Temperature Dependence of Chlorophyll \( \alpha \) Fluorescence in Relation to the Physical Phase of Membrane Lipids in Algae and Higher Plants

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ABSTRACT

The temperature dependence of the yield of chlorophyll \( \alpha \) fluorescence was measured at room temperatures in living algal cells and higher plant chloroplasts. \( 3-(3',4'-\text{Dichlorophenyl})-1,1\text{-dimethyleurea} \) was added to the samples during the measurements in order to eliminate the influence of photosynthetic photochemical reactions on the fluorescence yield.

In \textit{Anacystis nidulans} the maximum in the curve for the fluorescence yield versus temperature occurred near the temperatures at which the transition of physical phase of the membrane lipids changes from the liquid crystalline to the mixed solid-liquid crystalline states. These findings suggest that the occurrence of a fluorescence maximum in the temperature-dependence curve is an indication of the thermal transition of physical phase of membrane lipids. Similar but less distinct maxima were found in \textit{Cyanidium caldarium} and \textit{Euglena gracilis}. No maxima were seen in the curves for chlorophyll \( \alpha \) fluorescence versus temperature in \textit{Chlorella pyrenoidosa}, \textit{Plectonema boryanum}, \textit{Stichococcus bacillaris Phaeodactylum tricornutum}, or in chloroplasts of the higher plants, spinach, lettuce, tomato, and \textit{Tidestromia oblongifolia}.

The fluorescence yield of allophycocyanin in \textit{Anacystis} did not show such a maximum, but did show a steep increase with decreasing temperature below 3 C. The fluorescence yields of chlorophyll \( \alpha \) and phycobilins extracted from \textit{Anacystis} increased monotonously with decreasing temperature.

Intensive studies on isolated chloroplasts and intact cells have revealed that changes in fluorescence yield are affected by factors such as quenching produced by the functioning of the photochemical reaction center of photosystem 2 (5, 11, 14, 20); configurational changes of thylakoid membranes induced by cations (7, 16, 18, 19); pigment state 1 to state 2 shifts (2, 15, 17); and quenching caused by the high energy state of thylakoid memt branes (21, 29). In the present study we investigated the effect of temperature on the yield of Chl \( \alpha \) fluorescence and found that the fluorescence of Chl \( \alpha \) is a native probe for detecting the transition of the physical phase of lipids in the thylakoid membrane.

In biological membranes as well as in model membranes, Träuble and Overath (23, 27, 28) used artificial fluorescent probes such as 1-anilinonaphthalene-8-sulfonate and \( N \)-phenyl-1-naphthalene to detect the transition of the physical phase of membrane lipids. The fluorescence yield of these reagents responds to the phase transition by showing a characteristic feature in the temperature versus fluorescence curve. When the membrane lipids are either in the solid or in the liquid crystalline state, the fluorescence yield increases monotonously with decreasing temperatures. In a model membrane composed of a single lipid species, the fluorescence yield showed a drastic decrease at the phase transition from the liquid crystalline to the solid state. Because the phase transition occurs over a range of temperatures (within several degrees centigrade), a maximum appears when the lipid begins to solidify and a minimum appears when the solidification is completed. Colbow (3) found that Chl \( \alpha \) in phospholipid model membranes also functioned as a fluorescent probe for detection of the transition of the lipid phase.

The lipid composition of the biological membranes is complex. There seems to be a temperature region of mixed solid-liquid crystalline state in which the solid as well as the liquid crystalline states both exist but are separated laterally (13). Although the temperature versus fluorescence curve is not simple in these cases, a characteristic feature of these curves is the appearance of a maximum and a minimum (23).

It was found in the previous study (22) that the transition of the membrane lipids from the liquid crystalline to the mixed solid-liquid crystalline state occurred at room temperatures in the blue-green alga \textit{Anacystis nidulans}. The transition detected by the ESR signal of a spin label was found to occur near 24 C and 13 C in cells grown at 38 C and 28 C, respectively. Some biological activities showed transitions in the Arrhenius plots near these temperatures. The yield of Chl \( \alpha \) fluorescence showed a maximum near the temperature of phase transition. It was assumed that Chl \( \alpha \) would be a native fluorescent probe to detect the transition of physical phase of membranes.

This study was undertaken to define better the temperature dependency of fluorescence yield in relation to the physical state of membrane lipids.

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MATERIALS AND METHODS

Algal cells were grown in inorganic media under 4000 lux of fluorescent light illumination. *Anacystis nidulans* was grown at 19, 28, and 38 °C in Kratz and Myer's medium (12). *Plectonema boryanum* was also grown in the same culture medium at 23 °C. *Phaeodactylum tricornutum* and *Stichococcus bacillaris* were grown at 23 °C in artificial sea water medium (10). *Cyanidium caldarium* was grown at 38 °C in Allen's medium (1). *Euglena gracilis* and *Chlorella pyrenoidosa* were grown at 28 °C in Cramer and Myer's medium (4) and Spoehr and Milner's medium (26), respectively.

Romaine lettuce (*Lactuca* sp. var. Romaine, purchased at Germain's Seed Co., Los Angeles, Calif.) and tomato (hybrid tomato N-65, University of Hawaii) were cultivated at day and night temperatures of 25 and 15 °C in growth chambers with 14-hr light and 10-hr dark periods. *Tidestromia oblongifolia*, collected in Death Valley, California, was grown in growth chambers kept at 44 °C during the day and at 30 °C night. Spinach leaves were purchased at a local market.

To prepare the phycobilin solution, *Anacystis* cells, grown at 38 °C, were washed once and resuspended in a medium containing 0.4 mM sucrose, 10 mM NaCl, and 15 mM HEPES buffer, pH 7.3. Then the cells were ruptured with the French pressure cell. The homogenate thus obtained was centrifuged at 104,000g for 45 min. The supernatant containing phycocyanin and allophycocyanin, but not Chl a, was used for the fluorescence measurement after an adequate dilution with the same medium. Chlorophyll a was extracted from *Anacystis* cells grown at 38 °C with 80% acetone. This solution was used for the fluorescence measurement after an adequate dilution with 80% acetone.

For the preparation of higher plant chloroplasts, the leaves were homogenized in a Waring Blender in 0.4 mM succrose, 10 mM NaCl, and 50 mM phosphate buffer, pH 7.3. After being filtered through eight layers of cheesecloth, the homogenate was centrifuged at 1,000g for 5 min. The precipitate was suspended in the same medium. The suspension was centrifuged at 200g for 1 min, and the supernatant was recentrifuged at 1,000g for 5 min. The precipitate thus obtained was resuspended in the same medium, and after centrifugation at 200g for 1 min to remove the remaining contaminants, it was used for the measurements. Chloroplasts of *Euglena gracilis* were prepared in a similar way, but after the *Euglena* cells were ruptured with a French pressure cell.

Fluorescence spectra of *Anacystis* cells and phycobilin solutions were measured at various temperatures in a Perkin-Elmer spectrofluorometer (MPF-3) fitted with a temperature regulation unit.

For the automatic recording of the temperature dependence of fluorescence, a cuvette was designed in which the fluorescence yield could be measured while changing the temperature in a round from −5 °C to 40 °C. The round cuvette contained about 30 ml of sample and was placed horizontally in the excitation light beam which came from above. Fluorescence was detected from underneath the cuvette. Coolant flowing through stainless steel tubing placed around the inside of the cuvette but out of the light path provided the desired rate of temperature change. For acceleration of heat exchange a small propeller turning at 120 rpm was immersed in the sample. The temperature of the sample was measured by a copper-constantan thermocouple, whose signal after amplification was put into the X-axis of an X-Y recorder. The fluorescence yield was detected by a photomultiplier (EMI 9558B) and was put into the Y-axis. By changing the sample temperature continuously, the temperature versus fluorescence yield curve could be recorded automatically. The rate of temperature increase was 2 °C/min and the rate of decrease was 0.5 °C/min or 2 °C/min.

Chlorophyll a fluorescence was measured at 684 nm in intact cells and chloroplasts and at 675 nm in an acetone-water (80:20, v:v) solution with a Bausch and Lomb interference filter combined with a glass optical filter Schott RG-5. The fluorescence of allophycocyanin was measured at 655 nm in a similar way. Monochromatic excitation light was obtained from incandescent light that was passed through a Bausch and Lomb grating monochromator with an added band-pass filter. Chlorophyll a was excited with 430 nm light, Chl b with 480 nm light, and phycocyanin with 560 nm light.

Fluorescence measurements were done in the presence of 10 μM DCMU in order to eliminate the influence of the photo-chemical reactions of photosynthesis on fluorescence yield.

RESULTS

The effects of temperature on the yield of Chl a fluorescence were investigated in *Anacystis* that was grown at different temperatures. Figure 1 shows the curves for temperature versus fluorescence yield when Chl a was excited with 430-nm light. In cells grown at 38 °C, a maximum appeared at 16 °C on decreasing and at 21 °C on increasing the temperature. Although it was only occasionally observed, Figure 1 shows a shoulder at 24 °C on the curve for decreasing temperature. No such shoulder appeared in the other curves obtained from the two cultures grown at the lower temperatures. In the organism grown at 28 °C the maximum appeared at 11 °C on decreasing and at 14 °C on increasing the temperature. In cells grown at 19 °C the maximum appeared at 9 °C on decreasing and at 12 °C on increasing the temperature. A minimum in the fluorescence yield was not found in the temperature versus fluorescence curves measured in the room.
temperature region. Even in the experiment with the 38 C-grown cells, in which the temperature was lowered to -5 C, a distinct minimum was not observed.

The fluorescence maxima appeared at lower temperatures upon decreasing the temperatures than upon increasing the temperatures in all of the cultures (Fig. 1). This hysteresis effect tended to be more distinct when higher rates of temperature change were used. The most pronounced case was seen in cells grown at 38 C in which the maximum was found at 13 C using a cooling rate of 2 C/min and at 16 C with a cooling rate of 0.5 C/min. The dotted line for the cells grown at 28 C in Figure 1 shows the extent of the hysteresis effect. It is of interest that Triebüle and Overath observed such a hysteresis in the case of artificial fluorescent probes in model and biological membranes (23, 28).

The temperature dependences of sensitized fluorescence of Chl a and allophycocyanin were also measured upon the excitation of phycocyanin in Anacystis. Figure 2, upper part, shows a maximum of Chl a fluorescence at 25 C upon decreasing the temperature and at 26 C upon increasing the temperature in the 38 C-grown cells. A maximum appeared at 14 C upon decreasing the temperature and 17 C upon increasing the temperature in the 28 C-grown cells (not shown on the figure). It can be noted that the maxima of Chl a fluorescence appeared at higher temperatures when phycocyanin was excited than when Chl a was excited (cf. Fig. 1). Figure 2, lower part, also shows the temperature dependence of the fluorescence yield of allophycocyanin in Anacystis grown at 38 C. No maxima were seen in this case but instead there was a steep rise in fluorescence yield when the temperature was decreased below 5 C.

No such characteristic changes in fluorescence yield upon changing temperature were observed in Chl a or in phycobilins extracted from Anacystis grown at 38 C, as can be seen in the temperature versus fluorescence curves for Chl a in an acetone-water mixture (80:20, v/v) or for phycobilins in an aqueous solution (Fig. 3). In this case the curves showed only a monotonic decrease of fluorescence yield upon increasing the temperature. These findings suggest that the characteristic changes of yield of Chl a fluorescence and allophycocyanin fluorescence in the intact cells are attributable to the interaction of these pigments with the thylakoid membrane.

Maxima in the fluorescence yield of Chl a versus fluorescence curves were also observed in the thermophilic unicellular alga Cyanidium caldarium and in Euglena gracilis. A maximum and a minimum appeared at 29 C and 24 C, respectively, in Cyanidium upon increasing the temperature, although these were not seen when the temperature was decreased (Fig. 4). The maximum in the temperature versus fluorescence curve appeared in Euglena gracilis at 11 C on decreasing the temperature and at 21 C on increasing the temperature (Fig. 5). In the isolated chloroplasts of Euglena, a maximum, though less distinct than in the intact cells, appeared at 8 C on decreasing the temperature (not shown in the Fig.). It should be noted that the decline of fluorescence

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**Fig. 2.** Temperature dependence of sensitized fluorescence of Chl a and allophycocyanin in Anacystis grown at 38 C upon excitation of phycocyanin. Phycocyanin was excited at 560 nm, and the fluorescence of Chl a and allophycocyanin was measured at 684 and 655 nm, respectively. Rates of temperature change were 2 C/min.

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**Fig. 4.** Temperature dependence of Chl a fluorescence in Cyanidium caldarium grown at 38 C. Fluorescence was excited at 430 nm and measured at 684 nm. Rates of temperature change was 2 C/min.
yield below the temperatures of the maximum is much less distinct in *Cyanidium* and *Euglena* than in *Anacystis*. These findings suggest that a solidification of membrane lipids, even though only in a local area, occurs in the thylakoid membrane of these organisms in the range of room temperatures.

In *Chlorella pyrenoidosa*, Chl a fluorescence did not show a maximum or a shoulder in the temperature dependence curves upon increasing and decreasing the temperature. Figure 6 shows the temperature versus fluorescence curve upon excitation of Chl a in *Chlorella* at 430 nm. The fluorescence yield became higher as the temperatures approached 0°C. The two curves followed each other rather well upon decreasing and increasing the temperature. The fluorescence of Chl a sensitized upon excitation of Chl b at 480 nm gave rise to the same results in the temperature versus fluorescence curve. The lack of a maximum of fluorescence yield in the temperature dependence curves suggests that the maximum and thus the phase transition of membrane lipids occurs at temperatures below 0°C.

In *Plectonema boryanum*, *Stichococcus bacillaris*, and *Phaeodactylum tricornutum*, the fluorescence yields did not show maxima but increased upon decreasing the temperature towards 0°C. These results indicate that the phase transition of the lipids in the thylakoid membranes occurs below 0°C in these algae as well.

The temperature dependence of Chl a fluorescence was also investigated in isolated chloroplasts of a number of higher plants such as spinach (chilling resistant), lettuce (chilling resistant) grown at 15°C and 25°C, tomato (chilling sensitive) grown at 15°C and 25°C, and *Tidestromia oblongifolia* (a high temperature plant growing in Death Valley). In all of these plants no maxima appeared over the temperature range from about 0 to 20°C (Fig. 7). These findings suggest that the phase transition of the lipids in the thylakoid membranes also occurs below the freezing point in these plants. This conclusion is in contrast to an inference by Shneyour et al. (25) that the phase transition of membrane lipids of chloroplasts of chilling sensitive plants, but not that of the chilling-resistant plants, occurs around 10°C.

**DISCUSSION**

The present study indicates that in some algae, the fluorescence yield of Chl a shows characteristic features of the temperature-dependence curves that are related to the transition of the phase of the lipids of the thylakoid membranes. This is consistent with the finding by Colbow (3) that Chl a in a phospholipid model membrane functions as a fluorescent probe for the detection of the temperature-induced phase transition of lipids. Colbow found that the fluorescence yield was drastically decreased when the temperature passed through the phase transition point from the high to the low.

In our previous study (22), we found that the transition of the spin probe signal in membrane fragments of *Anacystis* occurred at 24°C in cells grown at 38°C and at 13°C in cells grown at 28°C; we suggested that these temperatures corresponded to the transition of the physical phase from the liquid crystalline to the mixed solid-liquid crystalline states. The transition temperature corresponds to a beginning of solidification of membrane lipids. The temperatures of the maximum of fluorescence yield was close to this phase transition temperature especially upon increasing the temperature.

Holton et al. (9) in their study of the fatty acid composition in *Anacystis* found that the degree of saturation of fatty acids was lower in the cells grown at lower temperatures. It is known that
the temperature of phase transition is lower with a lower degree of saturation. It was noted in this study that the fluorescence maximum occurred at a lower temperature when Anacystis was grown at a lower temperature. This would appear to be related to the change in the fatty acid composition of thylakoid membranes with the growth temperature. These relationships seem to explain the dependence of growth temperature and the temperature of maximum fluorescence.

In the biological membranes the lipid composition is complex with respect to the lipid species and fatty acids. The thylakoid membrane of Anacystis, for example, contains, as major lipid components, monogalactosyl diglyceride (\(\sim 30\%\)), digalactosyl diglyceride (\(\sim 10\%\)), sulfoquinovosyl diglyceride (\(\sim 10\%\)), and phosphatidyl glyceride (\(\sim 10\%\)). The fatty acid composition is also complex in each lipid species.

Philips et al. (24) studied the transition of the physical phase in a mixture of two kinds of lipids. In a thermal analysis of a mixture of dioleoylphosphatidylcholine and distearoylphosphatidylcholine, the endothermic transition occurred in two temperature regions, although the transitions became less sharp, if they were compared to the transitions of the individual lipid components. In some other cases, the mixture of two kinds of lipids, for example dipalmitoyl lecithin and distearoyl lecithin, caused the two endothermic phase transitions to fuse into one apparent endothermic transition.

Figure 8 represents two extreme cases for the phase transitions of model membranes composed of two lipid species. In A the endothermic transition occurs in two distinctly separated regions designated at \(t_1\) and \(t_2\). In B the transition occurs continuously over a wide range of temperatures bounded by \(t_1\) and \(t_3\). In the temperature region above \(t_3\), in both cases, the lipids are in the liquid crystalline state, and in the region below \(t_1\), the lipids are in the solid state. Between \(t_3\) and \(t_1\), the lipids consist of a mixture of the liquid-crystalline and solid states. It is reasonable to assume that the fluorescence yield of fluorescent probes reflects the phase transitions of lipids as shown in the lower part of Figure 8. In case A the fluorescence will show two sharp sigmoids giving rise to two maxima and minima. In case B the fluorescence will show a single broad sigmoid with maximum and minimum that are widely separated.

Much more than two species of lipids are present in biological membranes. This implies a complex behavior of fluorescence as a function of the phase transition of membrane lipids. In some cases, it can be assumed that more than one transition occurs at different temperature regions (similar to case A of Fig. 8). In other instances there is apparently only one broad transition occurring over a wide range of temperature (similar to case B in Fig. 8).

In the case of Cyanidium the appearance of a maximum at 29 C and a minimum at 24 C in the Chl a fluorescence versus temperature curve would seem to correspond to a minor phase transition that is separated from a major one which will appear at temperatures below those used for these experiments. This behavior seems to be an example of case A in Figure 8. In Anacystis and Euglena the maximum can but not the minimum appeared at room temperatures. A minimum will probably occur below 0 C corresponding to the situation suggested by case B.

The study of artificial fluorescent probes in model membranes suggests that they are partitioned between the hydrophobic region of the membrane and the aqueous phase, and fluoresce when they are bound to the membrane (23). The hydrophobicity of membranes is higher in the liquid crystalline than in the solid state. When the temperature passes through phase transition point from the high (liquid-crystalline state) to the low (solid state), the number of bound probe molecules and thus the fluorescence yield are abruptly reduced. This model can be applied to Chl a fluorescence in the model and biological membranes. The Chl a molecule is composed of a porphyrin ring which contains a hydrophilic and hydrophobic region, and a long chain alcohol, phytol, which is strongly hydrophobic. According to thermodynamics, the most stable condition of the Chl a molecule in the lipid bilayer is achieved when the phytol is associated with the hydrocarbon region of membrane lipids and the porphyrin ring is situated at the surface of the membrane. Because of a strong interaction between the phytol and the hydrocarbon region, the Chl molecules are always bound to the thylakoid membranes regardless of the change in hydrophobicity of the membrane produced by a change in the physical phases of membrane lipids. The porphyrin ring of Chl may behave like 1-anilinonaphthalene-8-sulfonate or N-phenyl-1-naphthaleine (Fig. 9). More of the porphyrin ring is dissolved in the hydrocarbon region of membranes in the liquid-crystalline than in the solid state. It seems likely that only the Chl a bound to membrane lipids is influenced directly by the physical state of the lipids whereas the Chl bound to membrane proteins is not. However, excitation transfer between the lipid-bound and protein-bound Chl a molecules must be very fast and efficient. Thus, if molecules of lipid-bound Chl a interact within membrane lipids, the Chl a fluorescence from both kinds should respond to the transition of the physical phase in similar ways.

The fluorescence of Chl a is a better probe for detecting the transition of the phase of membrane lipids than is the fluorescence from artificial probes. In intact cells, in which many kinds of membranes and cell organelles are included, the added artificial probes are not necessarily bound only to chloroplast membranes. Chlorophyll a molecules are localized exclusively in the chloro-
plants and thus provide information about the physical state of thylakoid membranes. The artificial probes do not work well even in extracted chloroplasts or in thylakoid membranes because the efficient energy transfer from the fluorescent probes to Chl or carotenoids almost completely quenches the fluorescence of the membrane-bound probes.

The drastic increase of fluorescence yield of allophycocyanin in Anacystis below 5 C must be a reflection of the suppression of excitation transfer from the allophycocyanin to Chl a in pigments system 2. At temperatures near 0 C the lipid areas of the membrane in the solid state are probably large and this may influence the binding between the phycobilisomes and the thylakoid membranes. In relation to this it is interesting to note that Anacystis is an organism that is especially chilling sensitive. When the cells are stored at 4 C for 1 hr, they lose the activities of photosynthesis and the Hill reaction (6).

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LITERATURE CITED