $Mg(acac)_2$ and self-catalyzing Chl *a* or *a*′ may exhibit a similar effect, though in these cases the interaction of magnesium should be weaker since the formation of an enol complex was not observed. A faster epimerization catalyzed by $Mg(acac)_2$ than by $MgCl_2$ (Fig. 10) suggests that a neutral molecule promotes epimerization more effectively than an ionic form. It should also be noted that an alcoholic solvent and nucleophiles in benzene and hexane might have acted in mediating the intermolecular interaction, but this was not the case at least for the self-catalysis in diethyl ether. Although the question still remains for the underlying molecular mechanism, it is evident that these magnesium compounds, including self-catalyzing Chl *a* and *a*′, act as electrophiles or acid catalysts, contrasting to the usual base catalysts [5]. Among the reactions catalyzed by both acids and bases are the isomerization of amino acids [28], hydrolysis of esters, and so forth.

An interaction between Mg and the β-keto ester system is seen in the models for Chl *a* aggregates containing intermediate water molecule [12,29,30] or not [31]. Nevertheless the occurrence of epimerization has not been reported in any aggregates, and we verified that the Chl *a* water-adduct consists of Chl *a* alone (Figs. 4 and 5). This is supposedly due to a steric hindrance in the aggregates to restrict a conformational change or to the direct participation of C-10 $COOCH_3$ group in linkage between the molecules. For these reasons, epimerization is unlikely to occur even if an enolization of the β-keto ester system would take place in an aggregate transiently via the mode proposed by Fetterman et al. [32].

Chl *a/a'* and Pheo *a/a'* in methanol showed different transformations by the action of Mg^{2+}, namely allomerization and epimerization, respectively. This suggests that both reactions may involve a common process of enol formation, as proposed in Ref. 17. In addition, the features of previously reported allomerization in neat methanol resembles that of the self-catalyzed epimerization found in the present study, in terms of the reaction rate dependence on Chl *a* concentration [23] and the fairly low rate for Pheo *a* [18]. In the allomerization of Chl *a* in neat solvents, the central magnesium atom in an adjacent molecule may play a catalytic role as in the self-catalyzed epimerization.

Acknowledgments

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