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Difference between Chlorophylls *a* and *a'* in the Intermolecular Association Behavior. Visible Absorption and Circular Dichroism Spectral Features in Aqueous Methanol

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Visible absorption and circular dichroism (CD) spectroscopies on a series of chlorophyll (Chl) ***a/a'*** epimeric mixtures in aqueous methanol have unraveled a striking influence of the stereochemistry at carbon 13^2 on the mode of intermolecular association of these pigments.

There has been a long-standing view that the primary electron donor (P700) in the reaction center (RC) I of oxygenic photosynthesis is a chlorophyll (Chl) ***a*** dimer, based primarily on the $1/\sqrt{2}$-fold ESR line narrowing in going from monomeric Chl a^+ to flash-produced cation radical of P700.[1] This view, however, has not yet been supported by unequivocal evidence. Through chemical analyses we recently demonstrated that two molecules of Chl ***a'***, the $C13^2$-epimer[2] of Chl ***a*** (Fig. 1), are present exclusively at the RC I core of higher plants and cyanobacteria.[3] We also found[4] that the heliobacterial RC, which is supposedly an ancestor of higher plant RC I,[5] contains just two molecules of bacteriochlorophyll (BChl) ***g'***, being again the $C13^2$-epimer of the major pigment, BChl ***g***. These unprecedented findings now incite us to speculate that dimeric Chl ***a'*** constitutes P700.

To substantiate this speculation, studies on the intermolecular association, including dimerization, of Chl ***a'*** as compared with Chl ***a***, and on physicochemical characterization of their dimers and aggregates are of utmost significance. In the monomeric state, no difference exists between Chls ***a*** and ***a'*** as to the UV-visible absorption spectrum,[6] fluorescence spectrum and quantum yield, and redox potentials (unpublished results) since the $C13^2$-position is outside the π-conjugated ring system. However, the mode of intermolecular association is expected to depend heavily on the stereochemistry at $C13^2$ if the oxygen atom of the $C13^2$ methoxycarbonyl itself or that of the $C13^1$ keto-carbonyl ligates to the Mg ion of another molecule. So far, to our knowledge, there has been no report dealing with the difference between Chl ***a*** and Chl ***a'*** in this particular aspect. As a first step, the spectral features of Chl ***a*** and ***a'*** have been compared in aqueous methanol, a medium known to promote aggregation of Chl ***a***.[7]

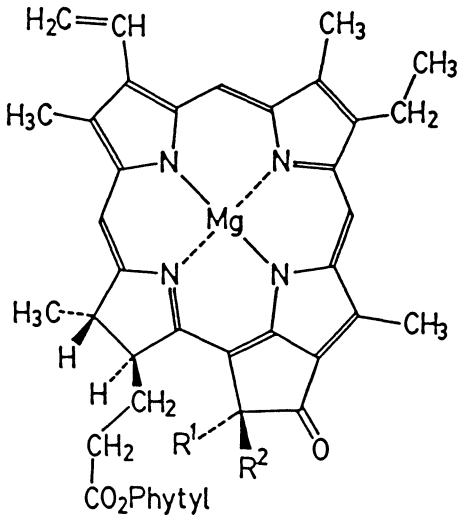
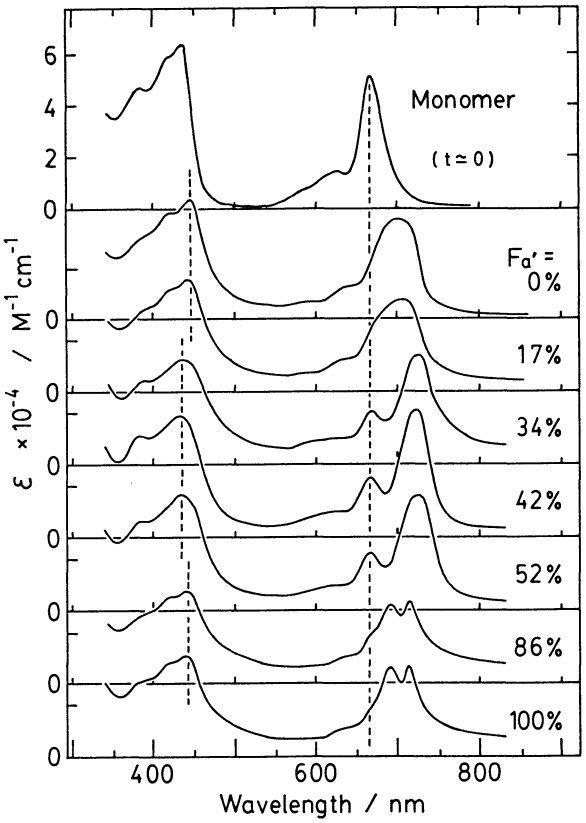


Fig. 1. Molecular structures of Chl ***a*** ($R^1 = COOCH_3$, $R^2 = H$) and Chl ***a'*** ($R^1 = H$, $R^2 = COOCH_3$). Phytyl = $C_{20}H_{39}$.

Chls *a* and *a'* of epimeric purity better than 99% were prepared according to the procedure established previously.[6] Addition of 2 parts (by volume) of doubly distilled water to 3 parts of spectrograde methanol containing Chl *a* and *a'* at a prescribed Chl *a'* mole percentage ($F_{a'}$) caused aggregate formation, as indicated by the spectral change. The final (total) concentration of the Chl *a*/*a'* pigment mixture was around 6.0 μM (M = mol dm^{-3}). The aqueous methanol solution in an air-tight cuvette, degassed by freeze-pump-thaw cycles, was placed in the sample compartment of a JASCO spectrophotometer Model UVIDEC-650, and the change of its visible absorption spectrum was monitored at appropriate time intervals. The absorption spectrum measurement was followed by CD measurement on a JASCO CD spectrometer Model J-500C. The cuvette was thermostated at 25 °C, and all the operations were conducted under dim green light. In total, 30 samples with $F_{a'}$ ranging from 0 (pure Chl *a*) to 100% (pure Chl *a'*) were submitted to the measurements.

The pigment integrity including epimeric purity was well maintained in aqueous methanol. Thus, a sample of initial epimeric composition of Chl *a*/*a'* = 44/56 showed only a slight increase in the Chl *a* fraction to become Chl *a*/*a'* = 46/54 in about 2 h of absorption/CD measurements, with practically no alteration products, as confirmed by analytical HPLC. This is in sharp contrast to the pigments in pure methanol, where nearly complete allomerization (oxidative degradation at the cyclopentanone ring) takes place in a few hours.[8]

Seven representative absorption spectra of Chl *a*/*a'* aggregates formed in aqueous methanol are displayed in Fig. 2 together with the monomer spectrum observed immediately after addition of water. The latter spectrum



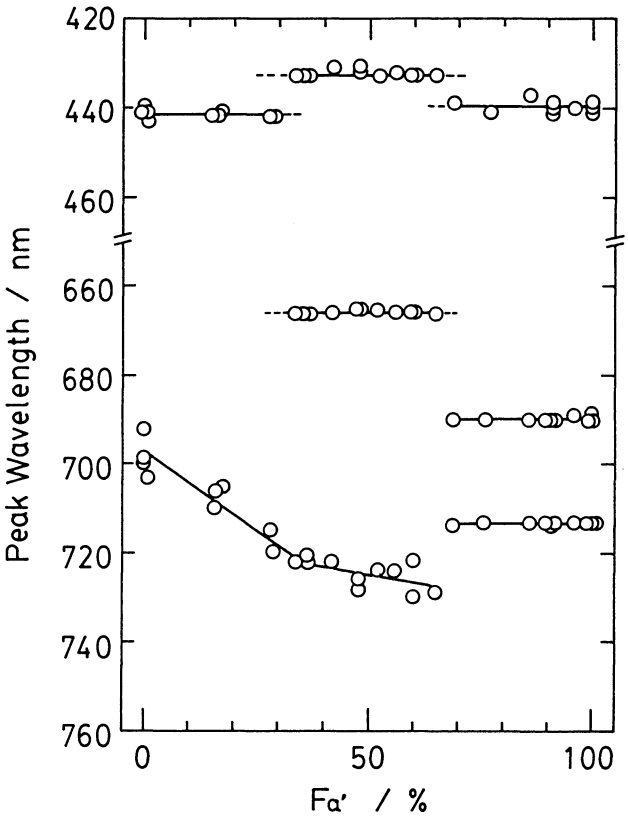


Fig. 2 (left). Dependence of the Chl *a*/*a'* aggregate spectrum on the Chl *a'* mole percentage ($F_{a'}$) in a methanol/water (3/2, v/v) mixed solvent at 25 °C. The top trace shows the monomer spectrum, which is independent of $F_{a'}$, at the instant of water admixing.

Fig. 3 (right). Relationship between the wavelengths of prominent peaks and $F_{a'}$, for a total of 30 samples.

is independent of $F_{a'}$, since the molar absorption coefficient is common between monomeric Chl *a* and *a*'.[6] The aggregate spectra in Fig. 2 were recorded at a quasi-stationary state, reached within 30 min, for each $F_{a'}$.

The following are noted in the red region (600-800 nm) of the aggregate spectra. At $F_{a'} = 0$ (pure Chl *a*), a bell-shaped broad spectrum appears peaking at 695 ± 5 nm, which is slightly different from that (710 nm) observed by Dijkmans[7] under identical conditions. The broad band suggests that the species formed here is a mixture of several Chl *a* oligomers. With increasing $F_{a'}$ up to *ca.* 33%, the peak wavelength shifts bathochromically to 722-725 nm. The latter value remains nearly constant for $F_{a'}$ = 33-65%. This composition range is featured also by the presence of residual monomer (666 nm), whose percentage over the entire pigment amounts to 25 ± 5% for $F_{a'}$ = 40-60%. On further increase in $F_{a'}$ beyond 65%, the spectral pattern changes significantly to give a sharp, double (692 and 713 nm)-peaked spectrum. This change accompanies the loss of the monomer peak at 666 nm.

The drastic influence of the epimer composition on the aggregate spectrum is more clearly depicted in Fig. 3, where the wavelengths of prominent peaks are plotted against $F_{a'}$. It is seen that the spectral pattern changes rather abruptly at two particular Chl *a*/*a*' molar ratios (roughly 2/1 and 1/2).

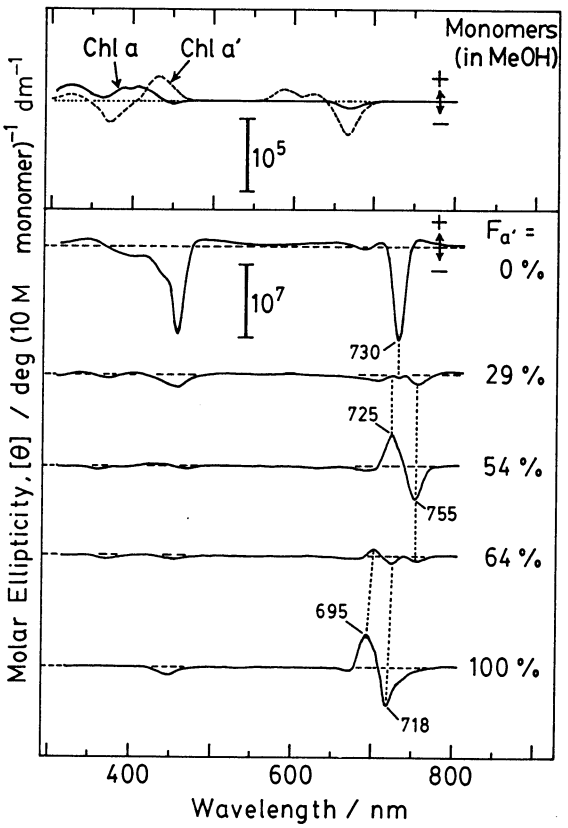


Fig. 4. Typical CD spectra of Chl *a*/*a*' aggregates in aqueous methanol, and of monomeric Chl *a* and *a*' in methanol.

Figure 4 shows representative CD spectra of Chl *a*/*a*' aggregates at five $F_{a'}$ values, together with those of monomeric Chls *a* and *a*' in methanol. Among the CD spectra for a total of 30 pigment samples with systematically varied epimeric composition, we note three typical patterns at $F_{a'} \approx 0$, 50, and 100%, and the spectrum at intermediate $F_{a'}$ values is apparently a weighted sum of two typical patterns. The three characteristic compositional regions are therefore the same as those noted for the visible absorption (Figs. 2 and 3). Another feature in the CD spectra is that the molar ellipticity, on a monomer basis, is about 100-fold larger for aggregates than for monomers. Qualitatively this is rationalized by invoking that the CD signal of a monomer arises from atomic level chirality, while that of an aggregate from molecular level chirality, as was discussed by Scherz and Parson[9] for bacteriochlorophylls.

For the moment, many problems remain to be clarified as to the structures of Chl *a*/*a*' aggregates in this solvent. Even the association numbers are not known for the three species manifesting themselves at $F_{a'} \approx 0$, 50, and 100%. The aggregate(s) formed at $F_{a'} \approx 50\%$ may involve some Chl *a*-*a*' heterodimers and oligomers, but the reason for the inability of as much as 25% of monomers to form aggregates is not clear. Whether the residual monomer is Chl *a* or *a*' cannot be unraveled by CD spectroscopy because of the much stronger CD signal from the aggregates.

Despite these uncertainties we have shown here for the first time that Chl ***a*** and Chl ***a***' are two entirely different chemical species with respect to intermolecular association. The principal cause for this discrimination lies in the fact that the bulky phytyl ester and methoxycarbonyl moieties are at opposite sides of the molecular plane in Chl ***a***, while they are at the same side in Chl ***a***'. This in turn indicates that the epimeric purity of the sample should be rigorously ensured in aggregation studies of chlorophyll derivatives. For instance, the peak wavelength (710 nm) observed by Dijkmans[7] for a "Chl ***a*** aggregate" in aqueous methanol may reflect a contamination with a 10-20% level of Chl ***a***', in view of the results illustrated in Fig. 3.

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