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Temperature dependent changes in absorption and fluorescence properties of the cyanobacterium *Anacystis nidulans**

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Abstract. Temperature dependent changes in absorbance and fluorescence of chlorophyll *a* (Chl *a*) were analyzed in membrane fragments and in a Chl-protein complex reconstituted with lipids isolated from the cyanobacterium *Anacystis nidulans*. Absorbance *versus* temperature curves measured at 656 nm showed an inflection point at 23–24°C and at 14–16°C in the membrane fragments prepared from *A. nidulans* cells, grown at 39° and 25°C, respectively. Temperature-induced absorbance changes measured at 680 and 696 nm did not show clear break points. The presence of lipids was essential in order to see a clear maximum in the fluorescence *versus* temperature curve of Chl *a* in a Chl-protein complex. It is suggested that a specific form of Chl *a* may be associated with lipids in the thylakoid membranes and that this form of Chl *a* may be responsible for temperature-induced absorbance and fluorescence yield changes in this cyanobacterium.

Introduction

Much of our knowledge regarding the location of Chl in photosynthetic membranes has been derived from studies relating solubilization of membranes with various detergents. These studies indicate that all of the Chl is bound to membrane proteins [18, 22]. However, there is evidence that suggests Chl is associated with lipids of thylakoid membranes. Murata et al. [19] and Murata and Fork [20] showed that Chl *a* fluorescence reflected the physical state of membrane lipids as evidenced by a change in fluorescence yield at the phase transition temperature of the membrane lipids. Recently, Vigh and Joó [28] showed that changing the saturation level of membrane lipids by catalytic hydrogenation of lipids shifted the change in

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; SDS, sodium dodecyl sulphate.

Chl *a* fluorescence yield to a higher temperature which suggests that Chl *a* is in some close association with membrane lipids.

Studies on reconstitution of Chl-protein complexes with lipids indicate that lipids play an important role in the organization of Chl in the membrane [16, 25, 26]. In addition, Eigenberg et al. [6] reported ^{13}C-NMR studies with intact spinach thylakoid membranes and suggested that at least 30% of the Chl was lipid-bound or at the most only loosely associated with proteins.

It is well known that fluorescence yield of Chl *a* in liposomes decreases markedly at the temperature of the gel to liquid crystalline phase transition of the lipid [15, 17]. Also, Chl *a* incorporated into liposomes exhibits temperature-induced absorbance changes upon passage through the phase transition temperature of the lipid [12]. Curve fitting analysis of the absorption spectrum of Chl *a* in liposomes showed that upon cooling below the phase transition temperature Chl *a* having absorption peak at 670 nm (Chl *a*-670) increased significantly at the expense of a species that absorbed maximally at 662 nm (Chl *a*-662) [13]. It is well known that various forms of Chl *a* occur *in vivo* [8]. Recently, Brown [5] suggested that each form may be associated with a specific thylakoid apoprotein.

In view of the observations that the physical state of membrane lipids influences the absorbance [4, 12], and fluorescence of Chl *a* [19, 20], we have analyzed temperature dependent changes in absorbance and fluorescence of Chl *a* in membrane fragments and in a Chl-protein complex reconstituted with lipids isolated from the cyanobacterium *Anacystis nidulans* with the hope of correlating the association of a particular Chl species with lipids in thylakoid membranes. Our results indicate that the temperature dependent properties of absorbance and fluorescence of thylakoid membranes are in some ways similar to those of Chl *a* incorporated into lipid, and that the temperature dependent fluorescence yield changes occurring at the phase transition temperature may be influenced by a change in the environment of a specific form of Chl *a*.

Materials and methods

Cell culture

Anacystis nidulans TX20 (UTEX 625, collection of Algae, University of Texas, Austin, TX 78712) was grown at 39 °C or 25 °C in nutrient medium as reported by Rao et al. [24]. (*A. nidulans*, a blue-green alga, is also classified as a cyanobacterium.) Cells were aerated with 3% CO_2 in air under constant illumination of fluorescent lamps.

Preparation of membrane fragments of A. nidulans

Cells in the logarithmic growth phase were harvested, suspended in 10 mM NaCl and 10 mM Tricine-NaOH (ph 7.8), and disrupted by passage through

a French pressure cell at 14 000 psi. The disrupted cells were cooled in an ice-water mixture and the following procedures were all done at about 4 °C. Unbroken cells and debris were removed by centrifugation at 30 000 × g for 30 min. The supernatant (25 ml) was layered onto a medium (8 ml) containing 0.4 M sucrose, 10 mM NaCl, and 10 mM Tricine-NaOH (pH 7.8), and centrifuged at 100 000 × g for 1 hr. The pellet was resuspended with 10 mM NaCl and 10 mM Tricine-NaOH (pH 7.8).

Preparation of Chl-protein complexes and glycerolipids from A. nidulans

Chl-protein complexes were isolated from thylakoid membranes of *A. nidulans* grown at 39 °C by a SDS-sucrose density gradient procedure as described previously [14]. Thylakoid membranes were obtained by centrifugation after lysozyme-treated spheroplasts were suspended with 10 mM NaCl and 10 mM Tricine-NaOH, pH 7.8, and disrupted by passage through a French pressure cell. The thylakoids solubilized with 4.5% SDS in 0.1 M Tris-HCl, pH 8.6, were layered onto a linear sucrose density gradient (5–35% sucrose in 0.1% SDS-50 mM Tris-borate, pH 9.5), and centrifuged at 258 000 × g for 16 hr at 15 °C.

Lipids were extracted from *A. nidulans* cells grown at 39 °C by the method of Bligh and Dyer [2], evaporated to dryness and dissolved with chloroform. The lipids were loaded onto a silicic acid column and washed with chloroform to remove large amounts of pigments. Glycerolipids with a small amount of carotenoid were eluted from the column with chloroform/methanol (1:1, v/v) and then with methanol. The lipids were further purified with thin layer plates covered with silica gel G (Supelco Inc.) using a solvent system of chloroform/methanol/acetic acid/water (170:25:25:6, v/v) or chloroform/methanol/water (65:25:4, v/v). The bands of glycerolipids (mono- and digalactolipids, sulfolipid, and phosphatidylglycerol) were detected by spraying 2% I_2 in ethanol at the edge of the plate. The bands were scrapped from the plate, eluted with chloroform/methanol (1:1, v/v), evaporated and dissolved with chloroform/methanol (1:1, v/v).

Reconstitution of a Chl-protein complex with glycerolipids

The Chl-protein complex, (10 μg Chl *a*, 0.12 ml) associated with photosystem I (Chl/P700 = 79, Band II in ref. 14) were mixed with liposomes (0–500 μg of glycerolipids) which were prepared in 10 mM NaCl and 10 mM Tricine-NaOH (pH 7.8) by sonication, and made up to 0.5 ml with the medium used for preparation of liposomes. The mixture was frozen at liquid nitrogen temperature for 90 sec and then thawed in a water bath (25–27 °C) with agitation as described by Larkum and Anderson [16], except that the sonication step was omitted. Chl concentration was measured spectrophotometrically as described by Arnon [1].

Fluorescence and absorbance measurements

Fluorescence emission spectra were measured with a microprocessor-based spectrofluorometer equipped with a fiber optic system as described previously [7]. Blue excitation light ($\lambda = 442$ nm) was provided by a HeCd laser (Liconix model 4240) fitted with a Corning C.S. 4-96 filter and a neutral density filter.

Temperature-dependent fluorescence changes were measured using the same fiber optic system as described previously [20]. Samples were excited by blue light (440 nm) produced by passing the white light obtained from a tungsten lamp (type DLS, 150 W) through an interference filter (440 nm, Balzers), a glass filter (Corning C.S. 4-96), a heat-reflecting filter (Balzers, Calflex C) and a neutral density filter. The rate of temperature decrease or increase was about 1 °C/min.

Absorption spectra were measured with a Perkin-Elmer 356 spectrophotometer connected to a Hewlett-Packard Computer (HP 1000, F series). Opal glass was placed between the cuvettes and the photomultiplier.

Absorbance *versus* temperature curves were measured as described previously [12]. The rate of change of temperature was the same as for fluorescence measurements, about 1 °C/min.

Results

Temperature-induced absorbance changes of membrane fragments of A. nidulans

Figure 1 shows absorption spectra of intact cells and membrane fragments of *A. nidulans*. The ratio of absorbance at 625 nm (phycocyanin absorption) to that at 678 nm (Chl *a* absorption) (A625/A678) of cells and membrane fragments was 1.23 and 0.23, respectively, indicating that most of the phycocyanin had been removed from the membrane fragments. The temperature-induced difference spectrum (A-B), between the absorbance of membrane fragments measured at 1 °C (A) and at 40 °C (B), showed two negative peaks at 656 nm and 696 nm, a positive peak at 680 nm, and a shoulder at about 670 nm in the red region of Chl *a* absorption, in addition to changes in the blue region of the spectrum (Fig. 1 bottom). These temperature-induced changes in the absorption spectrum were almost completely reversible after the temperature was raised from 1 °C to 40 °C. The spectral changes were very similar to those seen in thylakoid membranes by Omata and Murata [23]. The fluorescence emission spectrum of membrane fragments revealed three bands with peaks at 683, 725 and 748 nm (data not shown). It has been reported [21] that the emission band at 748 nm originates from P750 in the cell envelope [see Figs. 12 and 13 in ref. 11]. Thus, it seems that the membrane fragment preparation, which consisted mainly of thylakoid membranes, was contaminated with a small amount of cell envelope membrane

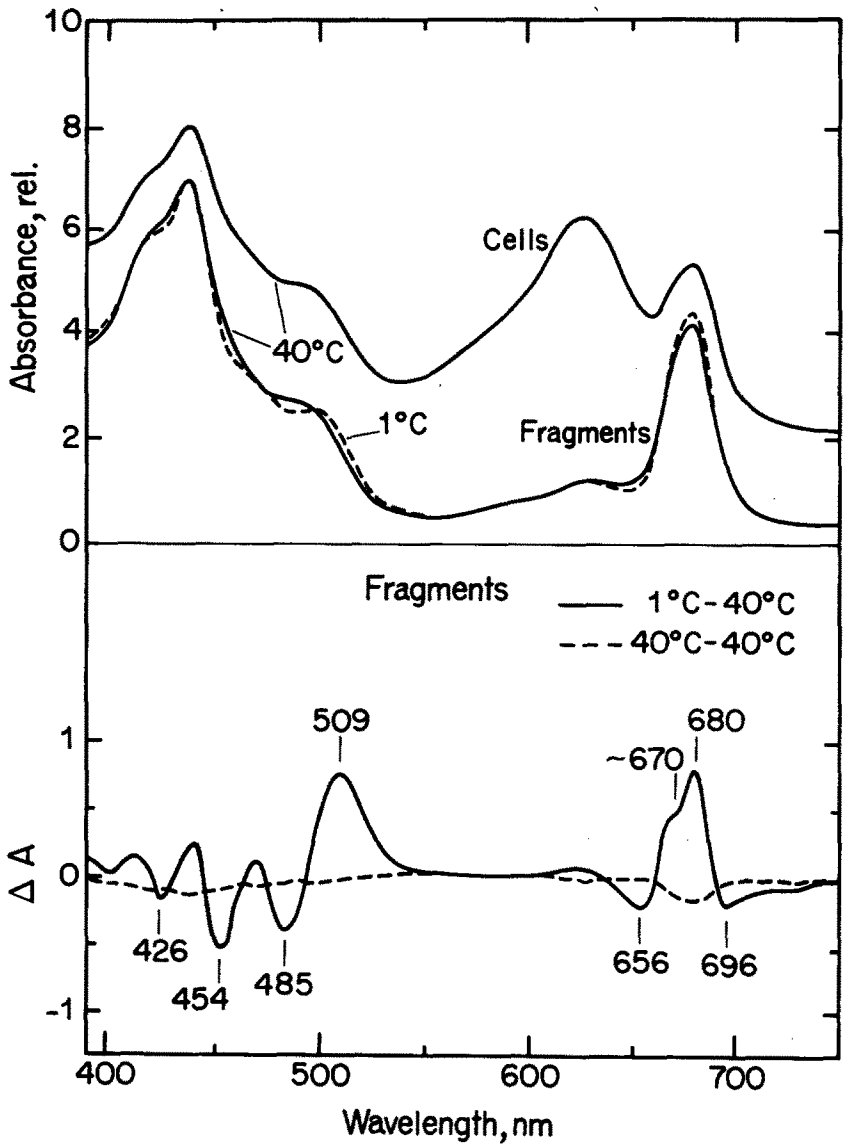
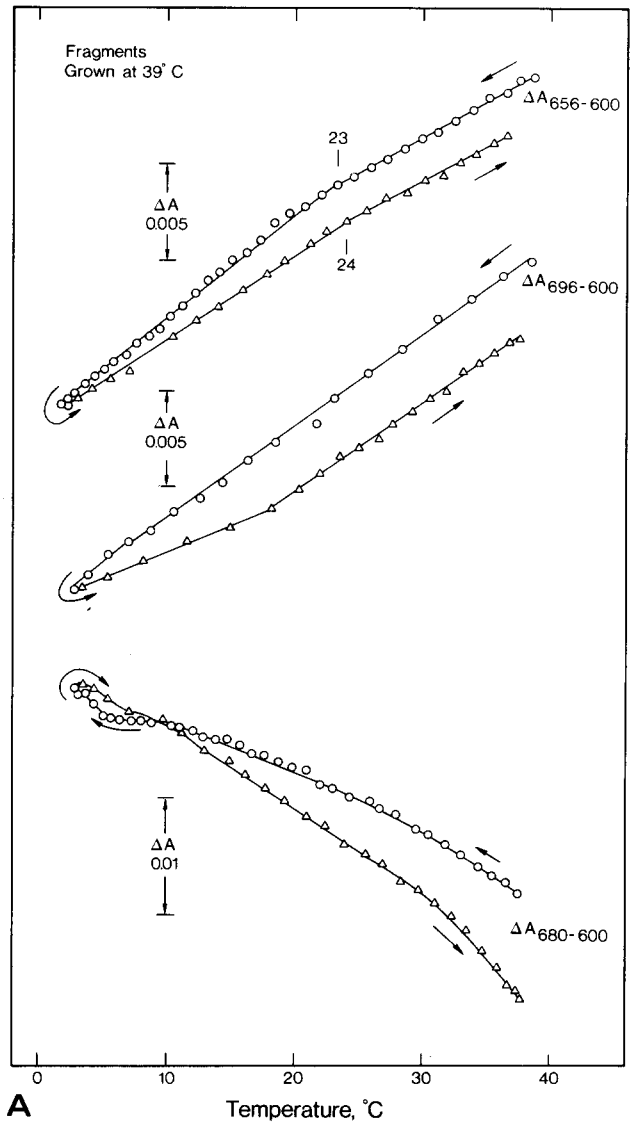


Figure 1. Absorption spectra of cells and membrane fragments (upper), and the temperature-induced difference absorption spectrum of membrane fragments (lower) of *Anacystis nidulans* grown at 39 °C. The sample, used for the measurement of the spectrum at 40 °C, was cooled to 1 °C and the absorption spectrum again measured. The cooled sample was warmed again to 40 °C.


Fragments
Grown at 39° C
ΔA656-600
23
ΔA
0.005
24
ΔA696-600
ΔA
0.005
ΔA
0.01
ΔA680-600
0
10
20
30
40
A
Temperature, °C

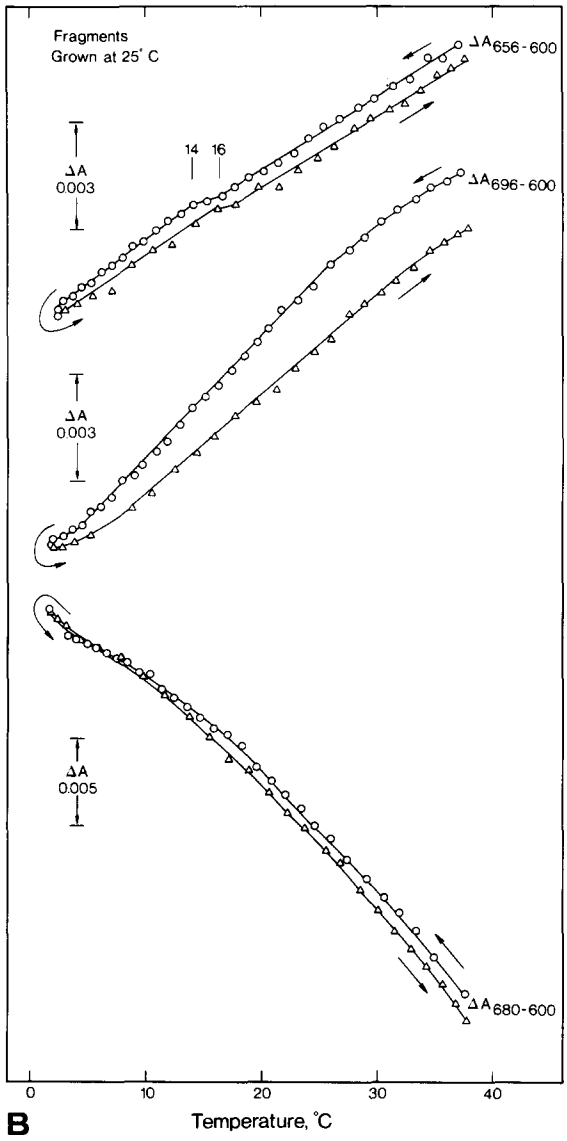


Figure 2. Temperature dependence of absorbance changes of membrane fragments isolated from *A. nidulans* grown at 39 °C (a) and at 25 °C (b). The spectrophotometer was operating in the dual-wavelength mode. Absorbance changes were monitored at 656, 680 or 696 nm, with the reference wavelength set at 600 nm.

fragments. A temperature-induced difference absorbance spectrum of intact cells also showed similar peaks near the same positions except that the extents of the changes in the blue-green region were much larger than those seen in red region, and the changes were only partially reversible as reported by Brand [3].

Figure 2a shows the temperature-dependence of absorbance changes, with reference to 600 nm, of membrane fragments obtained from 39 °C-grown cells. Upon cooling, the absorbance at 656 nm gradually decreased and showed a change of slope around 23 °C. Upon heating, the absorbance gradually increased and showed a slope change at 24 °C. The change in the latter case was less pronounced than in the former case. Absorbance measured at 696 nm did not show a break upon cooling from 39 °C to 3 °C. Upon increasing the temperature, however, a break point appeared below 18 °C. The absorbance change monitored at 680 nm showed a sigmoidal increase upon cooling, and it decreased in a similar fashion as the temperature was increased.

The temperature *versus* absorbance change curves of membrane fragments isolated from 25 °C-grown cells are shown in Fig. 2b. Upon cooling and heating, absorbance measured at 656 nm showed clear breaks at 14–16 °C. However, absorbance monitored at 680 nm, and at 696 nm did not show clear break points when the temperature was decreased or increased.

Temperature-dependent changes in Chl a fluorescence of membrane fragments and of a Chl-protein complex reconstituted with glycerolipids

Murata et al. [19] showed from measurements of electron spin resonance, fluorescence, electron transport activities and State I–State II transitions that Chl *a* in thylakoid membranes functions as an intrinsic fluorescent probe for the detection of phase transitions. As shown in Fig. 3, fluorescence *versus* temperature curves had maxima at 24–26 °C and at 14–16 °C in membrane fragments prepared from cells grown at 39 °C and 25 °C, respectively. There was little hysteresis in the measurements of temperature *versus* fluorescence of membrane fragments. Break points of absorbance at 656 nm *versus* temperature of membrane fragments (Figs. 2a and b) are very close to the maximum of fluorescence *versus* temperature in the same preparation (Fig. 3), which indicates that the absorbance change at 656 nm may be influenced by the fluidity of thylakoid membranes.

Figure 4 shows the fluorescence *versus* temperature curves of a Chl-protein complex reconstituted with glycerolipids. The Chl-protein complex associated with photosystem I and the lipids were isolated from 39 °C-grown cells. The Chl-protein complex without lipid did not show a temperature-induced maximum in the fluorescence yield. When the complex was reconstituted with lipids, the fluorescence *versus* temperature curves showed maxima at 22–26 °C. When the ratio of lipids to Chl *a* was 25, the fluorescence *versus* temperature curve showed the most distinct break at

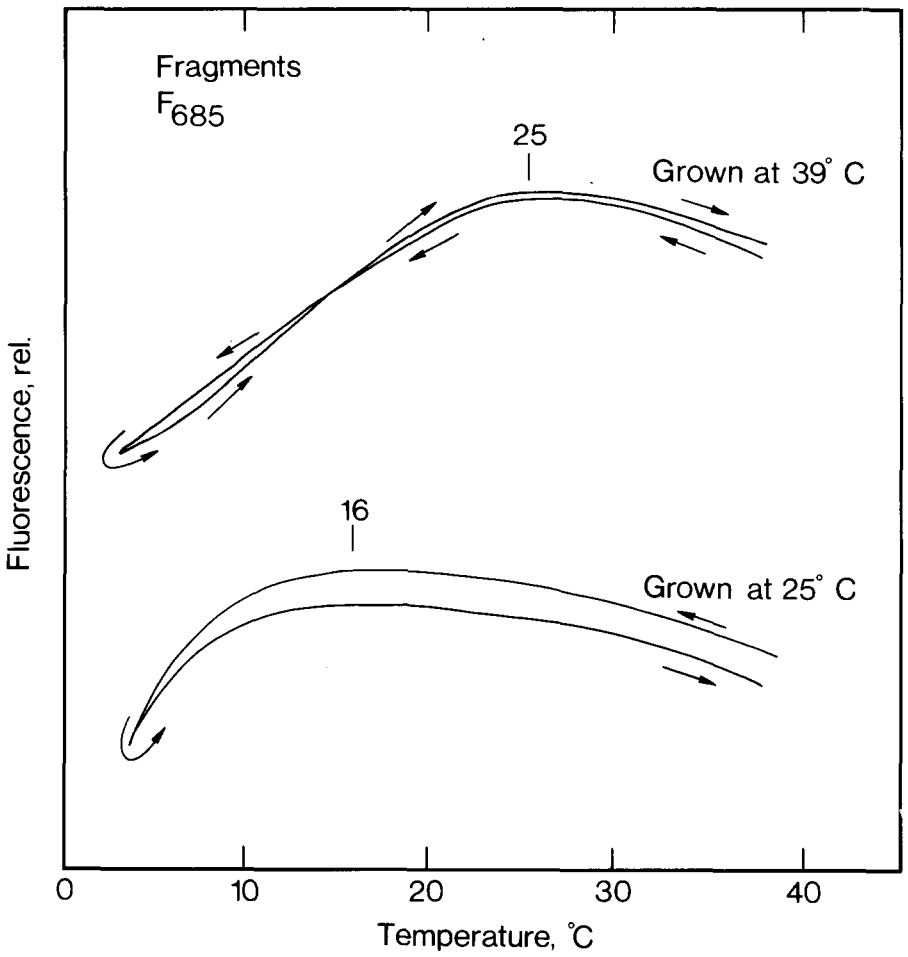


Figure 3. Temperature dependence of Chl *a* fluorescence in membrane fragments of *A. nidulans* grown at 39 or 25 °C. Fluorescence was excited at 440 nm and measured at 685 nm. The reaction mixture contained 10 mM NaCl, 10 mM Tricine-NaOH (pH 7.8), 10 μM DCMU and membrane fragments.

24–26 °C. This temperature is similar to the temperature obtained from the fluorescence *versus* temperature curve of 39 °C-grown membrane fragments (Fig. 3). The fluorescence emission spectra of the Chl-protein complex and reconstituted complex were measured at 77 K upon excitation with blue light (442 nm). The Chl-protein complex alone showed two emission peaks at 683 and 718 nm as reported previously [14]. The reconstituted Chl-protein complex also showed two peaks at 683 nm and 718 nm, in addition to a shoulder at 750 nm (data not shown). The ratio of F683/F718 in the Chl-protein complex alone was 0.38 and in the reconstituted complex was 0.33.

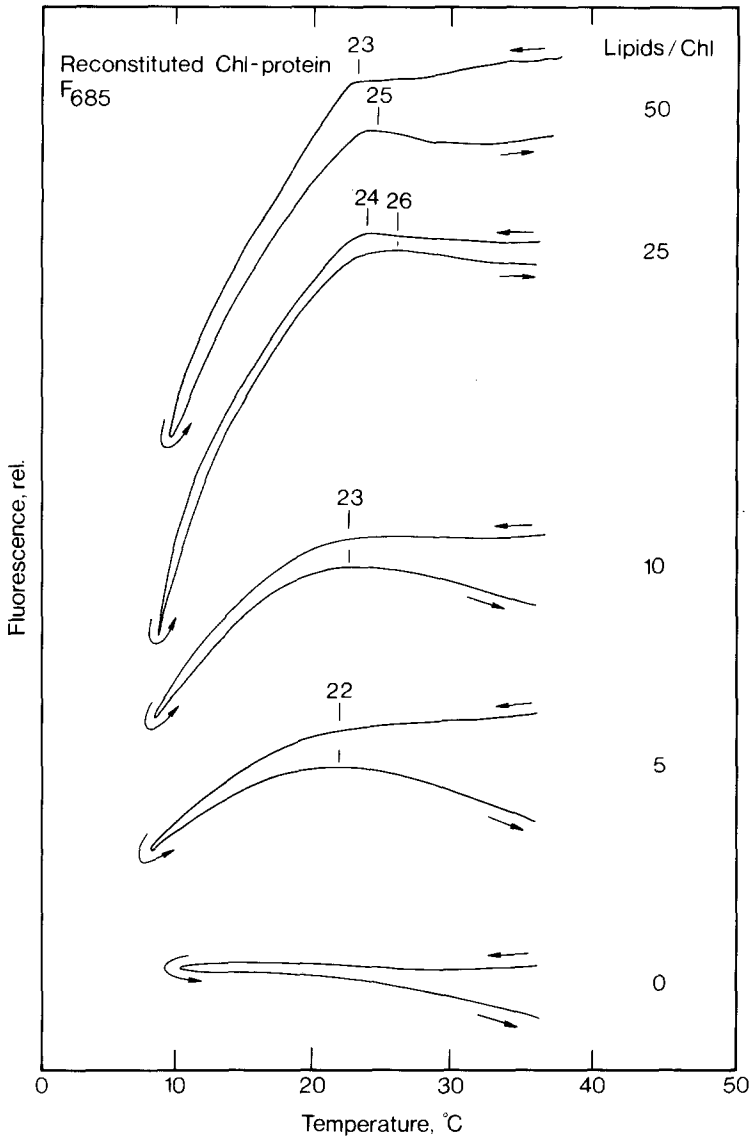


Figure 4. The temperature dependence of fluorescence of a Chl-protein complex reconstituted with glycerolipids. The ratios of lipid to Chl-protein were indicated by the weight ratios of lipid to Chl *a* when they were reconstituted. A ratio of zero means that the Chl-protein complex alone was measured but the freezing and thawing were performed the same way as for the Chl-protein complex in the presence of glycerolipids. Measurements were made in the same way as in Fig. 3.

Discussion

The Chl fluorescence *versus* temperature curve of the Chl-protein complex showed only a slightly monotonous change upon cooling or heating (Fig. 4). On the other hand maxima (22–26 °C) were observed for the Chl-protein complex reconstituted with glycerolipids from the 39 °C-grown cells, indicating that lipids were essential components for detecting the maximum in the fluorescence yield. When the reconstitution was done with a lipid to Chl *a* ratio of 25, the fluorescence *versus* temperature curve showed the most distinct maximum at 24–26 °C which was in good agreement with the value obtained for membrane fragments isolated from the 39 °C-grown cells (Fig. 3). In addition, the temperature was very close to that for the onset of the phase transition of thylakoids in 38 °C-grown cells as detected by the X-ray diffraction technique [27].

The temperature-dependent absorbance change at 656 nm showed a break point at the same temperature where maxima were seen in fluorescence *versus* temperature curves of membrane fragments of *A. nidulans*, although the break point of the absorbance change at 656 nm was not as clear as the maxima observed in the fluorescence yield changes. French et al [8] reported that four major forms of Chl *a* could be detected in the thylakoid membranes, *i.e.*, Chl *a*-662, Chl *a*-670, Chl *a*-677 and Chl *a*-684. Blue-green algae (cyanobacteria) contain phycobilins as accessory pigments [9, 10]. A detailed study has been done on phycobilisomes from *A. nidulans* [10] which are composed of phycocyanin (A_{max} 620 nm), allophycocyanin (A_{max} 650 nm) and the minor biliproteins, allophycocyanin B plus a high molecular weight biliprotein (also called allophycocyanin I), that absorb at 670 nm. Each of the core cylinders of the phycobilisomes is made up of four complexes that have absorption bands at 650–655 nm and fluorescence emission bands at 660–680 nm. The membrane fragments of *A. nidulans* used in this study contained a very small amount of allophycocyanin (F_{max} 660 nm) as was seen from the fluorescence emission spectrum of the fragments excited with blue-green light (mostly phycocyanin excitation) and might contain some of the phycobilisome core which fluoresces at 660–680 nm.

A question arises whether temperature-induced absorbance changes at 656 nm and fluorescence yield change at 685 nm derive from a specific form of Chl *a* or from allophycocyanin. It was previously reported that Chl *a* incorporated into liposomes showed temperature-induced absorbance changes upon passage through the phase transition temperature of the lipid [12]. The difference spectrum (absorbance spectrum at low temperature minus that at high temperature) had a negative peak at 657–668 nm and a positive peak at 675–685 nm. The position and extent of peaks depended upon the temperature and the molar ratio of the lipid to Chl *a*. The absorption spectrum of Chl *a* in liposomes was composed of four bands, and

Chl *a*-662 was transformed into Chl *a*-670 upon cooling below the phase transition temperature of the lipid [13]. If it is assumed that the amount of allophycocyanin is negligible compared to the amount of Chl *a* in membrane fragments, we can deconvolute the absorption spectrum into five components: Chl *a*-662, Chl *a*-670, Chl *a*-678, Chl *a*-685 and Chl *a*-694. Upon cooling, the amounts of Chl *a*-662, Chl *a*-685, and Chl *a*-694 appear to decrease while the amounts of Chl *a*-670 and Chl *a*-678 appear to increase (data not shown). It is possible, therefore, that the absorbance change at 656 nm may be produced by a change of Chl *a*-662, since a blue shift of Chl *a*-662 in the difference spectrum can be caused by an increase of Chl *a*-670. Thus, the transformation of Chl *a*-662 into Chl *a*-670 in thylakoid membranes may occur in a manner similar to that of the model system. This suggests that at least a part of Chl *a*-662 may be associated with lipids or perhaps very loosely associated with proteins in the thylakoid membranes. On the other hand, temperature-dependent absorbance changes measured at 680 and 696 nm do not seem to be associated with lipids.

Brody [4] reported a temperature-induced absorbance change in *A. nidulans* cells. He suggested that the change of Chl *a*-678, Chl *a*-690, and Chl *a*-703 was associated with a change of the physical phase of membrane lipids. Our results do not agree with this suggestion.

Murata and Fork [20] reported that the fluorescence yield at 684 nm showed a maximum at the temperature of the lipid phase transition but the fluorescence at 655 nm did not show a maximum when *A. nidulans* cells were excited at 560 nm. This indicates that allophycocyanin in the rods of the phycobilisomes was not influenced by a phase change of membrane lipids. Fluorescence at 684 nm is a mixture of fluorescence of Chl *a* and of the long wavelength form of allophycocyanin in the core of the phycobilisomes. Upon using excitation at 440 nm that was absorbed mostly by Chl *a*, a curve of temperature *versus* fluorescence was obtained at 685 nm that reflected fluorescence yield changes caused almost entirely by Chl *a* in the membrane fragments. However, it is difficult to rule out the possibility that some long wavelength forms of allophycocyanin in the core or phycobilisomes might participate in the temperature-induced absorbance changes seen at 656 nm in the membrane fragments used in this study.

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