By analyzing the steady state and time-resolved fluorescence anisotropy, the internal motions of chlorophyll $a$ of light-harvesting chlorophyll $a/b$-protein complex (LHClI) were characterized in a dimyristoylphosphatidylcholine (DMPC) liposome. Corresponding to the thermotropic phase of the membrane, chlorophyll $a$ showed an unique internal motion in LHClI. At the gel phase, two motional components, one fast and the other slow, were observed, which would originate in the heterogeneity of the mutual orientation and the binding site of the chlorophyll $a$ in LHClI. Interestingly, the faster motion was suppressed and only the slower segmental rotation with the larger motional amplitude was allowed on the phase transition to a liquid crystalline phase.

**Key words:** light-harvesting chlorophyll $a/b$-protein complex (LHClI); fluorescence anisotropy; phase transition; chlorophyll $a$

Light-harvesting chlorophyll $a/b$-protein complex (LHClI) acts as a light antenna for the oxygen evolving system (PSII). Coupling with 200–250 antenna chlorophylls of LHClI, the reaction center of PSII efficiently collects solar energy to drive the primary process of photosynthesis.\(^3\)\(^\text{1}\) Now, it is generally accepted that the well-regulated energy transfer from the antenna chlorophyll to the reaction center is an essential process to make the photosynthesis of higher plants efficient and the specific chlorophyll conjugating structure of LHClI carried out its essential parts. Recently, the three-dimensional structure of LHClI was measured with resolution of 3.4 Å.\(^2\)\(^\text{1}\)

The apo-protein of LHClI is a polypeptide chain with three trans-membrane $\alpha$-helices, and combines with 7 chlorophyll $a$ and 5 chlorophyll $b$. The center-to-center distances of the chlorophyll molecules to the nearest neighbour are in the range of 9–14 Å. Such a structure of LHClI is fit for creating the strong chlorophyll-chlorophyll interactions when LHClI is organized in the membrane. In fact, the absorption bands of LHClI are broadened and new bands are added around the Q transition band by the strong exciton interactions between the chlorophylls in organized LHClIs.\(^3\)\(^\text{1}\)–\(^8\)\(^\text{1}\) Although the excitation energy is transferred or relax through such the various exciton levels in the highly organized LHClI, it should be noted that the harmonious chlorophyll-chlorophyll interactions are generated by the mediation of LHClI apo-protein. The mutual orientation and stacking of chlorophyll molecules resulting from LHClI organization are determinant factors of the energy transfer and relaxation processes. These factors must be dependent on the properties of the occupation site of the chlorophyll in each LHClI. Therefore, more detailed information is required on the molecular behaviors as well as spectroscopic properties of chlorophylls in LHClI to understand deeply the energy transfer mechanism in the photosynthetic system. In this study, the internal motion of chlorophyll $a$ of LHClI in the lipid bilayer membrane was investigated through steady state and time-resolved fluorescence depolarization methods. Combining the picosecond laser system with the time-correlated single photon counting technique, this method was recently confirmed to be useful to
describe the internal motion of proteins.\textsuperscript{9-11}

Materials and Methods

Light-harvesting chlorophyll $a/b$-proteins (LHCCI) was prepared from spinach chloroplasts based on the method of Camm and Green.\textsuperscript{19} Solubilized proteins with $30$ mM $\beta$-d-octylglucopyranoside was separated by native-polyacrylamide gel electrophoresis. The gel band including LHCCI was cut out and these LHCCI was extracted into a buffer solution (10 mM Tris-HCl, pH 7.8, including 0.02% Triton X-100). For reconstitution of LHCCI with dimyristoylphosphatidylcholine (DMPC) membrane, McDonell and Strehelin’s method was used.\textsuperscript{12} The mixture of LHCCI with DMPC vesicles adjusted to the ratio of protein to lipid 1/1000 was frozen in liquid nitrogen and then thawed at room temperature. After annealing the samples for 90 min. and removing excess detergent by a Bio-beads SM-2 column, the freeze-thaw cycle was repeated three times to complete the incorporation of LHCCI into membrane.

Fluorescence and fluorescence anisotropy decay measurements were done using the time-correlated single photon counting methods. Excitation light ($W_{ex} = 630$ nm, pulse width 15 ps) was obtained from a combination of a sync-pumped argon ion dye laser and cavity dumper system (Spectra Physics model 2030, 375 and 344). The vertical ($I_{VV}$) and horizontal ($I_{VH}$) emission component against vertical excitation were detected after passing though a Gran-Thompson polarizer and a monochromator on a microchannel plate photomultiplier (Hamamatsu R1564-01). The fluorescence emission signals were accumulated in 1024 channels of a multichannel analyzer with channel width 10.8 ps. The instrumental response profiles were determined by scattering light from a suspension of rabbit liver glycerol in a cacodylate buffer. All fluorescence intensity/time profiles were acquired sequentially and were stored on floppy disks, converted to RT-11 format on a PDP 1134 computer, and transferred to an IBM 3270 VM computer for data analysis. To obtain the rotational correlation time, $\phi$, the fluorescence lifetime, $\tau$, and various pre-exponential terms, $I_{VV}$ and $I_{VH}$ were simultaneously fitted using the equations,

$$I_{VV} = (1/3)F(t)[1 + 2r(t)]$$
$$I_{VH} = (1/3)F(t)[1 - r(t)]$$

where $F(t) = \Sigma \alpha \exp(-t/\tau)$, $r(t) = \Sigma \beta \exp(-t/\phi)$, $\alpha$ and $\beta$ are pre-exponential factors of fluorescence and anisotropy decay, respectively. The quality of fit was judged by the serial variance ratio (SVR) and inspection of plot of weighted residuals/root mean square.\textsuperscript{13} The steady state fluorescence anisotropy were measured with Hitachi fluorescence spectrophotometer model 850. Samples were kept constant ($\pm 0.5$°C) by circulating water through a thermostatic bath.

Results and Discussion

The absorption maximum and two shoulders of LHCCI which can be attributed to the $Q_b$ band of chlorophyll $a$ and $b$, are located at 672 nm, 656 nm and 630 nm in the DMPC liposome membrane, respectively. Although the absorption spectrum was not so affected, the fluorescence spectrum was dependent on the LHCCI density in the membrane. The emission maximum shifted from 672 nm to 685 nm and the intensity was reduced by the increasing in the molecular ratio of [LHCCI]/[Lipid]. LHCCI contains 7 chlorophyll $a$, 5 chlorophyll $b$ and one carotenoid bound with apo-protein. When these photosynthetic pigments are isolated from the apo-protein and then incorporated into the membrane, the fluorescence of chlorophyll $a$ decayed with double exponential kinetics independently of their concentration. Their lifetimes were estimated to be $\tau_1 = 5.27$, $\tau_2 = 1.52$ ns at 15°C and $\tau_1 = 5.25$, $\tau_2 = 1.85$ ns at 25°C. On the other hand, the decay kinetics of the fluorescence of chlorophyll $a$ in LHCCI was definitely single exponential, of which the lifetimes were 5.27 ns at 15°C and 5.49 ns at 25°C at a low $P/L$ ratio. These suggest that the chlorophyll molecules are homogeneously distributed in the membrane, mediated by the apo-protein of LHCCI. This fluorescence decay property of LHCCI is very convenient for analyzing the internal motion of chlorophyll in LHCCI. Figures 1 and 2 show the fluorescence anisotropy decay curves of LHCCI in DMPC vesicles at 15°C and 25°C, respectively. At 25°C, the fluorescence anisotropy of LHCCI decayed with single exponential kinetics. On the other hand, two decay components, one very fast and the other slower, were observed at 15°C. It should also be noted that the fluorescence anisotropy of LHCCI very slowly converged to 0.05, and to 0.01 at 15°C and 25°C with the relaxation time larger than 10 $\mu$s, respectively. This extremely long relaxation time corresponds to the entire rotation of LHCCI in membrane. When the fluorescence anisotropy decay curves were analyzed with the linear combination of the exponential, some interesting parameters were characterized on the molecular motion of the chlorophyll. They are shown in Table 1 together with the fluorescence lifetime and the steady-state fluorescence anisotropy. Increased the temperature from 15°C to 30°C, the fluorescence lifetime transiently increased at 22°C and the steady-state anisotropy critically decreased at the same temperature. The fast decay component characterized with the larger $r_0$ value and smaller rotational correlation time, $\phi = 100$ ps was observed only in the lower temperature region. The fluorescence anisotropy decay at the temperature higher than 22°C was described by only one
component with the rotational correlation time compatible to the fluorescence decay time. It is also interesting to note that the fluorescence anisotropy of LHCII always gives $r_0 = 0.08$ and reaches 0.01 at the higher temperature side. These temperature dependences of the fluorescence depolarization properties of LHCII demonstrate that the molecular motion of chlorophyll $a$ is influenced by the thermotropic phase of DMPC vesicles, because the phase of DMPC vesicles changes from the gel to liquid crystalline phase at around 22°C. An Arrhenius plot for the rotational correlation rate of chlorophyll $a$ gave two straight lines at two temperature regions to suggest rotational motions of chlorophyll $a$ are under the influence of two activation energy 2.1 kcal/mol in the liquid crystalline phase, 15.9 kcal/mole in the gel phase (Fig. 3).
The steady state anisotropy of LHCII was consistent with one estimated by using the anisotropy decay parameters based on the relationship, 
\[ r_s = \frac{1}{\Gamma} \langle F(t) \rangle dt / \langle F(t) \rangle. \]
Therefore, the fluorescence anisotropy decay kinetics and their parameters decided here do not have any uncertainties and they should be reasonable. Based on those results, the rotational motions of chlorophyll \( a \) are doubtlessly restricted within LHCII. A number of theories are reported for describing the segmental motions of fluorophores bound to macromolecules. Most of them are well approximated by a simple equation 1, assuming the segmental motions of the fluorophore and whole rotation of macromolecules are mutually independent.\(^{19} \)

\[ r(t) = r_0[\Gamma \exp(-t/\phi_I) + (1-\Gamma)] \exp(-t/\phi_M) \quad (1) \]

where \( \phi_I \) and \( \phi_M \) are correlation times for the rotational motions of fluorophore and for overall rotation of macromolecules, respectively; \( r_0 \) is the anisotropy at \( t=0 \) and \( \Gamma \) is a parameter that indicates the freedom of the segmental motion which takes 1 if the segmental motion is completely free. According to equation 1, the anisotropy decay function of the fluorophore bound to the macromolecule is ordinarily double exponential. But it is not unreasonable to approximately neglect the contribution of macromolecules motion, when the correlation time of the entire rotation of macromolecules is far larger than one of the segmental motion. Indeed, the correlation time corresponding to the entire rotation of apoprotein of LHCII was estimated to be longer than 10 \( \mu \)s although it includes some uncertainty because the fluorescence lifetime (5–6 ns) is much shorter than the time for LHCII to rotate in the membrane. Cherry et al. also reported that the rotational relaxation time of an usual membrane protein in a membrane is around \( 10^{-7} \) sec.\(^{10} \)

Therefore, the fluorescence anisotropy decay component observed here could be attributed to the segmental motions of chlorophyll \( a \) in LHCII. Since two components were recognized at gel phase, the fluorescence anisotropy decay can be expressed summarily with equation 2 corresponding to the internal motion of chlorophyll \( a \).

\[ r(t) = \Sigma r_0_i[\Gamma_i \exp(-t/\phi_i) + r_\infty], \]

\[ r_\infty = \Sigma (1-\Gamma_i) \quad (2) \]

where \( \phi_i \), \( r_0_i \) and \( \Gamma_i \) are the rotational correlation time, \( r_0 \) and \( \Gamma \) of \( i \)-th rotational component, respectively. Although the internal motion of chlorophyll \( a \) is characterized with three parameters, \( \phi_i \), \( \Gamma_i \), and \( r_0_i \), \( r_0 \) is the intrinsic anisotropy decided with the orientation of the transition moments for the excitation and emission. Since the wavelength of the excitation light was 630 nm where the chlorophyll \( b \) was preferentially excited, the population of the fluorescence state of chlorophyll \( a \) is through the excitation energy transfer from chlorophyll \( b \) to \( a \). Therefore, the \( r_0 \) value obtained here indicates the mutual orientations of molecular axes of chlorophyll \( b \) and \( a \). The difference in \( r_0 \) between two components seen at the gel phase demonstrates that LHCII maintains a heterogeneous arrangement of chlorophylls in the gel phase. The rotational motion of chlorophyll \( a \) corresponding to the faster decay component is extremely fast (\( \phi_i < 100 \) ps, at 15°C) and the angular displacement (\( \mu \)) estimated by a relationship, \( 1-\Sigma \Gamma_i = (1/2)\cos \mu (\cos \mu + 1) \), was about 72°. On the other hand, the angular displacement of the slower rotational motion was 80° and it was larger than that of the faster rotational component. The anticipated motion of chlorophyll \( a \) of LHCII is shown in Fig. 4. Probably the subtle differences in the microenvironments of the binding site may give unique motions to the chlorophyll molecule. The single exponential decay kinetics at the liquid crystalline phase reveal that a homogeneous field is produced for the rotation and distribution of the chlorophyll by the phase transition. As far as we know, there is no report so far describing the segmental motion of a chlorophyll molecule in apo-protein. Therefore much attention should be paid to the rationalization for the segmental motion of chlorophyll molecules. But FAD molecule non-covalently bound with diaphorase II preserves a large motional freedom in apo-protein and its dynamics can be described by kinetics similar to this work.\(^{17,18} \)

No individual photophysical properties of chlorophyll \( a \) of LHCII has been reported yet, therefore, the more detail studies are demanded, but it is very interesting that at least one of 7 chlorophyll \( a \) is allowed to have the distinguishable internal motion(s) with the other(s) in the LHCII structure considering the regulation mechanisms of the energy transfer by the LHCII organization.

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**Fig. 4.** The Anticipated Internal Motion of Chlorophyll \( a \) in LHCII at the Gel Phase of Membrane.

The gross structure of LHCII is based on a model proposed by Kühlbrandt.\(^{21} \) Three rods indicate \( \alpha \)-helixes. The positions and transition moments of chlorophylls are shown in arbitrarily manner.
References


