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Spectral Changes in *Anacystis nidulans* Induced by Chilling[1]

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ABSTRACT

When *Anacystis nidulans*, strain TX 20 was grown at 39 C, then rapidly chilled to 0 C, a pigment with a carotenoid-like spectrum was bleached. This effect was not seen when cells which had been grown at 25 C were chilled. The effect seen in 39 C-grown cells was not reversible except under extreme conditions such as heating to near boiling for several minutes. Bleaching could be prevented by prior exposure of cells to glutaraldehyde, but could not be reversed by glutaraldehyde treatment following chilling. The effect occurred upon chilling 39 C-grown cells even after extensive heating at 85 C, a treatment which destroys phycocyanin and metabolic activities. 25 C-grown cells were induced to bleach by chilling when suspended in 50% glycerol. The results are interpreted as indicating a chill-induced change in aggregation state of a carotenoid, which changes its specific absorbance.

As described in the preceding paper (13), *Anacystis nidulans* exhibits several specific effects when 39 C-grown cells are chilled to near 0 C which are not seen when 25 C-grown cells are treated in the same manner. These results appear to reflect differences in membranes of cells grown at 39 and 25 C. The work described in this and in the previous paper extends previous observations which demonstrated cold sensitivity in *Anacystis* (2, 5, 6) and a change in the physical state of *Anacystis* membranes as they are chilled (10).

In this report, an additional effect of chilling *A. nidulans* is described. Bleaching of a pigment, observable spectrophotometrically, is shown to occur in cells grown at 39 C, but not those grown at 25 C. Evidence is presented that bleaching reflects physical changes within the photosynthetic membranes.

MATERIALS AND METHODS

A. nidulans, strain TX 20, was grown in continuous cultures as described in the preceding paper (13). Cells were routinely chilled to 0 C by placing them in a darkened 50-ml Erlenmeyer flask, then swirling in an ice water bath. The cell suspension was left in the bath for the duration of the chill time. Cell suspensions required approximately 2 min to reach below 4 C. For chill temperatures above 0 C, cells were placed in a darkened container and incubated in a water bath of appropriate temperature for the desired time. For temperatures below 0 C, cells were pelleted and resuspended in 50% (v/v) glycerol to give the original cell concentration. They were then placed in a darkened test tube and chilled to the appropriate temperature in a dry ice-acetone mixture. The sample was alternated between the cold bath and ambient temperature.

In experiments requiring glutaraldehyde fixation, cells were pelleted and resuspended in a 2% (v/v) glutaraldehyde solution in 0.05 M K-phosphate buffer (pH 6.8) of the same temperature as the cell suspension. Cell suspensions were kept in darkness during glutaraldehyde treatment.

Experiments requiring heated cells were held in a darkened 50-ml Erlenmeyer flask in a water bath of appropriate temperature. The bath was placed over a hot plate with a magnetic stirrer and the contents slowly stirred during heating. A marble was placed over the flask during heating to prevent excessive evaporation.

During chilling experiments, a control aliquot was always kept dark at the growth temperature to serve as a reference in measuring difference spectra. When chilling was coupled with another procedure, such as glutaraldehyde fixation or heating, the control was treated identically except that it was not chilled.

Difference spectra were measured at room temperature with a Varian model 635 spectrophotometer, using the 0 to 0.1 absorbance scale, and a slit width of 1 nm. Translucent scattering plates (Roehm & Haas No. 7328) were placed in 1-cm cuvettes for measuring spectra of whole cells. Difference spectra were obtained by placing chilled cells in the sample chamber and control cells in the reference chamber.

Chlorophyll concentration was estimated in 80% acetone using an absorption coefficient of 82 ml/mg at 663 nm.

All chemicals and solvents used were of reagent grade. Glutaraldehyde was purchased as highly purified 70% solution in 5-ml ampules from Ladd Research Industries, Inc.

RESULTS

A. nidulans, like other blue-green algae, is directly dependent on light for many metabolic activities other than photosynthesis (1). It responds to changes in light quality and intensity by modifying the relative concentration of various pigments (7, 11). However, when cells growing exponentially under steady-state conditions are placed in darkness for several hr, no change in any one pigment relative to the others is observed. This was shown by comparing spectra of dark-incubated cells with those of cells growing exponentially in the light. In fact, we observed no change in *A* of cells, measured from 400 to 700 nm, when kept dark for up to 6 hr. The ability to hold cells in a state of constant pigment concentration was very useful in the experiments described below, as this provided control for chilling experiments with no correction needed for cell growth during the treatment.

When *Anacystis* cells which have been growing actively under continuous culture conditions at 39 C are transferred to a low temperature (just above freezing), a characteristic bleaching pattern is seen. Figure 1 shows a difference spectrum between cells which had been chilled to 0 C for 0.5 hr and cells which were kept dark at the growth temperature during that same time. Cells grown at 39 C underwent a characteristic bleaching during chilling with an *A* minimum at 458 nm. A minor peak is seen at 490 nm and a shoulder at approximately 430 nm. This difference spectrum suggests that a specific pigment may have been destroyed, and is suggestive of the spectrum of a carotenoid.

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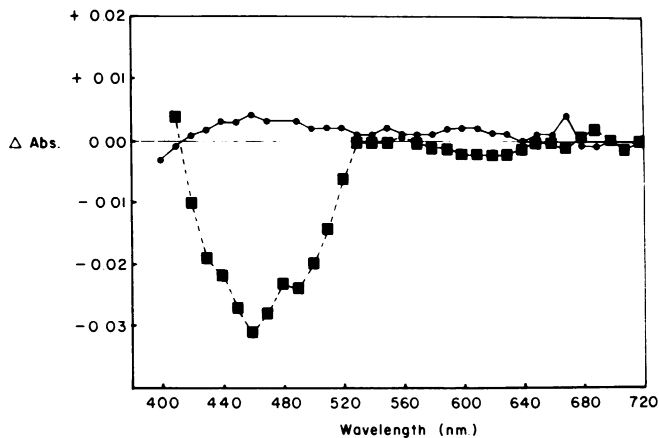

FIG. 1. Difference spectra of chilled *versus* nonchilled *Anacystis*. Chilling was to 0 C for 30 min. Absolute *A* at 678 nm was 0.67; absolute *A* at 458 nm was 0.65.

Previous estimates of the relative contribution of carotenoid to visible light absorption in *Anacystis* (6) indicate that at 458 nm approximately 50% of the total *A* is due to this class of pigments. We typically see an *A* decrease of about 0.053 at 458 nm upon chilling a cell suspension with an absolute *A* of 0.80 at this wavelength. This represents a decrease of 7% at 458 nm or an apparent decrease of approximately 14% of the total carotenoid. Figure 1 also indicates that cells grown at 25 C are not affected by chilling to 0 C. No decrease in *A* was seen, even after several hr at 0 C.

The bleaching was virtually complete within 20 min (Fig. 2). When cells were returned to 39 C in the dark following 0.5 hr chilling, the bleaching was enhanced, increasing gradually for approximately 5 hr. The spectrum was more difficult to interpret after a more extended period of incubation, as other pigments began to deteriorate.

The lipids of chilled and nonchilled cells were separately extracted with chloroform-methanol (1:2, v/v) (8). A difference spectrum of the two extracts revealed no differential absorption, indicating that the pigment composition was identical in the two samples. Furthermore, a difference spectrum of the residues remaining after lipid extraction also gave a flat spectrum. In other experiments, pigments were extracted with acetone; these extracts also showed no loss of pigment after chilling. Thus, bleaching does not appear to be due to the actual destruction of a pigment.

One possible explanation for the observed bleaching of 39 C-grown cells is that a pigment such as a carotenoid aggregates within the membranes. This aggregation might cause a decrease in *A* of that pigment due to the sieve effect (Duysens effect, absorption statistics) (12). If this interpretation is correct, the aggregation must be specific for one pigment, corresponding to the observed spectrum. Alternatively, the bleaching might be accounted for by a decrease in absorption due to an altered environment of the pigment resulting from an altered extinction coefficient. We favor the particle aggregation hypothesis since freeze fracture electron micrographs reveal large *de novo* particles on membrane surfaces following chilling (Kirchanski and Brand, unpublished).

One means of discovering if the bleaching is due to a change in membrane organization is to treat the cells in a way which prevents or limits particle mobility within the cells. Glutaraldehyde is a cross-linking agent which freely penetrates into cells. *Anacystis* cells were treated with glutaraldehyde to observe its effect on the ability of 39 C-grown cells to bleach (Fig. 3). Cells which are chilled prior to the addition of glutaraldehyde (- - -) show a greater change in *A* than cells not treated with glutaraldehyde (——). However, when cells are first incubated in glutaraldehyde, no bleaching is observed upon subsequent chilling (· · · · ·). These results are consistent with the interpretation that a pigment aggregates upon exposure of 39 C-grown cells to temperatures near 0 C, this aggregation being prevented by the addition of glutaraldehyde, which immobilizes components within the cells. When aggregation has already taken place, the addition of glutaraldehyde does not reverse it, but apparently holds the cells in a maximally bleached condition.

If bleaching in 39 C-grown cells is caused by a change in the state of aggregation, then it may reflect the physical state (fluidity) of the membranes, or alternatively, it may result from some enzymically induced aggregation. To test these alternatives, cells were heated to a temperature sufficient to inactivate enzymes and kill the cells without initially disrupting their internal organization. Heating the cells to 85 C for 7 min served this purpose.

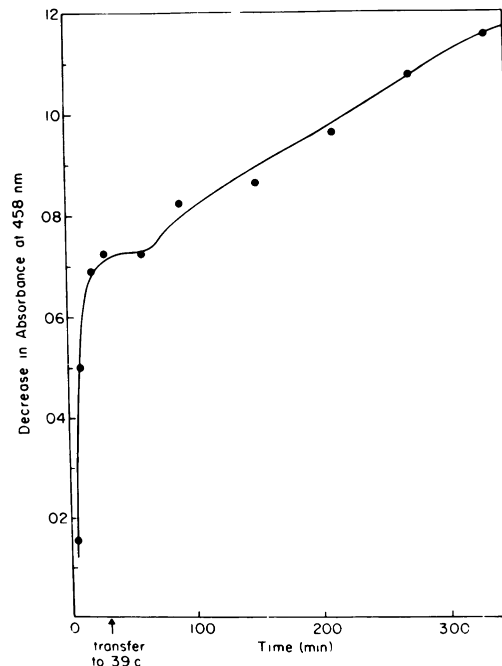

FIG. 2. Decrease in *A* with time after chilling 39 C-grown *Anacystis*. Difference spectra were determined at times indicated by (· · · · ·). Cells were kept at 0 C for the first 30 min, then transferred to 39 C for the remainder of the experiment.

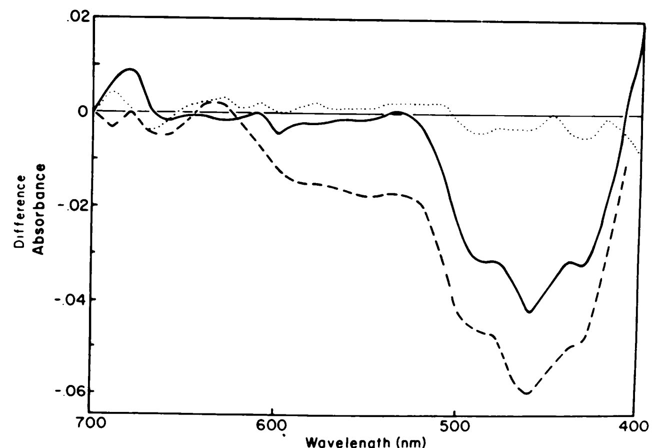

FIG. 3. Difference spectra of 39 C-grown *Anacystis* treated with glutaraldehyde and chilling. Cells represented in each spectrum were chilled for 60 min. Cells were either not exposed to glutaraldehyde (——), exposed to glutaraldehyde for 15 min prior to chilling (· · · · ·), or exposed to glutaraldehyde for 15 min immediately following chilling (- - -).

Although individual cells maintained their gross integrity (as observed by light microscopy), the phycocyanin was rapidly bleached by this treatment. Whole cell spectra of *Anacystis* before and after heating the cells to 85 C for 7 min are shown in Figure 4. These spectra show that virtually all of the phycocyanin is destroyed by heating, while virtually none of the Chl appears to be destroyed or altered by this treatment. Heating appears to be an effective means of destroying phycocyanin without destroying membrane integrity as viewed by the Chl spectrum.

Cells which were heated as described above were then chilled to determine if they were still capable of undergoing the characteristic decrease in *A*. Difference spectra of cells which were heated for 7 min, then chilled to 0 C for 0.5 hr, compared to cells which were heated for 7 min without chilling appeared identical to spectra of unheated cells which were chilled in the same manner (data not shown). The normal bleaching spectrum (Fig. 1) occurs in spite of the loss of phycocyanin and the probable loss of enzyme activities. Thus, bleaching is probably not directly related to the normal functioning of the phycobilosomes or to any other metabolic function. When 25 C-grown cells were heated, they also lost their phycocyanin, although at a significantly slower rate than did 39 C-grown cells. Subsequent chilling of the 25 C-grown cells did not result in any bleaching, indicating that the difference between cells grown at the two temperatures is maintained, even in metabolically inactivated cells which have lost most of their phycocyanin.

Heating for longer periods of time eventually prevented bleaching upon subsequent chilling.

When cells were first chilled, then heated to 85 C for 7 min, the bleaching pattern was almost completely eliminated, as seen in Table I. Subsequent chilling caused a recurrence of the bleaching pattern. Subsequent heating has little effect on reversing the pattern (Table I).

If the difference between 25 C and 39 C-grown cells is quantitative rather than a fundamental difference, then it might be possible to chill the 25 C-grown cells to a temperature below 0 C and observe bleaching similar to that seen in 39 C-grown cells. 25 C-grown cells were cooled to subzero temperatures to determine if the characteristic bleaching pattern would appear. To prevent ice crystal formation and accompanying gross cell disorganization, cells were pelleted and resuspended in a 50% (v/v) glycerol solution prior to chilling. A response very similar to that seen in chilled 30 C-grown cells occurred when 25 C-grown cells suspended in 50% glycerol were cooled to any temperature between approximately 0 C and −20 C. It was surprising that the 25 C-grown cells could bleach even at temperatures slightly above 0 C when suspended in 50% glycerol (Fig. 5). Glycerol was found to increase the temperature at which the effect occurs in 39 C-grown cells as well, as is seen in Table II. 39 C-grown cells normally do not bleach unless the temperature is lowered to approximately 10 C. In the presence of 50% glycerol, the cells bleach markedly, even at 15 C. Thus, in cells grown at 25 or 39 C, the of 50% glycerol causes an upward shift in the temperature at which cells bleach.

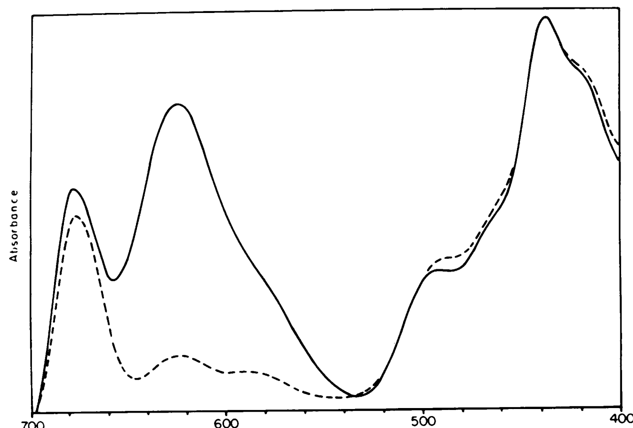

FIG. 4. Whole cell spectra of heated *Anacystis*. The visible spectrum of 39 C-grown cells after heating to 85 C for 7 min (- - -) is superimposed on an identical sample of cells which were not heated (——).

Table I. Effect of Alternate Chilling and Heating on the Extent of Bleaching in 39 C-grown *Anacystis*

Chill treatments were to 0 C for 30 min. Heat treatments were to 85 C for 7 min.

Treatment	Decrease in Absorbance at 458 nm
Chill	0.026
Chill → heat	0.005
Chill → heat → chill	0.044
Chill → heat → chill → heat	0.039
Chill → heat → chill → heat → chill	0.053
Chill → heat → chill → heat → chill → heat	0.046

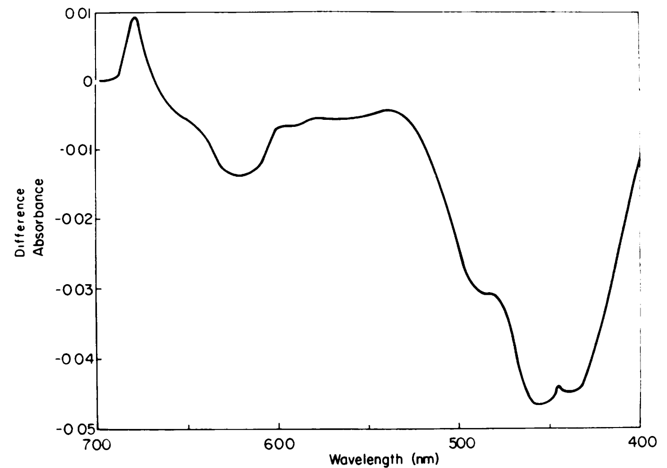

FIG. 5. Difference spectrum of 25 C-grown *Anacystis* after chilling to 0 C in the presence of 50% glycerol. Chilling time was 30 min.

Table II. Effects of Glycerol on the Chill Temperature Required to Bleach 39 C-grown *Anacystis*

Cells were pelleted and resuspended in either 0.05 M KH_2PO_4, pH 6.8, or in a solution of 50% glycerol plus the same buffer prior to chilling. Cell concentration was equivalent to 11.0 ug chlorophyll per ml. Cells were chilled for 30 min.

Chill Temperature	Change in Absorbance at 458 nm: Minus Glycerol	Plus Glycerol
15 C	-0.008	-0.023
10 C	-0.012	-0.041
5 C	-0.046	-0.049
0 C	-0.044	-0.045

DISCUSSION

The temperatures to which *Anacystis* grown at a high (39 C) or low (25 C) temperature had to be lowered to induce bleaching were considerably below those required for phase transitions in the membranes of cells grown at similar temperatures in the experiments of Murata *et al.* (10) and Murata and Fork (9). It appears that the pigment bleaching does not directly correlate with the temperature of the initial phase transition, but may relate to a second transition or aggregation which occurs at a lower temperature. The observed phase transition in *Anacystis* membranes above 0 C is considerably higher than the phase transition temperature of photosynthetic membranes in plants (14).

The observed bleaching of a pigment as a result of chilling might be accounted for in several ways. For example, chilling could cause the irreversible chemical destruction of a pigment, or it might induce a reversible change in the state of the pigment, such as a change in oxidation state or conformation. The observation that total extracted pigment per cell is identical spectrophotometrically before and after chilling argues against chemical destruction. Bleaching is induced upon chilling, even after the cells have been heated to 85° C for several min, which strongly suggests that it is not enzyme-induced.

Another possible explanation for the bleaching is that it is caused by a change in the state of aggregation of a particular pigment, perhaps within the membrane system. Since the difference spectrum is very similar to the absorption spectrum of carotenoids, one might predict that particles containing one or more carotenoids may change aggregation state upon chilling which would probably require lateral mobility of components within the membrane. The results showing that glutaraldehyde prevents bleaching support this interpretation.

The different response to chilling of cells grown at 39 and 25 C must be associated with some very stable property of cells since this difference remains even after cells are heated to near boiling. The most attractive hypothesis is that some compositional difference between cells grown at the two temperatures alters the capacity of one or more membrane components to change aggregation state. A class of components which does vary, depending on growth conditions of the cells, is membrane fatty acids. Cells grown at high temperature have more saturated fatty acids of longer chain length than those grown at a lower temperature (4). Membrane fatty acids of cells grown at the highest temperature would be expected to solidify at the highest temperature (10), and thus chilling would induce lateral displacement of the components at the highest temperature.

Alternatively, the difference between 39 and 25 C-grown cells may be due to the pigment which is seen to bleach. Halfen and Francis (3) demonstrated that the total carotenoid content of cells increased when the *Anacystis* cells were grown at increasingly higher temperatures. They also noted different relative concentrations of the major carotenoid pigments, depending on growth temperature. A considerable amount of the total carotenoid content of *Anacystis* occurred as glycoside, which also varied quantitatively with temperature. A carotenoid glycoside molecule would be amphipathic which might dictate an orientation within the membrane similar to that of galactolipids and phospholipids. Migration, and an accompanying change in state of aggregation of carotenoid glycoside might account for pigment bleaching.

Although the data appear to support the view that pigment bleaching is due to a change in the state of aggregation, the arguments are not yet conclusive. Electron microscopic observations of freeze-fractured membranes reveal the presence of large particles after chilling which may be correlated with the observed bleaching. Details of these observations will be reported soon. In other experiments now underway, we are isolating photosynthetic membranes from *A. nidulans* to determine if these, too, undergo the characteristic bleaching. If isolated membranes respond spectrally to chilling as whole cells do, it should be much easier to study the nature of this phenomenon.

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