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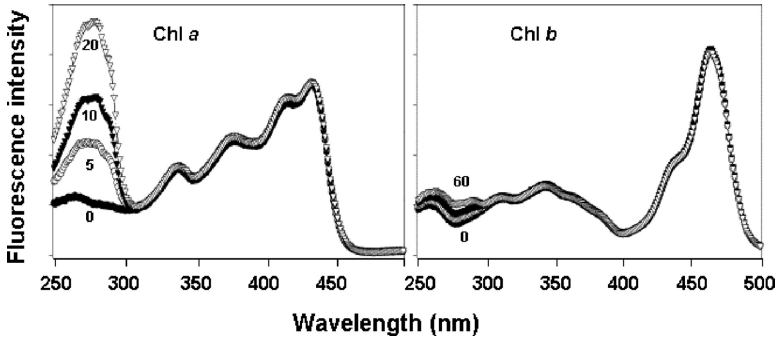

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Influence of Structure on Binding of Chlorophylls to Peptide Ligands

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Chlorophyll (Chl) is the essential pigment for oxygenic photosynthesis in cyanobacteria, algae, and plants. Chl occurs as four different species among these organisms, and each binds in a highly specific and ordered manner to proteins. Until recently, Chl *a* (see Figure 1 for structures) was thought to be the pigment in reaction centers in all of these organisms.[1] The accessory Chls, Chl *b* and Chl *c*, reside in specific sites in light-harvesting complexes (LHCs),[2] as exemplified by LHCII associated with photosystem II in plants.[3] In 1996, Miyashita et al.[4] discovered a cyanobacterium (*Acaryochloris marina*) in which over 95% of the Chl is Chl *d*. Chl *d* not only is the major light-harvesting pigment in *A. marina* but also occurs in reaction centers.[5]

In this study, we addressed the role of modifications at the periphery of Chl molecules on the chemistry of their interactions with protein-bound ligands. Introduction of the electronegative 3-formyl group in Chl *d* extends the electronic distribution along the Q_y axis, shifts the absorbance maximum to longer wavelengths, and increases the absorption coefficient as compared with those of Chl *a*. The 7-formyl group of Chl *b* withdraws electrons toward the periphery of the molecule in the Q_x direction, which reduces the dipole strength.[6] Consequently, the long-wavelength maximum of the spectrum is blue-shifted and the absorption coefficient is reduced relative to those of Chl *a*. This effect is intensified in Chl *c*, in which the C17−C18 double-bond of protochlorophyllide is retained and the ring conjugation system is extended to the unesterified, electronegative side-chain carboxyl group by introduction of the $C17^1$−$C17^2$ trans double bond.[7]

The Mg atom in Chl is pentacoordinate, with four ligands provided by the tetrapyrrole nitrogens. The fifth, axial ligand is provided by an amino acid side chain in a protein or water. It was proposed that the electronegative character of the 7-formyl group of Chl *b* and the *trans*-acrylate group of Chl *c* increases the Lewis acid strength of the Mg atom relative to Chl *a*, thereby requiring harder Lewis bases in proteins to displace a tightly bound water ligand.[8] These structural variations led to the question of whether the coordination chemistry of Chl *d* would be similar to that of Chl *a* or Chl *b*.

Eggink and Hoober[9] designed a synthetic peptide containing the sequence NH_2-GLLAW**RS**H**I**V**E**LAAGG-$CONH_2$, which was adapted from Chl-binding sites in the LHCII apoprotein (LHCP). Each of the Glu(E)-Arg(R) ion-pair and His(H) ligands binds a molecule of Chl *a*, as assayed by Förster resonance energy transfer from the adjacent Trp(W) residue to bound Chl. This assay was used to compare the ability of these ligands to interact, in addition, with Chls *b*, *c*, and *d*. *β*-*n*-Dodecyl maltoside (1 mM; critical micelle concentration, 0.18 mM) generated micelles that simulated a membrane environment.[10] The number of detergent molecules per micelle has been reported as 78−92 (Anatrace, Inc.) or 110−140,[11] which provided a micelle-equivalent concentration in the range of 8−10 μM. A 1 mM solution of the peptide was added with stirring to the reaction mixture containing 100 nM Chl,[12] buffered with 50 mM Na borate, pH 9.0, and the sample was allowed to equilibrate at 37 °C for 15 min after each addition prior to spectral analysis.

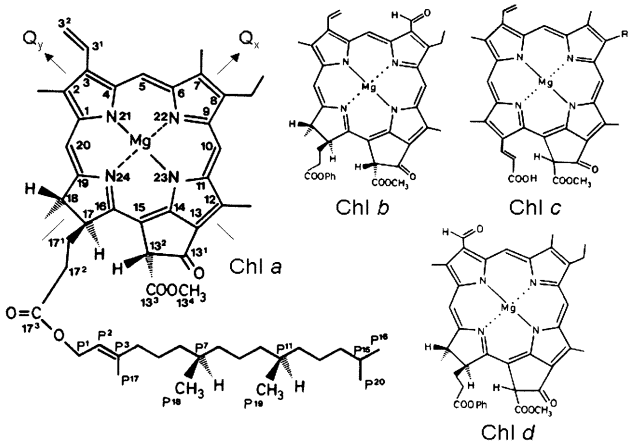

Figure 1. Structures of Chls *a*, *b*, *c*, and *d*. The esterified phytol on position 17^3 is shown in the structure of 3-monovinyl Chl *a*. Also shown are the Q_y and Q_x axes of the molecule. Chl *b* is identical to Chl *a*, except for oxidation of the 7-methyl group to a 7-formyl group. Chl *c* retains the C17−C18 double bond of the precursor protochlorophyllide and includes an additional double bond between $C17^1$ and $C17^2$. The 17^3-carboxyl group remains unesterified. Chl c_1, R = C_2H_5; Chl c_2, R = C_2H_3. Chl *d* is identical to Chl *a* except for oxidation of the 3-vinyl group to a formyl group. Ph = phytol.

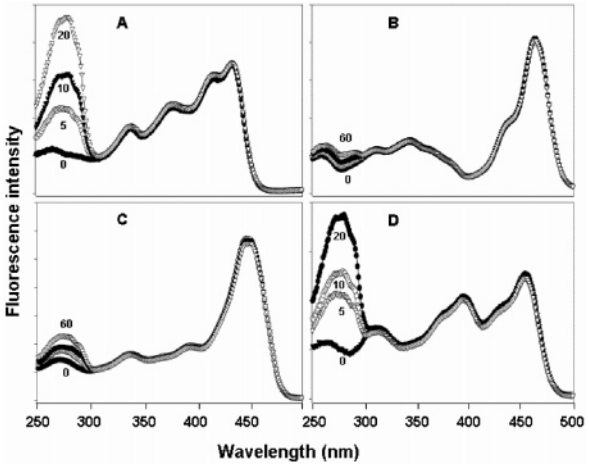

Figure 2. Excitation spectra of the Chls with various concentrations of the peptide. (A,D) Peptide was added to Chls *a* and *d*, respectively, to concentrations of 0, 5, 10, and 20 μM. (B,C) Peptide was added to Chl *b* to concentrations of 0, 10, 30, and 60 μM and to Chl *c* to 0, 20, 40, and 60 μM. Numbers under the peak at 280 nm refer to peptide concentration.

The Chls have nearly equal absorbance (excitation) maxima between 330 and 350 nm (Figure 2) that overlap the emission spectrum of Trp, a requirement for energy transfer.[9] When peptide

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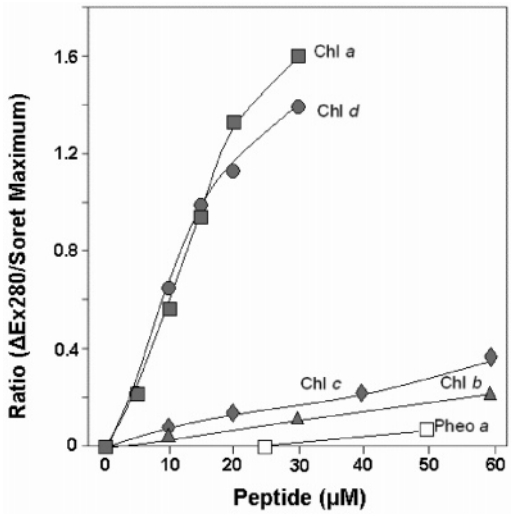

Figure 3. Graphical presentation of results as shown in Figure 2. Binding of the peptide is expressed on the ordinate as the ratio of the increase in fluorescence with excitation at 280 nm (ΔEx280, the excitation maximum of Trp) to the fluorescence intensity at the emission maximum of each type of Chl when excited at its Soret excitation maximum (Chl *a*, 434 nm; Chl *b*, 464 nm; Chl *c*, 447 nm; Chl *d*, 455 nm). Pheo *a* = pheophytin *a*.

was added to the reaction mixture, binding of Chl was detected by development of an excitation maximum at 280 nm, the absorbance maximum of Trp. A very low level of energy transfer from the peptide was detected with Chl *b* (Figure 2B) and Chl *c* (Figure 2C) as compared with Chl *a* (Figure 2A). Chl *d* provided results similar to those obtained with Chl *a* (Figure 2D). We cannot exclude the possibility that interactions occurred between Chls and the peptide that were not detected by energy transfer.

The results are presented graphically in Figure 3. Because fluorescence yield is influenced by the quantum yield of the specific Chl and its environment, energy transfer from the peptide to Chl, evidence of binding, was expressed as the ratio of the increase in fluorescence intensity of Chl with excitation at 280 nm relative to fluorescence intensity with excitation at the Soret maximum of each Chl. The Soret excitation maxima, between 430 and 470 nm, did not change as a consequence of peptide binding (see Figure 2) and thus served as an internal reference. As a control for the requirement of Mg for interaction with the peptide, pheophytin *a*, which lacks the central Mg atom, was prepared by addition of HCl to a sample of Chl *a* in ethanol immediately before addition to the assay. No energy transfer from Trp to pheophytin *a* was observed except at high peptide concentrations. This low level of nonspecific interaction with the peptide may have contributed to the slight increase in energy transfer observed with Chls *b* and *c* at relatively high peptide concentrations. Affinity values relative to Chl *a*, i.e., $K_{a/b}$, $K_{a/c}$, and $K_{a/d}$,[13] indicated that, within the precision of the assay, binding of Chl *d* was essentially the same as that of Chl *a* ($K_{a/d}$ = 1.1). Binding of Chl *a* was 13-fold greater than that of Chl *b* and 9-fold greater than that of Chl *c* at equal peptide concentrations.

Chls *a* and *d* form coordination bonds with the ligands in the synthetic peptide. Coordination of Chls *b* and *c* to such ligands is essentially negligible (Figure 3). Of the six Chl *b* molecules in LHCII, three retain a water ligand. Two are coordinated with peptide backbone carbonyl groups,[3] which have a dipole greater than that of water[14] and thus may compete effectively for the central Mg atom. As determined by circular dichroism, the synthetic peptide assumes a β-hairpin structure[9] that should have several exposed backbone carbonyl groups, yet no significant evidence of binding of Chl *b* was observed. The dielectric constant in the micellar system may be too high for effective competition with water, as compared with the low dielectric constant that occurs within a protein molecule or a membrane.[14] H-bonding to the 7-formyl oxygen of Chl *b* should enhance its electron-withdrawing effect and further strengthen the coordination bond between the Mg and a water ligand.

The conclusion that emerges suggests that sites provided by softer Lewis base ligands, which are apparently unable to displace bound water from Chl *b*, are filled rapidly with Chl *a* during assembly of LHCII. Ligands of Chl *a* in LHCII, such as the imidazole of His and Glu-Arg ion pairs, therefore, are not favorable ligands for Chls *b* and *c*. The kinetics of time-resolved reconstitution of LHCII in vitro,[15] and the positions of Chls *a* and *b* in the final product,[3] are consistent with this proposal. Complexes are apparently assembled in Chl *b*-less mutants with only Chl *a*[16] but are not sufficiently stable to survive purification. It is concluded that Chl *b* (and probably Chl *c*) stabilizes LHCs via H-bonding of the coordinated water molecules to the protein.[3]

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(12) Chls were extracted with 80% acetone, Chls *a* and *b* from light-grown cells of *Chlamydomonas reinhardtii*, Chl *c* from the diatom *Phaeodactylum tricornutum*, and Chl *d* from *A. marina*. Each was purified by HPLC in methanol (Maloney, M. A.; Hoober, J. K.; Marks, D. B. *Plant Physiol.* **1989**, *91*, 1100−1106), evaporated to dryness under a stream of argon, dissolved in ethanol, and added to the reaction mixture at a concentration of 100 nM. Millimolar extinction coefficients used were Chl *a*, 79.5 at 663 nm (Porra, R. J. In *Chlorophylls*; Scheer, H., Ed.; CRC Press: Boca Raton, 1991; pp 31−57); Chl *b*, 47.5 at 647 nm (Porra, R. J. In *Chlorophylls*; Scheer, H., Ed.; CRC Press: Boca Raton, 1991; pp 31−57); Chl *c*, 324 at 447 nm;[7] and Chl *d*, 98.6 at 696 nm (French, C. S. In *Encyclopedia of Plant Physiology;* Ruthland, W., Ed.; Springer-Verlag: Berlin, 1960; p 254). Fully corrected excitation spectra were obtained with a FluoroMax spectrofluorometer (Jobin Yvon-Spex Industries, Edison, NJ), with fluorescence emission measured at the experimentally determined maximum for each type of Chl (Chl *a*, 674 nm; Chl *b*, 654 nm; Chl *c*, 643 nm; Chl *d*, 704 nm). The emission maximum of Trp in the mixture was 326 to 330 nm, indicative of a hydrophobic environment.

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