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Primary Role of the Cytoplasmic Membrane in Thermal Acclimation Evidenced in Nitrate-Starved Cells of the Blue-Green Alga, *Anacystis nidulans*

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ZOLTÁN GOMBOS* AND LÁSZLÓ VIGH
Institute of Plant Physiology (Z.G.) *and Institute of Biochemistry* (L.V.), *Biological Research Center Hungarian Academy of Sciences, P.O. Box 521, 6701 Szeged, Hungary*

ABSTRACT

The lipid phase transition of the cytoplasmic membrane and the chilling susceptibility were studied in nitrate-starved *Anacystis nidulans* cells. Nitrate starvation resulted in the disappearance of the thylakoid membrane system, without any effect on chilling susceptibility. The chilling susceptibility of the algal cells depended on the growth temperature. Temperatures of lipid phase transitions of the cytoplasmic membranes were detected by chilling-induced spectral changes in the carotenoid region, *in vivo*. These values were identical to those of cultures containing intact thylakoid systems. Our results suggest that cytoplasmic membrane plays a determinative role in the thermal acclimation of the alga cells.

The blue-green alga, *Anacystis nidulans*, offers an effective experimental system for the study of the relationship between growth temperature, cold sensitivity, and thermotropic changes in membranes. Although this alga is a prokaryotic organism, it can be regarded as a model system for the study of the temperature effects on higher plants (18). As reported by Murata *et al.* (9, 10), photosynthetic evolution, Hill activity to benzoquinone, and the bleaching of membrane pigments in *Anacystis* cells show identical temperature dependences. The optimum temperatures associated with these phenomena are a function of the growth temperature of the cultures (13). These optimum temperatures, however, were found to be considerably lower than the phase transitions detected in the thylakoid membranes, but they were in very good agreement with those for release of potassium ions and ninhydrin-reacting substances (14). These data suggest that it is a phase separation occurring in the cytoplasmic membrane which leads to an enhanced permeability of the cells.

Changes in lipid phase with temperature have been detected within the membranes of *Anacystis* using spin probe (10), x-ray diffraction (22) methods, or freeze-fracture EM (5). These physical techniques however, yield conflicting results. Even using freeze-fracture technique, which allows a direct visualization of the segregation of smooth regions of gel-phase lipids within fractured faces, the onset of phase separation of the cytoplasmic membrane is found to be considerably higher in a study of Armond and Staehelin (2) than observed by Ono and Murata (15) using *Anacystis nidulans* cells grown at identical temperatures (38°C).

To prove the validity of the mechanism attributing primary role of cytoplasmic membrane in thermal adaptation and chilling susceptibility, we used nitrate-starved *Anacystis nidulans* cell system. As described elsewhere (6), growing the algal cells in nitrogen-free culturing medium results in a rapid degradation, or even a complete breakdown, of the photosynthetic apparatus. During this process, the lipids of the alga cells are catabolized, and concurrent changes in the composition of carotenoids can also be detected (6, 17). By adapting the method of nitrate starvation, we were able to obtain *Anacystis* cells, grown at various temperatures, which had disrupted endomembrane system, but possessed intact cytoplasmic membrane. Moreover, spectral changes of carotenoids during chilling, which are considered to be indications of the phase separation of the cytoplasmic membrane, were amplified by approximately three-fold as a consequence of enhanced zeaxanthin content of these cells.

MATERIALS AND METHODS

Anacystis nidulans cells IU 625 (ATCC 27144) were propagated axenically in Kratz and Myers' liquid medium C (8), containing 1 M KNO_3, at 28° and 38°C and illuminated with white light at an intensity of 3.6×10^4 mW m^{-2}. A mixture of sterile air and CO_2 (95:5) was bubbled through the medium. Nitrate starvation was induced by suspending the algal cells in nitrate-free culturing medium, following a 5000*g* centrifugation.

For the electron microscopy studies, algal cells were fixed in 3.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.0) for 4 h. After washing in this buffer, they were postfixed in 1% OsO_4 for 1 h. The material was dehydrated in a graded series of ethanol:water solutions and embedded in Araldit (Fluka). Ultrathin sections were stained with lead citrate and examined in a JEOL 100B electron microscope.

For chilling treatment, the alga cell suspensions were kept at appropriate temperatures between 0° and 27°C for 20 min. Before performing various measurements, the samples were rewarmed to 25°C.

To investigate the K^+ release in the chilling treated cells, the cultures were washed three times by resuspension and recentrifugation with 40 mM Na-phosphate buffer (pH 7.0) at the growth temperature. The suspensions were centrifuged at 5000*g* for 10 min and the amount of potassium in the supernatant was determined by atomic absorption spectrometry (AAS-1-N, Zeiss, GDR).

The separation of thylakoid and cell envelope in the normally grown and nitrate-starved *Anacystis* cells was carried out according to the method of Murata *et al.* (9). The cells were disrupted by French pressure cells following lysozyme digestion. Unbroken cells were removed by centrifugation at 10,000*g* for 15 min. The bulk of the membrane was collected at 40,000*g* for 60 min. The fragmented membranes were recovered from the supernatant by centrifuging at 120,000*g* for an additional 40 min. The combined

membrane fractions was further separated on a sucrose gradient (30 to 90 %/w/v) by a 6-h centrifugation at 180,000*g*. Carotenoids were separated by TLC according to the method of Hager and Meyer-Bertenrath (7) using the petroleum ether: 2-propanol (20/1 v/v) solvent system suggested previously (9). Chl *a* was eliminated in the case of the normal cells as described previously (9). The amounts of separated carotenoids were determined spectrophotometrically (9).

The absorption and difference spectra of the thermally acclimated control and nitrate-starved cells were recorded at 25°C after chilling treatment, described above, on a UNICAM SP 1800 spectrophotometer using an opal glass technique similar to that of Shibata (20). The cell density was adjusted to 3.8×10^8 cells/ml for spectroscopic studies and to 4.5×10^9 cells/ml for the measurements of the K^+ release. The results reported here are the means of a minimum of three replicate experiments.

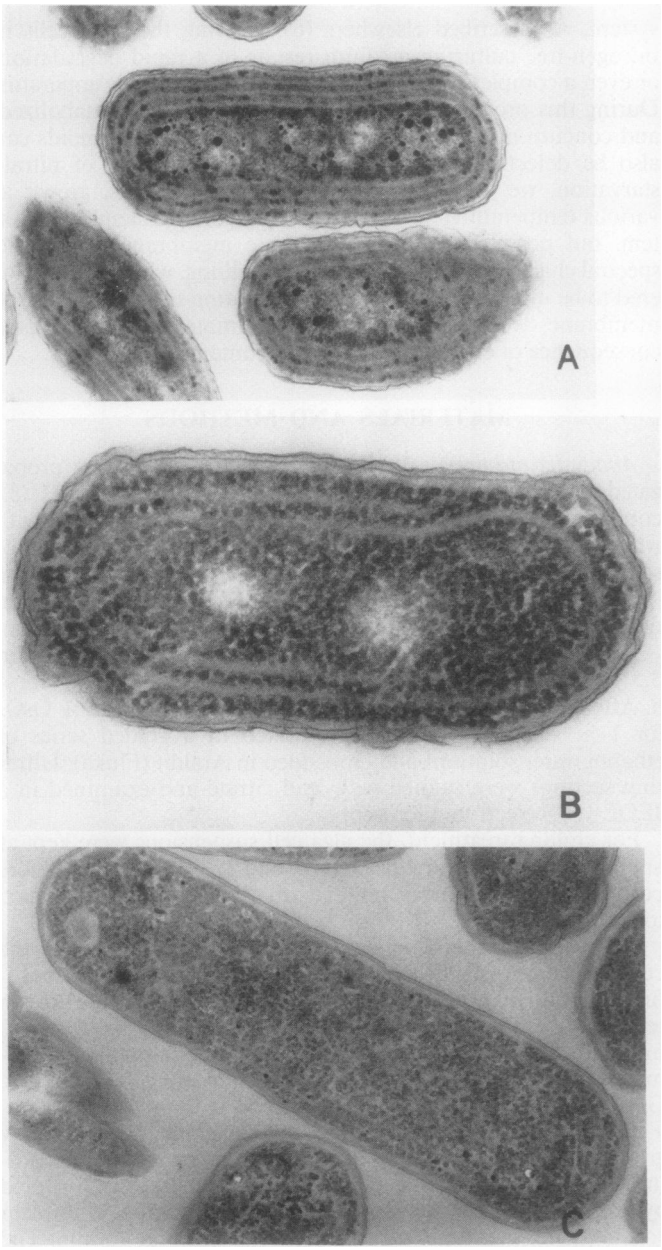

FIG. 1. Longitudinal section of the *Anacystis nidulans* grown at normal conditions (A), nitrate-free medium for 24 h (B), and for 48 h (C), respectively (magnification × 25,000).

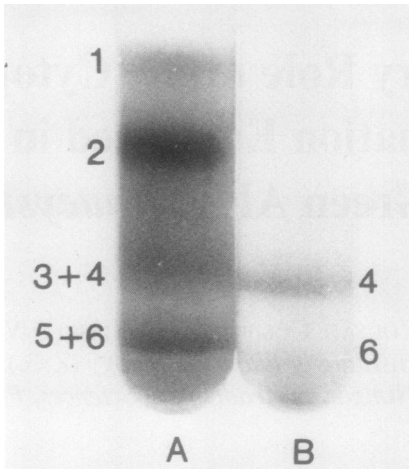

FIG. 2. Separation of thylakoid and cell envelope by centrifugation on a linear sucrose density gradient. The membrane fraction of control cells (A) and cells nitrate-starved for 48 h (B) were centrifuged at 180,000*g* for 6 h. Bands belong to: (1), phycobiliproteins (blue); (2), fragmented thylakoid (green); (3), thylakoid (green); (4), fragmented envelope (orange-yellow); (5), unbroken cells (green); (6), envelope (orange-yellow).

Table I. *Changes in the Carotenoid Percentage Compositions during Nitrate Starvation*

	Duration of Nitrate Starvation			
	0 h	24 h	48 h	72 h
β-Carotene	52	29	9	5
Cryptoxanthin	1	tr[a]	tr	tr
Zeaxanthin	38	53	68	69
Caloxanthin	7	14	19	20
Nostoxanthin	2	4	4	6

[a] tr, trace.

RESULTS

The fundamental membrane structures found in *Anacystis nidulans* cells are the cell envelope and the thylakoid. The former consists of an outer, lipopolysaccharide region and an inner, or cytoplasmic, membrane. The photosynthetic apparatus consists of lamellae made up from two closely apposed unit membranes (1, 23). Typical electron micrographs of normally grown *Anacystis* cells, showing details of their ultrastructure are presented in Figure 1A. There were however, dramatic changes in the fine structure of the membranes of cells grown in nitrate-free medium (Fig. 1, B and C). These figures show typical longitudinal sections of nitrate-starved alga cells cultured in nitrate-free medium for various times at 38°C. Although the size and the shape of the cells did not vary with nitrate starvation, there was an almost complete loss of thylakoid structure following a 24-h starvation period. To obtain further proof for the disappearance of the thylakoids, the putative thylakoid and cell envelope were separated by mechanical disruption of lysozyme-treated alga cells by differential and density gradient centrifugation. In agreement with the earlier study of Murata *et al.* (9), the thylakoids obtained from the cells grown at the normal nitrate level formed a green band at a sucrose concentration of 60% and the cell envelope an orange-yellow band at 80% sucrose concentration (Fig. 2A). Comparison of this pattern with that obtained from the cells grown in nitrate-free medium for 48 h supports the view that the thylakoid membranes are absent in cells (Fig. 2B). These cells yielded only two orange-yellow bands on the sucrose gradient

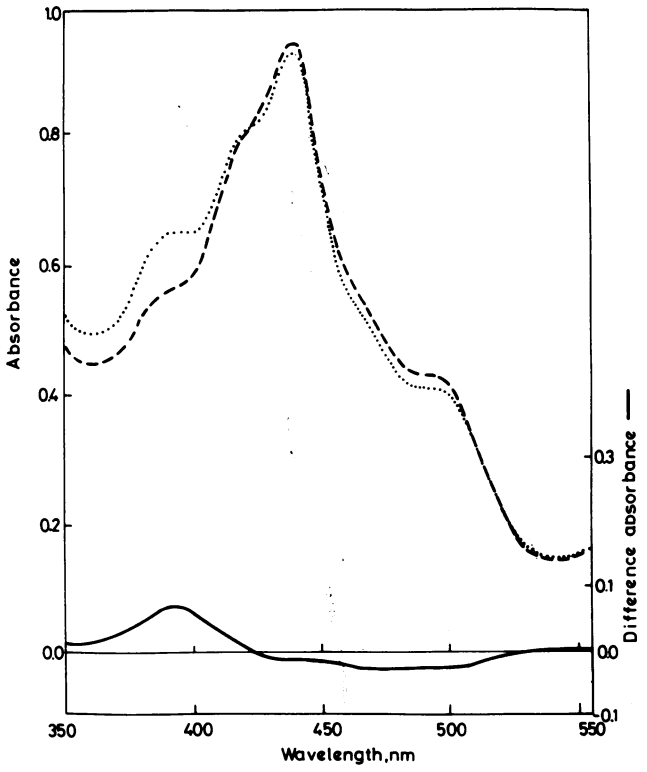

FIG. 3. Effect of chilling treatment at 0°C on the light-absorption spectrum in cells grown in the normal culture medium at 38°C. The cells were suspended in the culture medium to give a cell density 3.8×10^8 cells/ml. Absorption spectra were recorded after the cell suspension was rewarmed to 25°C. The symbols indicate the absorption spectrum of unchilled cells (– – – –), the cells treated for 20 min at 0°C (· · · · ·) and the difference spectrum between unchilled and chilled cells (——).

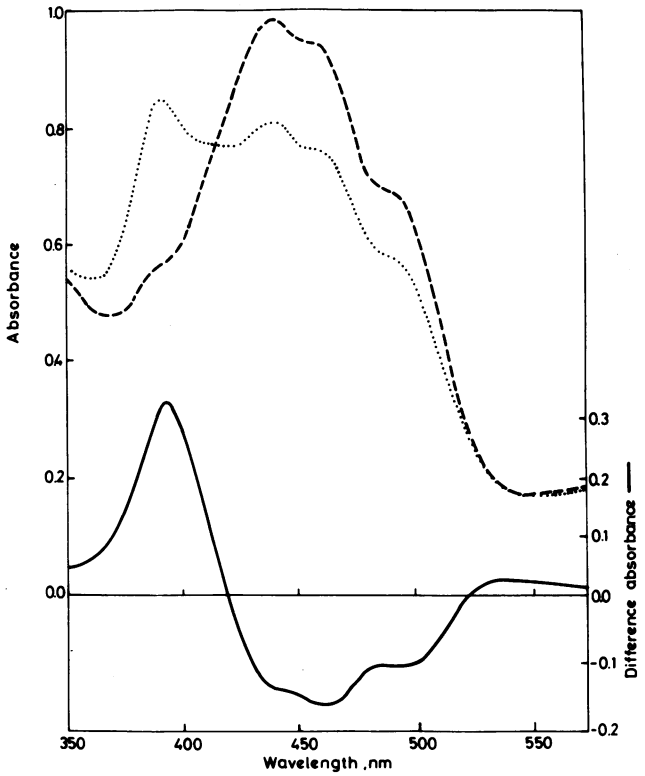

FIG. 4. Effect of chilling treatment at 0°C on the light-absorption spectrum in cells grown in nitrate-free medium for 48 h at 38°C. The cell density was 3.8×10^8 cells/ml. The symbols are identical to those of Figure 3.

and these were identified by EM as consisting solely of envelope membranes (data not shown). The alterations observed in the ultrastructure of nitrate-starved cells, associated with this manipulation also resulted in considerable changes in their carotenoid composition (Table 1). The relative content of β-carotene gradually decreased during starvation, whereas the levels of the xanthophylls were greatly enhanced in the 'yellow' cells. The proportion of zeaxanthin, which is the main xanthophyll component of the cytoplasmic membrane (12), is almost doubled following 48-h nitrate starvation. No significant changes were detected in the control cells grown in the normal culturing medium for a similar period. As might be expected, nitrate starvation induced characteristic changes in the light absorption spectrum of the alga cells (6). In comparison to normal alga cells, the absorbance in the carotenoid region increased markedly on nitrate starvation (see the band at 490 nm on Figs. 3 and 4).

In agreement with previous studies (3, 4, 13), we found that the absorption spectrum of the alga cells is greatly influenced by chilling pretreatment. An absorption increase occurs at 390 nm, which has been attributed to a conformational change or an aggregation of the zeaxanthin in the membrane, due to a lipid phase transition (13, 25, 26). The difference in spectra between the cells grown at 38°C that have been cooled to either 0° or 25°C is shown for normal and nitrate-starved populations in Figures 3 and 4, respectively. This comparison which was made at equivalent cell densities, showed that chilling-induced absorption increase at 390 nm is greatly enlarged following nitrate starvation.

The dependence of these changes on chilling temperatures in cells grown at 38°C in normal and nitrate-free culturing medium is shown in Figure 5A. Low-temperature-induced enhancement in the spectral shift of zeaxanthin was about tripled in nitrate-starved cells but the temperature range, between 9° and 15°C with a midpoint around 12°C, was not affected by nitrate starvation.

We also examined the way in which the critical temperature region depends on the growth temperature. We found that the midpoint value of the zeaxanthin shift in the cells grown at 28°C, was unrelated to the nitrate supply and shifted, parallel with the decrease in the growth temperature, to around 4°C (Fig. 5B). The midpoint values of the critical temperature regions established on the base of zeaxanthin shift, closely coincided with those observed for K^+ release. The temperature dependence of K^+ release in the cells grown in nitrate-free medium at 38° and 28°C is shown in Figure 6. The midpoint values for cells grown at 38° and 28°C were around 15° and 3°C, respectively.

DISCUSSION

The mechanism of thermoadaptation and chilling injury in plant cells is thought to be a membrane-related phenomenon, and has been intensively investigated using *Anacystis nidulans* as a model system (16, 18). Elucidation of the primary site of chilling damage requires identification of the specific membrane alterations responsible for the acclimation process. Murata *et al.* (9, 10) have suggested that chilling injury, *i.e.* irreversible damage to physiological activity at chilling temperature, in *Anacystis* cells is induced by a lipid phase transition of the cytoplasmic membrane from the liquid-crystalline to the phase separated state. We have recently reported that *in vivo* catalytic hydrogenation of the *cis*-unsaturated fatty acyl bonds results in a shift in the chilling susceptibility of the alga cells, which simulates the thermoadaptive modulation of membrane fluidity (24).

To check the role of the cytoplasmic membrane, compared to that of the thylakoid system, in the mechanism of thermoadap-

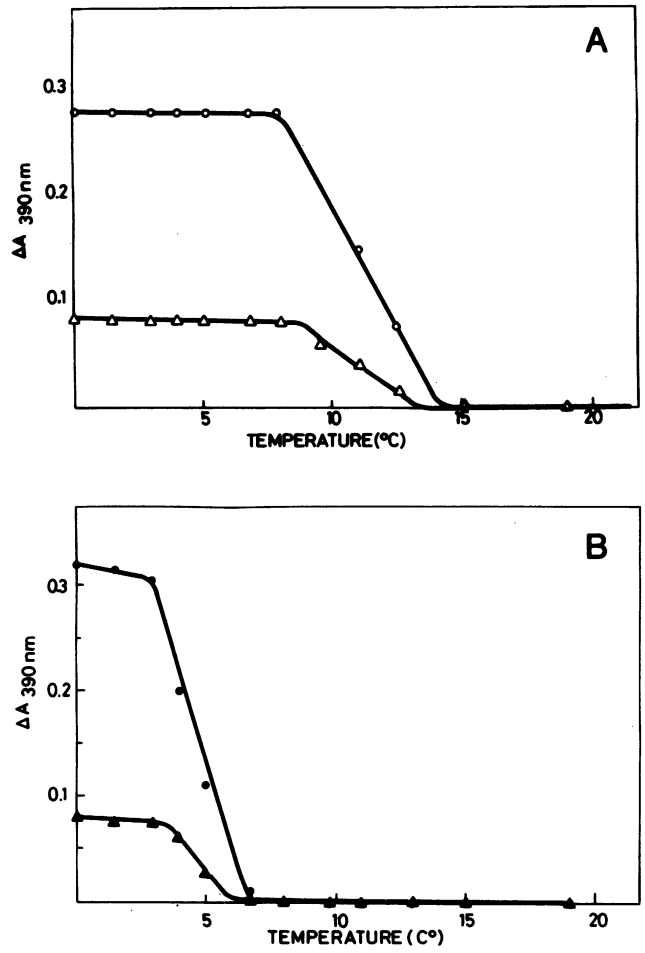

FIG. 5. Dependence of the absorption changes in cells grown at 38°C (A) in normal culture medium (Δ) and in nitrate-free medium for 48 h (O) on chilling temperatures and in cells grown at 28°C (B) in normal medium (▲) and in nitrate-free medium (●). Cell density was adjusted to 3.8×10^8 cells/ml.

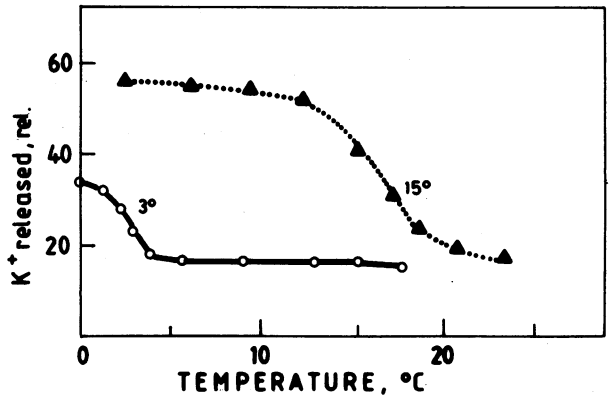

FIG. 6. Dependence of K^+ release on chilling temperatures in alga cells grown at 38°C (▲) and 28°C (O), in nitrate-free medium. The cell density was 4.5×10^9 cells/ml. The cells were treated for 20 min at chilling temperatures.

tation and chilling injury our present experiments were performed on nitrate-starved algal cells. In contrast to normally grown alga cells, which possess a well-developed system of peripheral photosynthetic lamellae, after a 48-h period of nitrate starvation, the cell interior became almost structureless. This breakdown of the thylakoid membranes caused a significant loss of cytoplasmic material. The absence of a normal thylakoid system in nitrate-starved cells was confirmed by gradient centrifugation. The Raman decay constants of some membrane-bound carotenoids have proved to be temperature-dependent in *Anacystis* cells and are believed to reflect the phase transition temperature of the thylakoid membranes. No such temperature dependence was observed in nitrate-starved alga cells, again suggesting that there is general destruction of the thylakoid membranes in such cells (21).

Nitrate starvation resulted in a relative enhancement of the level of zeaxanthin which was accompanied by a simultaneous decrease of β-carotene, and the overall composition of the carotenoids in the starved cells became similar to that of the cytoplasmic membrane isolated from normal 'green' cells. The increased extent of the chilling-induced spectral change associated with zeaxanthin, found in the spectrum of the nitrate-starved cells, is also characteristic of the isolated cytoplasmic membrane (12). On the basis of these data, we conclude that the algal cells grown in nitrate-free medium for at least 48 h offer a convenient experimental system for the study of the role of cytoplasmic membrane in thermal acclimation.

It is known that in higher plants the membranes of several organelles exhibit chilling-induced changes (11, 16, 19). However, as pointed out by Quinn and Williams (18), no clear correlation has been demonstrated between these changes in the endomembranes and chilling sensitivity of higher plants (16), and there is as yet no evidence of a causative role of these changes in primary chilling injury.

We have demonstrated that the removal of the thylakoid system by nitrate starvation does not influence the chilling susceptibility of *Anacystis*, which is determined only by the growth temperatures of the alga cells. This is confirmed by the similarity of the transition temperatures associated with the zeaxanthin shift or K^+ release in the thylakoidless and control *Anacystis* cells (14, 24). Our results thus strongly suggest that it is the cytoplasmic membrane that plays a determinative role in thermal adaptation and that it is also the primary site of chilling damage. Since the *Anacystis* cells may be considered a model system for the study of thermal adaptation in higher plants, our results imply that changes in the plasma membrane have similar primacy in thermal adaptation and chilling sensitivity in higher plants.

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