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Chlorophyll *a*/*a'* epimerization in organic solvents *

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Based on HPLC analyses of high resolution, the kinetic and mechanistic features of chlorophyll (Chl) *a* ⇄ *a'* epimerization (reversible stereoisomerization on the cyclopentanone ring) have been studied for the first time in several organic solvents, and in diethyl ether containing a series of basic compounds. The epimerization-promoting effect of a base well parallels its pK_a value, suggesting that epimerization proceeds via general base catalysis. Under favorable conditions the epimerization is completed within 1 min to give an equilibrium composition [Chl *a'*]/[Chl *a*] ≈ 1/3. With imidazole (Im) and its derivatives as bases, the epimerization rate constant is proportional to the squared base concentration, presumably due to the occurrence of a 'tandem general base mechanism' involving an Im dimer with enhanced basicity of one heteronitrogen atom. Chl *a*/*a'* epimerization is promoted also by histidine suspended in diethyl ether. These results are discussed referring to previous qualitative reports and to the epimeric composition of Chl-type pigments in photosynthetic apparatus.

Introduction

Our recent analytical results [1–3] evidence the close association of Chl *a'* (C-10 epimer of Chl *a*, see Fig. 1) with the reaction center of plant Photosystem (PS) I. The Chl *a'*/P-700 molar ratio had been assayed to be around 2 in the initial study [2], but a later work [3] with improved HPLC resolution and on a variety of PS I preparations (with Chl *a*/P-700 molar ratios ranging from about 8 to 1000) and blue-green algae yielded Chl *a'*/P-700 = 1 as a more plausible stoichiometry. Further, we were able to reconstitute a pigment-protein complex exhibiting spectral features reminiscent of P-700 by combining the 65 kDa PSI apoprotein with Chl *a'*, but not with Chl *a* [4]. These findings prompt us to investigate in vivo and in vitro properties of Chl *a'* in detail.

Since the first description of Chl *a'* by Strain and Manning in 1942 [5], and its identification with the C-10 epimer of Chl *a* a few decades later [6–8], it has occasionally been noted that Chl *a*/*a'* epimerization takes place quite easily in vitro [6,7,9–14]. Although the epimers cannot be distinguished from each other by ultraviolet-visible absorption or fluorescence spectroscopy, they behave as basically distinct chemical species in many other aspects (NMR and CD spectra, solubility, chemical reactivity, intermolecular aggregation, etc.) [15]. In any study where Chl *a*, Chl *a'* and

* Epimerization of chlorophyll derivatives. Part 3 (Part 2: Ref. 17).

Abbreviations: Chl, chlorophyll; Pheo, pheophytin; 2-MeIm, 2-methylimidazole; EtIm, 2-ethylimidazole; *N*-MeIm, *N*-methylimidazole; HPLC, high-performance liquid chromatography.

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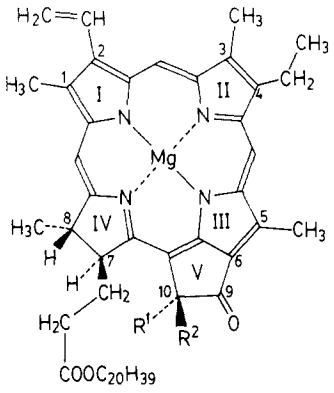

	R^1	R^2
Chl a	$COOCH_3$	H
Chl a'	H	$COOCH_3$

Fig. 1. Structure and carbon numbering for chlorophyll (Chl) *a* and *a'*. Replacement of the central Mg atom with two H atoms gives pheophytin *a* and *a'*.

their derivatives are involved, it is hence of crucial importance to pay proper attention to the rate of epimerization under given experimental conditions. However, due mainly to the lack of an analytical tool enabling rapid and unambiguous determination of the epimer pairs, knowledge of the epimerization rates as well as of the equilibrium epimer compositions has long remained on a qualitative level. For instance, no data are available in the literature on the rate constant for Chl *a*/*a'* epimerization. Quantitation of the epimerization kinetics has become possible only by the recent implementation of normal-phase silica HPLC [16]. Following preliminary works on the pheophytin (Pheo) *a*/*a'* epimerization kinetics [15,17], we present here the results of a similar investigation on the Chl *a*/*a'* epimerization in organic media.

Materials and Methods

Chl *a* (epimeric purity > 99.9%) and Chl *a'* (about 99.5% unless otherwise noted) were prepared by means of preparative-scale HPLC specified in Ref. 15, except that Chl *a'* was obtained here by treating Chl *a* with triethylamine. For comparative measurements, Pheo *a'* (> 80% in epimeric purity) was prepared in a similar manner. Each pigment was dissolved at a concentration from 0.02 to 0.1 mM in an organic solvent of 3 to 10 ml with or without an additive. Diethyl ether, pyridine, *N*,*N*-dimethylformamide, benzene, chloroform, acetone, hexane (containing 1.5 vol% 2-propanol for reasons of pigment solubility), methanol, and ethanol were used as solvents. For two epimerization-promoting solvents, pyridine and dimethylformamide (see below), the results before and after their distillation were compared to check for the effect of impurities. Imidazole, 2-methylimidazole, 2-ethylimidazole, *N*-methylimidazole, triethylamine, piperidine, pyrrolidine, pyrrole, pyridine, dimethylformamide, histidine, arginine, and adenine were employed as additives in diethyl ether solutions. The last three compounds, being insoluble in diethyl ether, were used in a suspended state. All these chemicals were of reagent grade. A deoxygenated solution (or suspension) containing Chl *a* or Chl *a'* was left standing in darkness under nitrogen atmosphere in a thermostated water bath, and the temporal evolution of the Chl *a*/*a'* molar ratio was measured by the analytical HPLC specified in Ref. 1. By keeping the column temperature at 0 °C and the total elution time within 15 min, any pigment degradation or epimerization during passage through the silica HPLC column is negligible, as evidenced by our success in recording a chromatogram of 99.99% pure Chl *a* [1].

The Chl *a*/*a'* epimerization is described by the following formula:

$$\text{Chl } a \underset{k'}{\overset{k}{\rightleftarrows}} \text{Chl } a' \qquad (1)$$

Here k and k' are pseudo-first-order rate constants under the condition, as in the present experiments, that the Chl *a*/*a'* concentration is much lower than that of a coexisting substance. The kinetics are analyzed as follows. We denote the mole fraction of Chl *a'* (ratio of Chl *a'* concentration to Chl *a* + Chl *a'* total concentration) at the onset of measurement, at time t, and at equilibrium by $[a']_0$, $[a']_t$ and $[a']_\infty$, respectively. Then formula (1) is equivalent to the kinetic equation:

$$\frac{[a']_t - [a']_\infty}{[a']_0 - [a']_\infty} = \exp[-(k + k')t] \qquad (2)$$

with

$$[a']_\infty = k/(k+k') \quad (3)$$

Thus, starting from any off-equilibrium epimer composition, the values of k and k' are calculated by fitting an experimental $[a']_t$ vs. t profile to Eqn. 2. When epimerization is sufficiently fast, the value of $k/(k+k')$ is obtained readily via Eqn. 3 by measuring $[a']_\infty$. The epimerization time constant is given by $(k+k')^{-1}$, and the degree of epimerization DE at time t, defined such that $DE = 100\%$ at equilibrium and $DE = 0\%$ for any off-equilibrium composition at the start of measurement, is given by $100 \times ([a']_0 - [a']_t)/([a']_0 - [a']_\infty)$.

Results

General features of Chl a/a' epimerization

HPLC traces showing the time-course of epimerization under three different conditions are given in Fig. 2, for the case of 99.5% Chl a' as the starting composition. In neat diethyl ether at 10 °C, only about 7% of Chl a' is converted to Chl a in 150 h. Raising the temperature to 25 °C results in an increase in the epimerization rate. Addition of imidazole 100 mM markedly enhances the rate of epimerization, and an equilibrium molar ratio [Chl a']/[Chl a] ≈ 1/3, or $[a']_\infty \approx 0.25$, is attained within 20 h. In terms of the degree of epimerization defined above, $DE \approx$ 10% (10 °C) and ≈ 28% (25 °C) at 150 h in neat diethyl ether, but as high as ≈ 66% (25 °C) at 6 h in the presence of 100 mM imidazole. In deoxygenated diethyl ether, and in other solvents employed here excluding methanol and ethanol (see below), very few molecular alterations other than epimerization were noted on HPLC charts up to 100 h.

Fig. 3 illustrates typical time-courses of Chl a/a' composition, starting from either Chl a or Chl a' at two temperatures in neat dimethylformamide. The curves are drawn according to Eqn. 2, and best-fit k and k' values, applicable commonly to the two experimental $[a']_t$ vs. t profiles at each temperature, are thus obtained. As is seen,

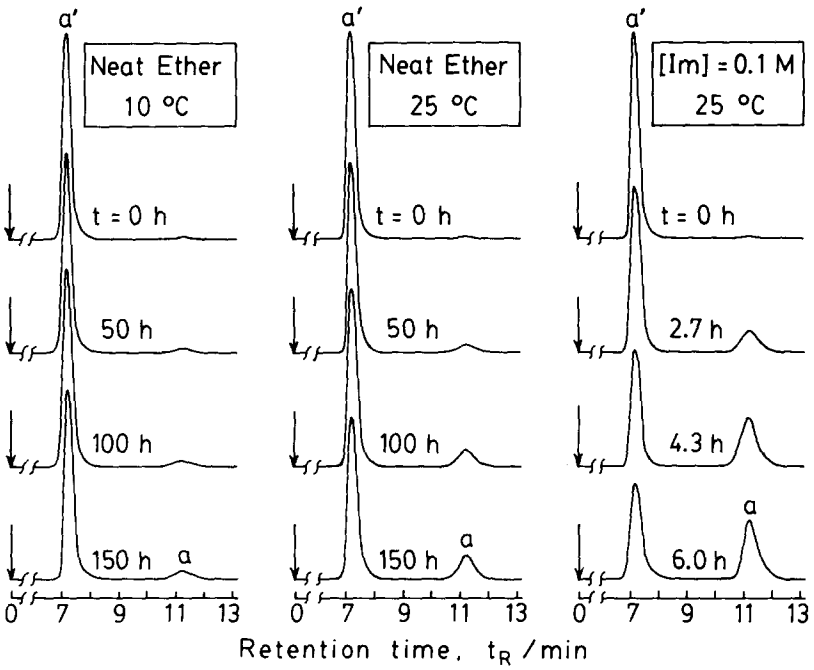

Fig. 2. HPLC traces for the time-courses of Chl a/a' epimerization under three different conditions in diethyl ether. The initial composition is Chl a'/Chl a = 99.5/0.5, and the equilibrium composition (not yet reached here) is Chl a'/Chl a = 24.1/75.9 in each case. See Refs. 1 and 16 for details of HPLC analytical conditions.

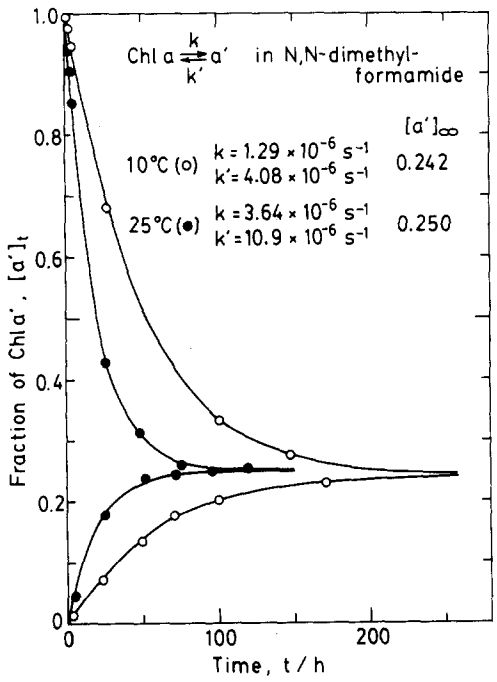

Fig. 3. Temporal evolution of the Chl a/a' composition in neat N,N-dimethylformamide at two temperatures.

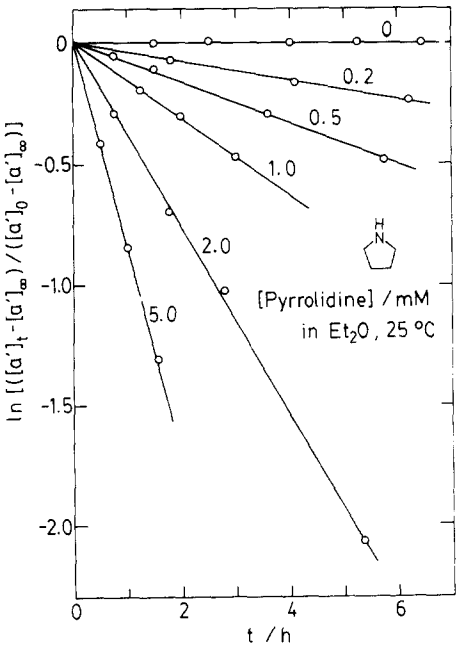

Fig. 4. Kinetic plots, according to Eqn. 2, for Chl a/a' epimerization as a function of pyrrolidine concentration in diethyl ether.

the simulation is satisfactory, and the epimerization rate constant exhibits a significant temperature dependence. The equilibrium mole fraction $[a']_\infty$ does not change much with temperature, suggesting that the enthalpy difference is small between the Chl a/a' pair, as was also the case for the Pheo a/a' system [17].

Base catalysis of Chl a / a' epimerization

With diethyl ether as a common, substantially inert solvent (cf. Fig. 2), the effects of a series of additives in promoting Chl a/a' epimerization have been examined. Out of ten nitrogen-containing compounds soluble in diethyl ether, six (pyrrolidine, piperidine, triethylamine, imidazole, 2-methylimidazole and 2-ethylimidazole) showed remarkable epimerization-promoting activities at concentrations below about 100 mM. Figs. 4 and 5 illustrate, as typical examples, the temporal evolution of the natural logarithm of the left-hand side of Eqn. 2 at different concentrations of pyrrolidine and imidazole, respectively. The slope of a line gives the $-(k + k')$ value. Visual inspection of Figs. 4 and 5 suggests that the epimerization rate depends roughly linearly on pyrrolidine concentration, but more strongly on imidazole concentration.

This feature is made conspicuous in Fig. 6, where the rate constant $k + k'$ is plotted against molar concentration C_{base} for the six epimerization-promoting compounds. For pyrrolidine, piperidine and triethylamine, the Chl a/a' epimerization rate is proportional to C_{base}, and the order of rate constants apparently parallels that of pK_a values of their conjugate acids in aqueous solutions. This strongly suggests that epimerization proceeds via general base catalysis, in which abstraction of the C-10 proton by a base (cf. Fig. 7), yielding transiently an enol form with the C-10–$COOCH_3$ bond oriented coplanarly to ring V, is followed by reattachment of the proton, thereby regenerating either Chl a or Chl a' to a ratio determined by the free energy difference between them.

An upper limit of the $k + k'$ value readily accessible in these (slow) measurements is around 10^{-3} s^{-1}, which corresponds to an epimerization time constant of about 20 min. Extrapolation of

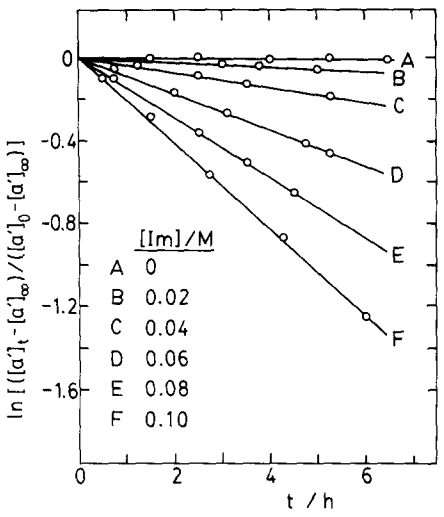

Fig. 5. Same as Fig. 4 as a function of imidazole (Im) concentration.

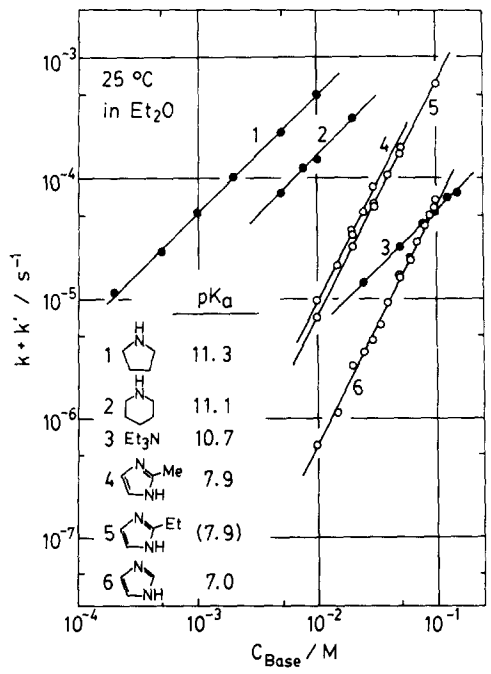

Fig. 6. Dependence of the Chl a/a' epimerization rate constant, $k + k'$, on the concentration of six basic compounds. The slopes are 1.0 for lines 1–3, and 2.0 for lines 4–6. The pK_a for 2-ethylimidazole, not available in the literature, is assumed to be the same as that of 2-methylimidazole.

curve 1 in Fig. 6 to $C_{base} = 1$ M gives $k + k' = 5 \cdot 10^{-2}\ s^{-1}$, or an epimerization time constant of 20 s. Indeed, in diethyl ether containing 1 M pyrrolidine, the near-completion of epimerization, as verified by establishment of a stationary composition [Chl a']/[Chl a] ≈ 1/3, was confirmed within 1 min, irrespective of the starting Chl a/a' composition.

Fig. 6 shows that imidazole and its derivatives also promote Chl a/a' epimerization, though they possess pK_a values much lower than those of the three bases mentioned above. Here again, the order of rate constants seems to parallel that of pK_a. However, the slope of $\log(k + k')$ vs. $\log C_{base}$ plots is 2.0 in these cases. This unexpected finding could be accounted for by invoking a 'tandem general base mechanism' (Fig. 7), which had been originally proposed to elucidate imidazole-catalyzed ester hydrolyses [18–20]. A high tendency of imidazole to form dimers and larger aggregates via N ··· H-N hydrogen bonding is well known [21,22], and a recent ab initio molecular orbital calculation on hydrogen-bonded imidazole dimer [22] has unraveled the occurrence of a partial electron flow from one molecule to the other, thereby increasing the electronic density (and hence the basicity) on a heteronitrogen atom of the acceptor-side molecule. We thus suspect that the imidazole and its derivatives can effectively base catalyze the Chl a/a' epimerization only in the state of dimers.

Under identical conditions, the Pheo a/a' system undergoes epimerization at a rate 50- to 200-fold higher than that for the Chl a/a' system. Thus, at 10 °C, the $k + k'$ values for Pheo a/a' epimerization are $1.23 \cdot 10^{-3}$ and $1.42 \cdot 10^{-4}\ s^{-1}$ in neat *N,N*-dimethylformamide and pyridine, respectively [17], while the corresponding figures for

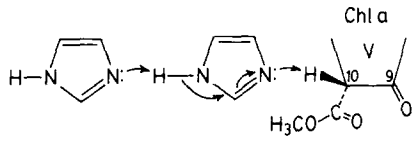

Fig. 7. Schematic illustration of a 'tandem general base mechanism' [18–20] for the initiation of Chl a/a' epimerization by imidazole. Only the cyclopentanone ring V (cf. Fig. 1) of Chl a is drawn.

Chl a/a' epimerization are $5.5 \cdot 10^{-6}$ and $2.8 \cdot 10^{-6}$ s^{-1} (see below). In view of this, we expected that the Pheo a/a' epimerization would be catalyzed by monomeric as well as dimeric imidazole. The result given in Fig. 8 shows this is indeed the case; the apparent slope of 1.60 is indicative of the operation of both mechanisms in Pheo a/a' epimerization.

In the course of epimerization, the transient proton removal from carbon 10 must accompany some deformation, or a change in the degree of strain, of the cyclopentanone ring V (cf. Fig. 1). This process would be facilitated if such an additional strain could be readily dissipated toward the main body of the molecule, namely the tetrapyrrole ring. Therefore, the higher epimerization rate constant for the Pheo a/a' as compared to the Chl a/a' system is most likely a consequence of the much higher flexibility of the tetrapyrrole ring in the former by virtue of the absence of the central Mg atom.

N-Methylimidazole ($pK_a = 7.1$–7.3) is a slightly stronger base than imidazole ($pK_a = 7.0$), but did not show noticeable epimerization-promoting activity at concentrations below 100 mM. Only at much higher concentrations did it catalyze Chl a/a' epimerization, with rate constants roughly proportional to concentration ($k + k' = 2.3 \cdot 10^{-5}$ s^{-1} at [*N*-MeIm] = 1.0 M, and $1.2 \cdot 10^{-4}$ s^{-1} at [*N*-MeIm] = 5.0 M in Et_2O at 25 °C). This is certainly due to the inability of *N*-MeIm to form a hydrogen-bonded dimer as the one illustrated in Fig. 7. Pyrrole, while possessing an -NH moiety, is not a base ($pK_a = -3.8$); no Chl a/a' epimerization was noted in diethyl ether containing 100 mM pyrrole. Pyridine ($pK_a = 6.0$) and dimethylformamide (pK_a unknown) did not, within experimental error, promote Chl a/a' epimerization when added in diethyl ether at concentrations below 100 mM, whereas they are promoters when used as solvents (see below). Extrapolation of the rate constants obtained at 25 °C in neat liquids (formal molarity: 12.9 M for dimethylformamide and 12.4 M for pyridine) to a concentration of 100 mM yields the $k + k'$ values of $1.2 \cdot 10^{-7}$ and $9.7 \cdot 10^{-8}$ s^{-1} for dimethylformamide and pyridine, respectively; these are indeed well below the $k + k'$ value ($6.0 \cdot 10^{-7}$ s^{-1}) for Chl a/a' epimerization in neat diethyl ether employed as solvent.

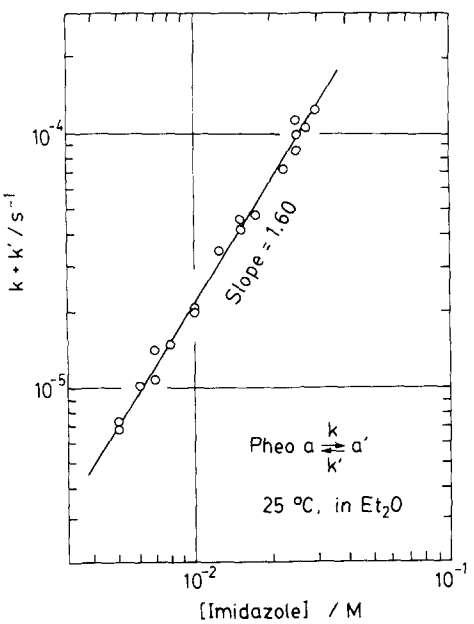

Fig. 8. Dependence of the Pheo a/a' epimerization rate constant $k + k'$ on imidazole concentration.

Chl a/a' epimerization in neat solvents

The stability, and its temperature dependence, of chlorophyll derivatives against epimerization as well as other molecular degradations should be of primary concern in handling the pigments in vitro and also in treating plant tissues with solvents. In view of this, measurements were performed on the rate and its temperature dependence of Chl a/a' epimerization in nine organic solvents listed in Materials and Methods. Fairly rapid epimerization took place in dimethylformamide and pyridine, for which the kinetic plots according to Eqn. 2 at three temperatures are given in Fig. 9. These data are for reagent-grade solvents used as received, but in freshly distilled solvents the slopes of the kinetic plots agreed with those in Fig. 9 to within 2% for dimethylformamide and to within 10% for pyridine. The possibility that any basic impurities are acting as epimerization promoters in these solvents, could thus be excluded. In other seven organic solvents employed, the Chl a/a' epimerization rates were at least an order-of-mag-

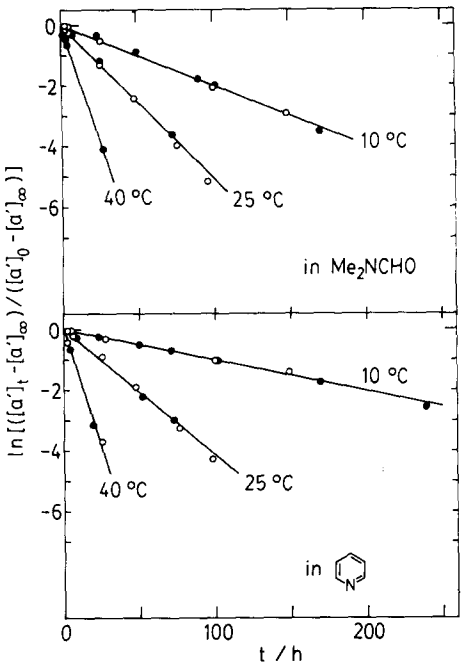

Fig. 9. Kinetic plots for Chl a/a' epimerization in neat *N*,*N*-dimethylformamide and pyridine at three temperatures. The initial species were either Chl a (●) or Chl a' (○). The data points for 10 and 25 °C in dimethylformamide are the same as those already given in Fig. 3.

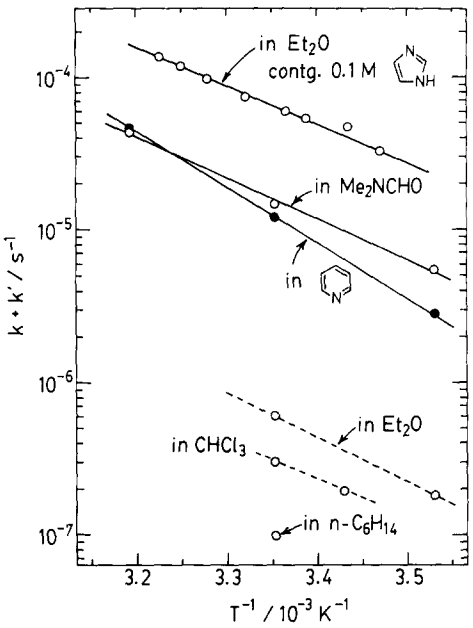

Fig. 10. Arrhenius plots over a temperature range 10–40 °C for Chl a/a' epimerization. See text for the rate constants in acetone, benzene, methanol, and ethanol.

nitude lower than those in dimethylformamide and pyridine.

Some Arrhenius plots for the rate constant $k + k'$ are displayed in Fig. 10, together with that in diethyl ether containing 100 mM imidazole for comparison. In most cases investigated here, the equilibrium Chl a' mole fraction, $[a']_\infty$, is 0.24 ± 0.01, or $k/k' = 0.32 \pm 0.02$ (cf. Eqn. 3), with little temperature dependence. In pyridine alone, the $[a']_\infty$ value is somewhat smaller, 0.19 ± 0.01. The activation energy, E_a, of Chl a/a' epimerization is 46.5 ± 0.5 kJ · mol^{-1} in most media. Here again, the result in pyridine is exceptional, $E_a = 69.1$ kJ · mol^{-1}. The smaller $[a']_\infty$ and higher E_a in pyridine, than in dimethylformamide, are what were observed also for Pheo a/a' epimerization [17]. Elucidation of such peculiarity of pyridine is however beyond the scope of the present paper. The epimerization rate constants in benzene and acetone are not plotted in Fig. 10 because of a poor reproducibility noted from one measurement to another, for some unclarified reason. Nevertheless the $k + k'$ values in these two solvents were usually between those in hexane and in diethyl ether, namely from 10^{-7} to 10^{-6} s^{-1} at 25 °C.

In the seven organic solvents mentioned thus far, practically no molecular alterations other than Chl a/a' epimerization were observed up to a period of 50–100 h at 25 °C, as long as the solution was kept under nitrogen atmosphere and in darkness. In contrast, the occurrence of drastic molecular degradations was noted in methanol and ethanol. Thus, formation of at least seven alteration products was clearly discerned on HPLC traces already at 10 h after dissolution of Chl a or Chl a', and at 26 h the fraction of the Chl a/a' pair over the total amount of pigments became only 10–20%; this strongly interfered with precise determination of epimerization rate constants. Use of methanol for plant pigment extraction also led to substantial alteration of chlorophylls (unpublished results). Most of such degradation products are eluted in HPLC after Chl a, indicating that they are of elevated polarity. A most plausible mechanism for the formation of these products is

allomerization [23–25], in which the isocyclic ring V (cf. Fig. 1) is oxidatively cleaved and/or rearranged, by an attack of trace amounts of residual oxygen. The non-occurrence of Pheo *a*/*a'* allomerization in methanol and ethanol up to a period of 150 h [17], is suggestive of the importance of alcohol coordination to the central Mg atom in initiating Chl *a*/*a'* allomerization. We speculate that such coordination furnishes the chlorin macrocycle with a strain, which is then imparted to the isocyclic ring V, thereby enhancing its chemical reactivity.

All of the nine organic solvents described above were employed without special drying treatments. However, the epimerization-promoting activity of water appears to be negligibly small, since no clear difference was noted for the Chl *a*/*a'* epimerization rate constant measured in reagent-grade acetone (water content, max. 0.3%) and 50% aqueous acetone.

Chl a/a' epimerization in suspension systems

Three diethyl ether-insoluble, nitrogen-containing compounds (histidine, arginine and adenine) were tested for the Chl *a*/*a'* epimerization-promoting property. The compounds were ground in a mortar and suspended in diethyl ether solutions of Chl *a'* (97.5% in epimeric purity) under magnetic stirring. Histidine, carrying an imidazole moiety, alone showed a significant catalytic activity, as displayed in Fig. 11. After an induction period of 1–2 h, Chl *a*/*a'* epimerization neatly sets in with a rate depending on, though not proportional to, the amount of histidine suspended. The Chl *a*/*a'* compositional evolution beyond 3 h is well analyzed by Eqn. 2, and the values of $k + k'$ are $5.1 \cdot 10^{-6}$ and $1.4 \cdot 10^{-5}$ s^{-1} for suspended amounts of 10^{-4} and 10^{-3} mol/10 ml, respectively. The existence of the induction period suggests that Chl *a*/*a'* epimerization proceeds on the histidine particle surface, which has to be conditioned in some way via a slow interaction with diethyl ether as solvent. Practically no Chl *a*/*a'* epimerization was noted in arginine and adenine suspensions up to a period of 12 h.

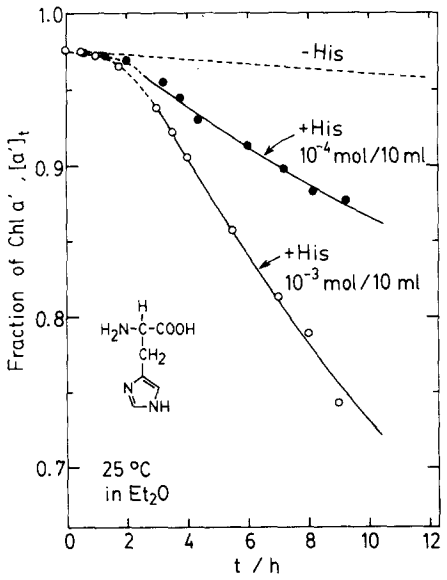

Fig. 11. Promotion of Chl *a*/*a'* epimerization by powdery histidine suspended in diethyl ether. Solid curves are the best-fit ones according to Eqn. 2.

Discussion

The present results demonstrate quantitatively that the Chl *a*/*a'* system is liable to epimerize in environments (solvents or coexisting substances) that are occasionally employed for in vitro studies of Chl derivatives and for treatments of plant materials. For example, in neat dimethylformamide at 25 °C, originally pure Chl *a'* and Chl *a* will degrade respectively to Chl *a'*/Chl *a* = 92/8 and Chl *a*/Chl *a'* = 97/3 mixtures in 2 h. A similar degree of epimerization will be reached in about 30 min in a 1 mM solution of a compound comparable in basicity to pyrrolidine. These features are in sharp contrast to those for amino acids, of which the L/D racemization time constant is of the order of 10^8 h in aqueous solutions at room temperature [26,27]. As seen in Fig. 10, hexane and chloroform are two of the most inert solvents with regard to Chl *a*/*a'* epimerization. The use of hexane and chloroform as an HPLC eluent and a solvent for extraction, respectively, in our plant pigment composition analyses [1–3] could thus be rationalized.

Several previous workers reported, though mostly in a qualitative or semi-quantitative manner, on the rates and equilibrium compositions for Chl *a*/*a'* epimerization in vitro. Based

on NMR peak intensity measurements, an epimer half-life of 2 h in pyridine and an epimerization near-completion period of 24 h in benzene were noted by Katz et al. [6], and an epimerization time of 1 h in acetone was assayed by Hynninen et al. [7]. These rates are obviously much higher than those determined in this work; we suspect, though not conclusively, that their solvents might have been contaminated with traces of potential bases. A very slow epimerization in benzene and a very rapid one in triethylamine [7] are in line with the present results. Omata and Murata [13] studied the Chl a/a' epimerization as a function of adsorption time in a DEAE (diethylaminoethyl)-Sepharose CL-6B column. To our knowledge, this is so far the sole report dealing with the time-courses of Chl a/a' composition. From their composition vs. time profiles, $k + k'$ is evaluated to be $1.45 \cdot 10^{-4}$ s^{-1} at 4°C. Extrapolation of this value to 25°C, by assuming an activation energy of 46.5 $kJ \cdot mol^{-1}$ (see above), yields $k + k' = 6.0 \cdot 10^{-4}$ s^{-1}, or an epimerization time constant of 28 min; this is consistent with their observation that Chl a/a' epimerization was completed within 90 min in the column. This rapid epimerization was caused most likely by the action of strongly basic diethylaminoethyl moieties bound to the gel. The use of such an epimerization-promoting column should be avoided if one intends to prepare chlorophyll derivatives with high epimeric purity.

The equilibrium Chl a' mole fraction $[a']_\infty$ reported in the literature ranges in general from 0.15 to 0.20 [6,7,13], which is comparable to but somewhat smaller than those obtained here. Hynninen and co-workers evaluated $[a']_\infty$ to be 0.38 in petroleum ether by low-resolution column chromatography [11], and 0.40 in neat triethylamine by NMR peak intensity measurements [7]. These values are evidently too large; we have measured repeatedly the $[a']_\infty$ value in triethylamine with the present high-resolution HPLC (cf. Fig. 2) to obtain 0.241 ± 0.005 at 25°C. This figure in turn means that, in terms of Gibbs free energy, Chl a' is less stable than Chl a by 2.8 ± 0.1 $kJ \cdot mol^{-1}$ or 29 ± 1 meV per molecule.

For quite a long time most workers a priori supposed that higher plant photosynthetic apparatus contained only Chl a and Chl b as chlorophyll-type pigments. A work of Klimov et al. reported in 1977 [28] showed for the first time the involvement of Mg-free Pheo a in the primary charge separation within PS II. Several studies performed thereafter have demonstrated that one Chl a' molecule [3] and two Pheo a molecules [29] are closely associated with the PS I and PS II reaction center, respectively. There is no indication for the presence of Pheo a' in leaves [1–3]. Hence, the epimeric compositions in vivo are expressed as Chl a'/Chl $a \simeq 1/300$ to $1/500$ and Pheo a'/Pheo $a = 0$; these are, for such fairly epimerizable pairs (Pheo a/a' in particular), surprisingly far from the equilibrium compositions Chl a'/Chl $a \simeq 1/3$ and Pheo a'/Pheo $a \simeq 1/4.5$ [17] attained easily in vitro. This makes us suppose that every Chl a/a' or Pheo a molecule is, already from the moment of its biosynthesis, perfectly sequestered from contact with basic compounds (purine and pyrimidine derivatives, basic amino acids including histidine, etc.) that are ubiquitous in chloroplasts. No previous works have ever discussed, or even touched upon, the molecular-level mechanism of such sequestering.

Recent X-ray crystallographic studies on bacterial pigment-protein complexes [30,31] have revealed that every bacteriochlorophyll or bacteriopheophytin molecule is assembled in a protein cage with strictly regulated mutual orientation as well as spatial distribution. Even the orientations of peripheral substituents on the bacteriochlorin macrocycle appear to be rigorously fixed, probably through subtle coordinative interactions with polypeptide chains. Such a picture may hold also for higher plant photosynthetic organs, though not experimentally verified yet. Epimerization, namely the inversion of H and $COOCH_3$ moieties at carbon 10 (Fig. 1), should be enough to drastically affect the mode of such interactions, as evidenced indirectly by a significant difference in the visible absorption spectrum between Chl a and Chl a' dimers or aggregates [2]. Epimerization of any chlorophyll-type molecules working as key components at or in the very vicinity of a reaction center, would be fatal to the sound functioning of a photosynthetic unit. Pheophytin a, of which two molecules constitute the primary electron acceptor of PS II, is particularly liable to epimerize, with a rate roughly two orders-of-magnitude higher than that for Chl a/a'

(see above). Incited by these considerations, studies are currently in progress on a correlation between epimerization-promoting effects and herbicidic activities of a variety of artificial substances. Preliminary measurements showed that Simetryne (*N*,*N*′-diethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine; a commonly used herbicide) and *N*-methylpropionamide (a compound with a -CONH- moiety borne by many herbicides) are effective epimerization-promoting agents for Pheo *a*/*a*′. Also, a correlation is qualitatively noted between epimerization-promoting effects and herbicidic actions of the nitrogen-containing compounds employed in the present work. We thus speculate that, at least for a small group of herbicides, promotion of Pheo *a* or Chl *a*/*a*′ epimerization might be one of the molecular-level mechanisms in their photosynthesis-inhibiting actions. Details of these results will be published elsewhere.

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