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TEMPERATURE DEPENDENCE OF OPTICAL PROPERTIES OF CHLOROPHYLL *a* INCORPORATED INTO PHOSPHATIDYLCHOLINE LIPOSOMES

SATOSHI HOSHINA

Department of Biology, Faculty of Science, Kanazawa University, Marunouchi, Kanazawa 920 (Japan)

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Temperature-dependent spectral changes of chlorophyll *a* (Chl *a*) incorporated into liposomes of two types of phosphatidylcholine are studied. When Chl *a* incorporated into the liposomes is cooled down to 5°C from the temperature of the gel-to-liquid crystalline phase transition of the lipid, the red shift as well as the increase in half-bandwidth of the red peak of Chl *a* are only slight. By measuring the difference spectra produced by substracting the absorption spectrum at the phase transition temperature of the lipid from that at lower temperature, it is shown that the component absorbing at longer wavelength (675–685 nm) than the peak of the red maximum (about 670 nm) significantly increases at the expense of the component absorbing at shorter wavelength (657–668 nm). The positions of positive and negative peaks depend on the temperature and the molar ratio of the lipid to Chl *a*. The absorbance change is most pronounced on cooling below the phase transition temperature of the lipid. The temperature-induced absorbance change is almost completely reversible. The results indicate that the aggregated forms of Chl *a* in liposomes can be spectrophotometrically detected in the gel phase of the lipid.

Introduction

The thylakoid membranes contain a high concentration of chlorophyll molecules [1] which absorb light quanta in the primary process of photosynthesis. It is well known that the red absorption band of Chl *a* in vivo is broadened and red shifted as compared with the absorption spectrum in polar organic solvent. Observations using a computer suggested that the width of the red band in vivo was due to the presence of several kinds of absorption spectra of Chl *a* [2]. To understand the mechanism of photosynthesis, it is necessary to have detailed information about chlorophyll organization in thylakoid membranes.

The aggregated forms of Chl *a* were detected by measuring the absorption spectrum of Chl *a* in organic solvents [3–6]. These spectra had a band with a peak at longer wavelength than that of the monomeric form [4–6]. Aggregated Chl *a* is formed by the interaction between the keto C = O of Ring V of Chl *a* and the central magnesium atom of another Chl *a* [4,6,7].

Chl *a* in lipid bilayer vesicles and liposomes is often used as a model system for thylakoid membranes [8–12]. The fluorescence intensity of Chl *a* in liposomes markedly decreases at the temperature of the gel-to-liquid crystalline phase transition of the lipid [13,14]. This has been interpreted as being the result of an increase in the proportion of aggregated Chl *a* when the lipid is in the gel state. However, it has been reported that change in the absorption spectrum of Chl *a* in liposomes is hardly found at temperatures below and above the phase transition of the lipid, and that the aggregated forms are not spectrophotometrically detected in the gel state of the lipid [13–15]. On the other hand, Nicholls et al. [16] reported that the absorption bands of Chl *b* in phosphatidylcholine liposomes showed a red shift in both the blue and red regions on cooling below the

Abbreviations: Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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transition temperature, and the change, as measured by the 460–490 nm absorption difference, increased at temperatures below the phase transition.

The present study shows that the aggregated forms of Chl *a* were spectrophotometrically detected in liposomes prepared from dipalmitoyl- or dimyristoylphosphatidylcholine by measuring the difference between the absorption spectrum at the phase transition temperature and that at lower temperature. The proportion of aggregated Chl *a* began to increase on cooling below the phase transition temperature of phosphatidylcholine.

Materials and Methods

Preparation of Chl a

Chl *a* was prepared from fresh spinach leaves according to two different methods as described below. First, acetone-soluble pigments were transferred in diethyl ether and were separated on a cellulose column as described by Ikemori [17]. β-Carotene, lutein and violaxanthin were eluted with hexane and then hexane/chloroform (9 : 1, v/v). Chl *a* was then eluted with hexane/chloroform (8 : 2, v/v). Secondly, Chl *a* was purified from dioxane-precipitated pigments on a Sepharose CL-6B column according to the method of Omata and Murata [18] but without the DEAE-Sepharose column.

The purity was checked by the absorption spectrum in diethyl ether or acetone [19], paper chromatography using hexane/chloroform (6 : 4, v/v) or hexane/diethyl ether/*n*-propanol (70 : 30 : 0.5, v/v) [17], and thin-layer chromatography on silica gel G plates [20]. Other pigments (carotenoids, Chl *b* etc) and glycerolipids were not detectable.

Chl *a* concentrations were measured by the method of Strain et al. [21].

Preparation of liposomes containing Chl a

Chl *a* (between 0.15 and 0.20 μmol) and various amounts of phosphatidylcholine dissolved in chloroform/methanol/benzene (4 : 2 : 1, v/v) were mixed in a test-tube and evaporated to dryness under reduced pressure. Aqueous buffer (7 ml of 0.1 M NaCl/20 mM Tricine-NaOH, pH 7.8) was added and the mixture was then sonicated for 2–6 min at temperatures near the phase transition of phosphatidylcholine with a Branson Sonifier Cell Disruptor 185 equipped with a microtip (20 kHz, 60 W). The almost transparent solution was centrifuged at 10000 × *g* for 20 min to remove titanium fragments from the probe tip and undispersed lipid, and the supernatant solution was used as described below. The molar ratios of phosphatidylcholine to Chl *a* in organic solvent before formation of liposomes were 4 : 1–420 : 1.

Measurements

All spectrophotometric measurements were performed with a Shimadzu Recording Spectrophotometer UV-300 connected to a Shimadzu Spectral Data Processor Sapcom-1. The absorbance of the red absorption band in each sample was about 0.7 with a 1 cm cuvette, unless otherwise noted. The difference spectrum of an identical sample at different temperatures was measured by substraction of the absorption spectra of Chl *a* incorporated into liposomes at a given temperature from that at other temperatures using the Sapcom-1 instrument.

Changes in temperature were achieved with circulation of water from a water bath to the cuvette chamber of the spectrometer, and the temperature in the cuvette was measured directly. The rate of increasing or decreasing temperature was 1–2°C/min.

Reagents

Solvents of analytical grade were used without redistillation. Dipalmitoyl- and dimyristoylphosphatidylcholine were purchased from Sigma. Their purity was checked by thin-layer [20] and gas-liquid chromatography [22] as described previously.

Results

Fig. 1 shows absorption spectra of Chl *a* in dipalmitoylphosphatidylcholine liposomes at 22°C. The absorption spectrum had a red-band peak at 667–670 nm, with a half-bandwidth of 23–34 nm. These were dependent on the ratio of lipid to Chl *a*, and on the temperature. When the molar ratio of lipid to Chl *a* was 4, the position of the red-band peak was at 668 nm with a half-bandwidth of 28 nm, at 668 nm with 30 nm, and at 670 nm with 34 nm at 41, 22 and 5°C, respectively. When the ratio was 42, the position of the red-band peak was at 668 nm with a half-bandwidth of 23 nm, at 669 nm with 25 nm, and at 670 nm with 25 nm at 41, 22 and 5°C, respectively. When

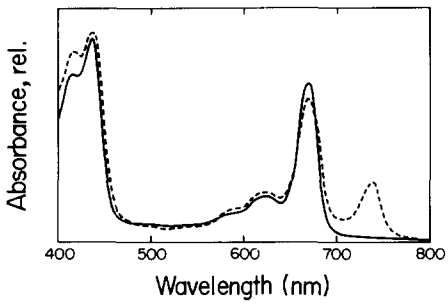

Fig. 1. Absorption spectra of Chl *a* incorporated into dipalmitoylphosphatidylcholine liposomes. Measurements were performed at 22°C at lipid-to-Chl *a* ratios of 42 (———) and 4 (------). The absorbance at 668 nm was 0.376 at a lipid-to-Chl *a* ratio of 4.

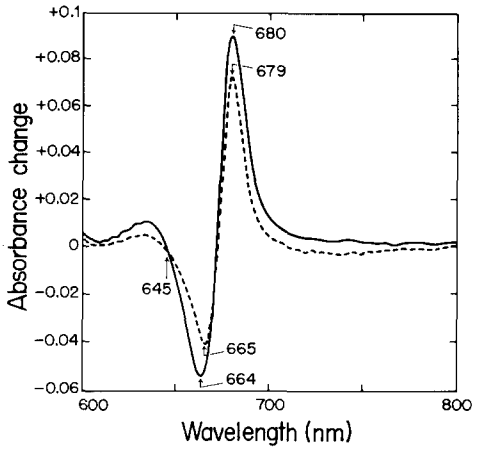

Fig. 2. Difference spectra produced by substracting the absorption spectrum of Chl *a* incorporated into dipalmitoylphosphatidylcholine liposomes at 41°C from that at 22 (------) or 5°C (———). The lipid-to-Chl *a* ratio was 42. After the absorption spectra were measured at 22°C, the temperature of the sample was increased to 41°C and then decreased to 5°C. The difference spectrum obtained by substracting the absorption spectra at the different temperatures was multiplied by 10 and was carried out by smoothing.

the ratio was 420, the position was at 668 nm with a half-bandwidth of 23 nm, and at 669 nm with 24 nm at 41 and 5°C, respectively. Thus, the temperature-induced changes in the absorption spectra were only slight, as described in other literature [13–15,23].

When the ratio of lipid to Chl *a* was 4, the additional peak, other than that at about 670 nm, appeared at 737–739 nm with a half-bandwidth of 30–32 nm. This peak and half-bandwidth were also only slightly changed by the temperature of Chl *a* in liposomes. It was shown that the absorption spectrum of the Chl *a* microcrystal had a red peak at about 740 nm [3,4,12]. This indicates that the microcrystal of Chl *a* is formed when the amount of lipid is lowered as compared with the chlorophyll concentration.

When the ratio of dipalmitoylphosphatidylcholine to Chl *a* was 42, the difference spectrum produced by substracting the spectrum of Chl *a* in the liposomes at 41°C from that at 22°C had a positive peak at 679 nm and a negative peak at 665 nm as shown in Fig. 2. Upon cooling the Chl *a* in liposomes to 5°C, the difference spectrum obtained showed that the absorbance change became larger and that the positions of peaks were slightly shifted (Fig. 2). The difference in absorption spectra between the liposomes at 48 and 41°C was slight. After the absorption spectrum of the liposomes was measured at 41°C, the temperature of the sample decreased to 5°C and then increased to 41°C. The difference between the spectra at the first 41°C point and the latter 41°C point was negligible. Thus, the changes in the temperature-induced absorption spectrum were almost completely reversible. The isosbestic points were changed in the difference spectra between the liposomes at various temperatures and at 41°C, although one is shown at 645 nm in Fig. 2. A temperature-induced difference spectrum in the Soret region of Chl *a* was also observed.

When the ratio of dipalmitoylphosphatidylcholine to Chl *a* was 125, the difference in absorption spectra between the liposomes at 5 and 41°C was a positive peak at 676 nm and a negative peak at 657 nm (Fig. 3). The positions of peaks were at shorter wavelengths as compared with those obtained at the ratio of 42. Upon increasing the ratio of lipid to Chl *a* to 420, the difference spectrum showed the positive and negative peaks at the same positions as shown at the ratio of 125.

At a lipid-to-Chl *a* ratio of 4, the temperature-induced difference spectra were complicated because the absorption spectrum had two peaks in the red region as shown in Fig. 1. The temperature-induced difference spectra showed two positive peaks at 685 nm and 733–740 nm and a negative peak at 666–668 nm. Upon cooling the liposomes, the component

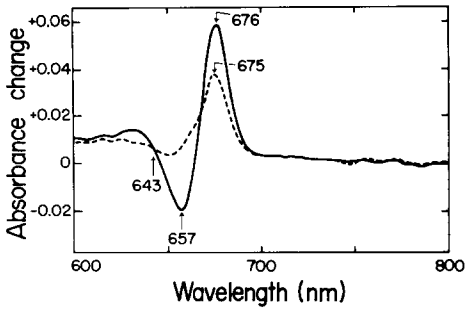

Fig. 3. Difference spectra produced by substracting the absorption spectrum of Chl *a* incorporated into dipalmitoylphosphatidylcholine liposomes at 41°C from that at 23 (- - - - -) or 5°C (———). The lipid-to-Chl *a* ratio was 125. The methods of measurements were the same as that described in Fig. 2, except that the initial temperature was 23°C.

at about 740 nm first began to increase, and then that at 685 nm increased (Fig. 4).

The lower the ratio of lipid to Chl *a*, the greater the increase in temperature-induced difference spectra except for the extent of the positive peak of the difference spectrum at between 22 and 41°C (Fig. 5).

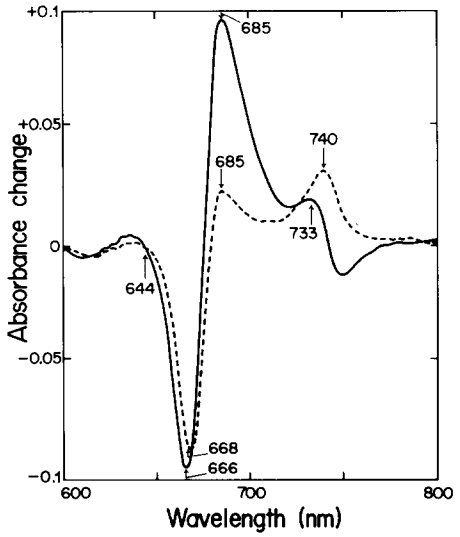

Fig. 4. Difference spectra produced by substracting the absorption spectrum of Chl *a* incorporated into dipalmitoylphosphatidylcholine liposomes at 41°C from that at 22 (- - - - -) or 5°C (———). The lipid-to-Chl *a* ratio was 4. The methods of measurements were the same as that described in Fig. 2. The absorbance at 668 nm was described in Fig. 1.

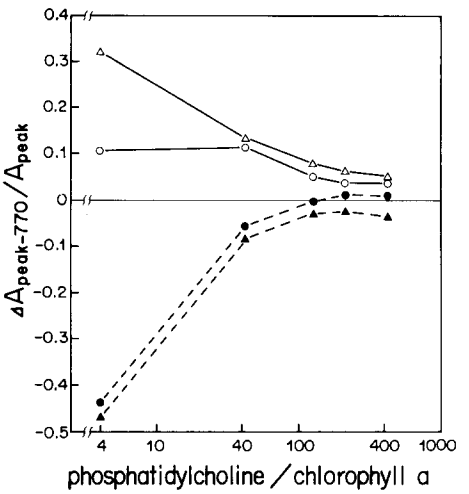

Fig. 5. Temperature-induced absorption change of Chl *a* incorporated into dipalmitoylphosphatidylcholine liposomes measured as a function of the ratio of lipid to Chl *a*. The data were calculated from the difference spectrum as shown in Figs. 2–4. The vertical axis is the ratio of $\Delta A_{positivepeak-770}$ to A_{peak} (○———○, △———△) or $\Delta A_{negativepeak-770}$ to A_{peak} (●- - - - -●, ▲- - - - -▲). ΔA values were obtained from the difference spectrum at 5 and 41°C (△———△, ▲- - - - -▲) and the difference spectrum at room temperature and at 41°C (○———○, ●- - - - -●). The absorbance values at 667–669 nm were measured at room temperature (19–24°C).

Although the temperature-induced spectrum changes became greater upon lowering the ratio of lipid to Chl *a*, it is presumed that the physical state of the lipid molecules is influenced by the incorporated Chl *a* molecules.

Figs. 6 and 7 show the absorbance changes for Chl *a* in dipalmitoylphosphatidylcholine liposomes upon increasing or decreasing temperature. When the temperature of the sample was decreased from 50°C, the change, as measured by the 680 – 770 nm difference, was first slightly decreased until the temperature reached 41°C. Upon further decreasing the temperature, a marked increase in the absorbance change was observed, and the absorbance increment continued during temperature decreases to 5°C, but the rate of the absorbance increment was gradually decreased at temperatures below about 33°C. When the temperature of the sample was raised from 5 to 43°C, the absorbance change was almost completely reversible (Fig. 6). Absorbance measured at other wavelengths

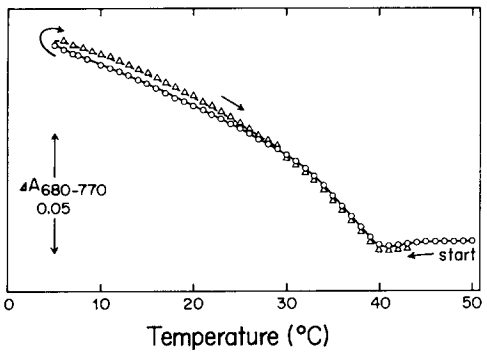

Fig. 6. Absorbance change at 680 nm of Chl *a* in dipalmitoylphosphatidylcholine liposomes as a function of temperature. The ratio of lipid to Chl *a* was 48. The spectrophotometer was operating in the dual-wavelength mode. The reference wavelength was 770 nm. The liposomes were first cooled from 50 to 5°C (o———o), and then heated to 43°C (△———△).

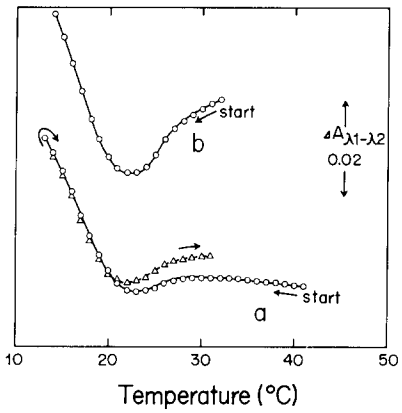

Fig. 8. Absorbance changes at 678 nm of Chl *a* in dimyristoylphosphatidylcholine liposomes as a function of temperature. The ratio of lipid to Chl *a* was 48. a, $\Delta A_{678-770}$; b, $\Delta A_{678-664}$. The temperature was decreased (o———o) or increased (△- - - - -△).

also markedly changed at temperatures below 40–42°C (Fig. 7), and this is shown as the temperature of the phase transition of dipalmitoylphosphatidylcholine measured with calorimetry, ESR or fluorescence methods [24].

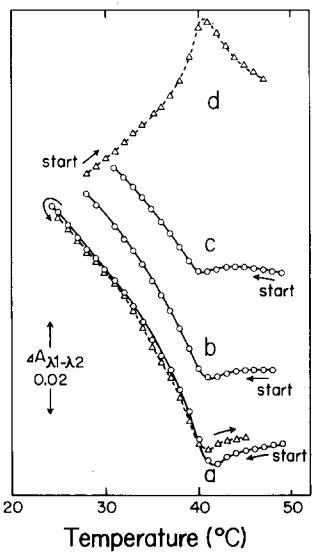

Fig. 7. Absorbance changes at various wavelengths of Chl *a* in dipalmitoylphosphatidylcholine liposomes as a function of temperature. The ratio of lipid to Chl *a* was 42. a, $\Delta A_{680-770}$; b, $\Delta A_{678-770}$; c, $\Delta A_{676-770}$; d, $\Delta A_{665-770}$. Temperature was decreased (o———o) or increased (△- - - - -△).

At ratios of lipid to Chl *a* higher than 100, absorbance changes, as measured by the 675 – 770 or 678 – 770 nm differences, were significantly smaller than those shown in Figs. 6 and 7. When the ratio of lipid to Chl *a* was 4, changes in the 685 – 770 and 666 – 770 nm differences were detected at 38 and 44°C, respectively. Thus, the absorbance change for Chl *a* depended on the physical state change of phosphatidylcholine and was effectively observed only at lipid-to-Chl *a* ratios of 40–50.

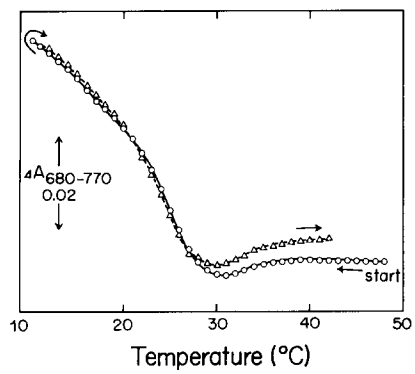

Fig. 9. Absorbance change at 680 nm of Chl *a* in liposomes of an equimolar mixture of dipalmitoyl- and dimyristoylphosphatidylcholine as a function of temperature. The ratio of lipid to Chl *a* was 48. The reference wavelength was 770 nm. The temperature was decreased (o———o) or increased (△- - - - -△).

The difference between the absorption spectra for Chl *a* incorporated into dimyristoylphosphatidylcholine liposomes at 5 and 22°C was a positive peak at 678 nm and a negative peak at 664 nm when the ratio of lipid to Chl *a* was 48. As shown in Fig. 8, the temperature-induced absorbance change showed a minimum at about 23°C. When liposomes containing Chl *a* were prepared with an equimolar mixture of dimyristoyl- and dipalmitoylphosphatidylcholine, the absorbance change for the liposomes showed a minimum at 30–31°C (Fig. 9). These values are also in good agreement with the previously reported phase transition temperatures of phosphatidylcholines obtained by calorimetry, ESR or fluorescence methods [24,25].

Discussion

When liposomes of phosphatidylcholine containing Chl *a* were transferred from high to low temperatures or vice versa, it was clearly shown that temperatures below the phase transition of the lipid induced an increase in the component of 675–685 nm for Chl *a* and a decrease in that of 657–668 nm. Murata and Sato [12] showed that the absorption spectrum of Chl *a* was composed of a single major band with a peak at 671.3 nm and additional minor bands with peaks at 650.1, 660.6 and 685.6 nm from the results of analysis according to the curve-fitting method for the absorption spectrum of Chl *a* in an aqueous dispersion of a mixture of sulfoquinovosyldiacylglycerol and monogalactosyldiacylglycerol. They pointed out that the minor band with a peak at 685.6 nm might have been produced by aggregated forms of Chl *a*. It was also reported that the absorption spectrum of aggregated Chl *a* in organic solvents was composed of a band with a peak at longer wavelength than those of its monomeric forms [4–6]. The present study shows that the proportion of aggregated Chl *a* in the phosphatidylcholine liposomes began to increase at the expense of the monomeric form in the gel phase of the lipid upon lowering the temperature below the phase transition of the lipid. At higher ratios of lipid to Chl *a*, i.e., lower concentrations of Chl *a* in the lipid, the distance between adjacent chlorophyll molecules is considerable, and this makes it difficult to increase the proportion of aggregated Chl *a* at temperatures below the phase transition of the lipid. At lower ratios of lipid to Chl *a*, the physical state of the lipid molecules is influenced by the incorporated Chl *a*. When the ratio of lipid to Chl *a* was changed, the temperature-induced difference spectra had bands with peaks at different wavelengths (Figs. 2–4). The present study suggests that a monomeric form of Chl *a* is the compoment having a band with a peak at 660.6 nm and that the aggregated forms are the components having bands with peaks at 671.3 and 685.6 nm as shown by Murata and Sato [12]. It is known that aggregated Chl *a* is formed by a keto carbonyl group-Mg interaction and that Chl *b* aggregates by a keto carbonyl group-Mg and a formyl group-Mg interaction [7]. One may interpret the data reported by Nicholls et al. [16] as being that aggregated forms of Chl *b* are detected more easily than those of Chl *a*.

Lee [13] reported that in bilayers of dipalmitoylphosphatidylcholine at a lipid-to-Chl *a* molar ratio of 430, there was a main transition (a marked drop in fluorescence intensity) at 40.5°C, and a pretransition at about 29°C. He pointed out, however, that the main transition had broadened and the pretransition was abolished in dipalmitoylphosphatidylcholine at higher concentrations of Chl *a* (greater than 1 mol Chl : 100 mol lipid). Nicholls et al. [16] also reported that incorporation of Chl *b* into liposomes of dipalmitoylphosphatidylcholine broadened the phase transition. However, Knoll et al. [14] reported that in dipalmitoylphosphatidylcholine vesicles at 3 mol% Chl *a* in the lipid, i.e., a lipid-to-Chl *a* ratio of 32, the main transition as well as the pretransition were clearly demonstrated by Chl *a* fluorescence, and even at 10 mol% Chl *a* in the lipid, the main broadened transition was evident. In this study, the physical state of phosphatidylcholine may be somewhat influenced by the Chl *a* molecules incorporated into the lipid phase because the phase transition had actually broadened and the pretransition could not be detected as shown in Figs. 6–9.

It has been reported that the phase transition of phosphatidylcholine can be detected by measuring the turbidity changes of liposomes [23,26–28]. This method is available for high concentrations of lipid. In this study, turbidity changes were hardly detected at the lipid-to-Chl *a* ratio of about 40. At higher ratios of lipid to Chl *a*, turbidity was significant, and a peak at about 650 nm obtained from the tempera-

ture-induced difference spectrum did not appear negative as seen in Fig. 5.

French et al. [2] showed a large number of spectra of in vivo systems at −196°C by using a curve-fitting method. There are four major forms of Chl *a*, viz., Chl *a*-662, Chl *a*-670, Chl *a*-677 and Chl *a*-684, in which the numbers represent the wavelengths (in nm) of the peak position. Sugiyama and Murata [29] showed that the four major Chl *a* forms existed in the water-soluble chlorophyll proteins and they assumed that these chlorophyll forms could be produced by the interaction of Chl *a* with protein. Murata and Sato [12] also suggested that the Chl *a* form of Chl *a*-670 was produced by the interaction of Chl *a* with lipid from the result of the curve-fitting analysis of the absorption spectrum of Chl *a* in an aqueous dispersion of lipids. The findings in this study suggest that many forms of Chl *a* can also be produced by the interaction of Chl *a* with lipid and by Chl *a*-Chl *a* interaction in a lipid bilayer.

Brody and Singhal [30] reported the temperature dependence of spectral changes of barley thylakoid membranes. They concluded that the temperature-dependent change in the phase of the membrane lipids modified the state of chlorophyll aggregation. However, it is entirely unknown what kind of lipid state causes the complicated changes in the absorption spectrum of thylakoid membranes. We also observed that the absorption spectrum of thylakoid membranes isolated from tomato leaves reversibly changed as a function of physiological temperature (unpublished data). Future studies on the temperature-induced spectral changes of thylakoid membranes will be made.

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References

1 Rabinowitch, E.I. (1954) Photosynthesis and Related Processes, vol. 1, pp. 411–412, Interscience Publishers, New York
2 French, C.S., Brown, J.S. and Lawrence, M.C. (1972) Plant Physiol. 49, 421–429
3 Jacobs, E.E., Vatter, A.E. and Holt, A.S. (1954) Arch. Biochem. Biophys. 53, 228–238
4 Anderson, A.F.H. and Calvin, M. (1964) Arch. Biochem. Biophys. 107, 251–259
5 Brody, S.B. and Brody, S.S. (1965) Biochim. Biophys. Acta 94, 586–588
6 Cotton, T.M., Trifunac, A.D., Ballschmiter, K. and Katz, J.J. (1974) Biochim. Biophys. Acta 368, 181–198
7 Katz, J.J., Dougherty, R.C. and Boucher, L.J. (1966) in The Chlorophylls (Vernon, L.P. and Seely, G.R., eds.), pp. 185–251, Academic Press, New York
8 Chapman, D. and Fast, P.G. (1968) Science 160, 188–189
9 Trosper, T., Raveed, D. and Ke, B. (1970) Biochim. Biophys. Acta 223, 463–465
10 Steinemann, A., Alamuti, N., Brodmann, W., Marschall, O. and Läuger, P. (1971) J. Membrane Biol. 4, 284–294
11 Cherry, R.J., Hsu, K. and Chapman, D. (1971) Biochim. Biophys. Res. Commun. 43, 351–358
12 Murata, N. and Sato, N. (1978) Plant Cell Physiol. 19, 401–410
13 Lee, A.G. (1975) Biochemistry 14, 4397–4402
14 Knoll, W., Baumann, J., Korpium, P. and Theilen, U. (1980) Biochem. Biophys. Res. Commun. 96, 968–974
15 Luisetti, J., Möhwald, H. and Galla, H.J. (1979) Z. Naturforsch. 34c, 406–413
16 Nicholls, P., West, J. and Bangham, A.D. (1974) Biochim. Biophys. Acta 363, 190–201
17 Ikemori, M. (1973) Bull. Jap. Sea Res. Inst., Kanazawa Univ. (in Japanese) 5, 25–87
18 Omata, T. and Murata, N. (1980) Photochem. Photobiol. 31, 183–185
19 French, C.S. (1960) in Handbuch der Pflanzenphysiologie (Ruhland, W., ed.), vol. 5, pp. 252–297, Springer, Berlin
20 Hoshina, S., Kaji, T. and Nishida, K. (1975) Plant Cell Physiol. 16, 465–474
21 Strain, H.H., Thomas, M.R. and Katz, J.J. (1963) Biochim. Biophys. Acta 75, 306–311
22 Hoshina, S. (1979) Plant Cell Physiol. 20, 1107–1116
23 Colbow, K. (1973) Biochim. Biophys. Acta 318, 4–9
24 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 237–281
25 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 285–344
26 Yi, P.N. and MacDonald, R.C. (1973) Chem. Phys. Lipids 11, 114–134
27 Abramson, M.B. (1971) Biochim. Biophys. Acta 225, 167–170
28 Blok, M.C., Van Deenen, L.L.M. and De Gier, J. (1976) Biochim. Biophys. Acta 433, 1–12
29 Sugiyama, K. and Murata, N. (1978) Biochim. Biophys. Acta 503, 107–119
30 Brody, S.S. and Singhal, G.S. (1979) Biochem. Biophys. Res. Commun. 89, 542–546